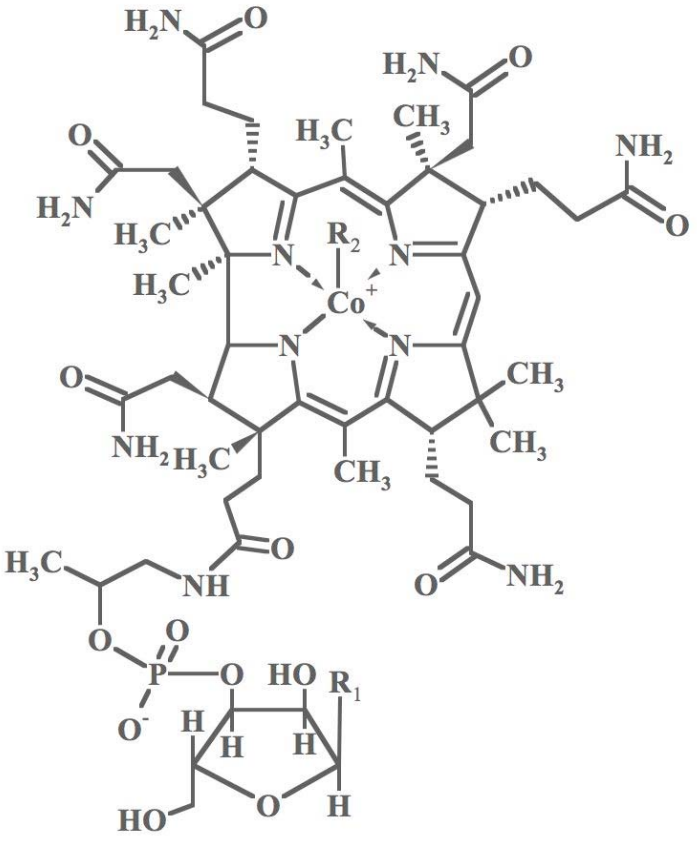
VITAMIN B_{12} SYNTHESIS IN *Lactobacillus reuteri*



Filipe Santos

Promotor:
Prof. Dr. W. M. de Vos
Hoogleraar Microbiologie Wageningen Universiteit

Co-promotor:
Prof. Dr. J. Hugenholtz
Hoogleraar Industrial Molecular Microbiology Universiteit van Amsterdam

Samenstelling Promotiecomissie:
Prof. Dr. John R. Roth
University of California, Davis, USA

Prof. Dr. Douwe van Sinderen
University College Cork, Ireland

Dr. Paul G. Bruinenberg
DSM Food Specialties, Delft

Prof. Dr. René H. Wijffels
Wageningen Universiteit

This research has been conducted at the VLAG Graduate School

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Filipe Santos
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For my parents,

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Abstract

Vitamin B_{12} is a fascinating molecule that acts as a co-factor in processes crucial to many living things. It is an essential constituent of our diet and an industrially important co-factor used in biocatalysis processes, such as the production of 1,3-propanediol. *De novo* synthesis of vitamin B_{12} is limited to a few representatives of bacteria and archaea. *Lactobacillus reuteri* has been reported to produce vitamin B_{12}, but before this study, our knowledge about this process was very limited. The work presented here aimed at characterizing all aspects related to vitamin B_{12} production in *L. reuteri* using a multidisciplinary approach, and exploring its potential applications.

The native corrinoid produced by *L. reuteri* under anaerobiosis was analyzed by HPLC, MS and NMR and found to contain adenine, instead of 5,6-dimethlbenzimidazole, in the Coα-ligand. Commonly known as pseudovitamin B_{12}, this compound could play a role in assessing the capability of vitamin B_{12}-dependent enzymes to utilize alternative cofactors, and in understanding the impact of analogues in vitamin B_{12} metabolism.

The anaerobic biosynthetic pathway converting glutamate into vitamin B_{12} was found to be encoded in one single stretch of the chromosome, neighbored by clusters of genes encoding glycerol reduction, metabolosome assembly and cobalt transport. The control of expression of these clusters appears to be part of the same regulon, in which a single regulatory protein plays a pivotal role. Genome-scale analysis tools such as cDNA microarrays and metabolic network modeling, exposed unsuspected liaisons between glycerol utilization, vitamin B_{12} synthesis and amino acid metabolism. This provided new leads that resulted in a 20-fold increase in vitamin B_{12} production without the usage of genetic manipulation. The possibility of combining the production of folate and vitamin B_{12} in the same *L. reuteri* cell was also illustrated and applied to the fermentation of substrates of plant origin.

The findings reported here, besides their scientific relevance, can be applied to the improvement/development of fermentation process with increased vitamin B_{12} production. Ultimately, the work presented here might also be used to ensure an adequate vitamin B_{12} intake in humans.

Keywords: vitamin B_{12}, *Lactobacillus reuteri*, lactic acid bacteria, genome-scale model, omics, biosynthesis.

CHAPTER 1

GENERAL INTRODUCTION

Abstract

Vitamin B_{12} is a captivating molecule that is associated with several fundamental processes that sustain life. For humans, it is an essential nutrient in our diet and a co-factor of great relevance in biotechnological transformations. In this Chapter, a brief description of the illustrious history of B_{12} research will be presented, followed by some general aspects relative to its chemistry and biochemistry. We will review our current understanding of how this complex molecule is synthesized in Nature and its importance in human nutrition, describing a particular species of lactic acid bacteria, *Lactobacillus reuteri*, which unlike others in this group can synthesize B_{12}. A brief account of the most modern research approaches is provided. The Chapter ends with a short overview of the thesis.

Brief historical background of vitamin B_{12} research

In resemblance to so many other discoveries, vitamin B_{12} was first stumbled upon while studying a condition caused by its absence. Minot and Murphy reported in 1926 that by submitting their patients to a diet including whole liver they were able to cure pernicious anemia (67). They explained their remarkable finding by postulating the presence of an "extrinsic factor" in liver, whose identity was going to remain elusive for more than 20 years to come. For their efforts related to liver therapy in cases of anemia, Minot and Murphy (along with Whipple) were attributed the first Nobel Prize related to vitamin B_{12} research in 1934.

The race to identify the mysterious "extrinsic factor" culminated in the isolation of a red crystalline compound from liver that was able to cure pernicious anemia. Two principal pharmaceutical companies separated by the Atlantic Ocean achieved this independently within the same year. The discovery was carried by a group at Glaxo in the UK headed by Smith and a group at Merck in the USA lead by Folkers, and at this stage the "extrinsic factor" was designated vitamin B_{12} (78). Within the same year, vitamin B_{12} was also found to be present in milk powder, beef extract and culture broth of several bacteria (77), and was reported to be a growth factor for *Lactobacillus lactis* that lead to the development of a vitamin B_{12} bioassay (90, 91). Soon, the presence of cobalt was recognized (79), along with the identification of analogues that retained biological activity (29, 36), namely pseudo-B_{12} (72).

The isolation of vitamin B_{12} along with the realization that this molecule was structurally much more complex than anything that had previously been solved, triggered research on the determination of its exact crystal structure. Hodgkin pioneered X-ray crystallography of complex molecules and in 1955 she revealed the intricately complex three-dimensional structure of vitamin B_{12} (cyanocobalamin) with a very high level of accuracy (48, 49). Shortly after Barker had crystallized the first biologically active coenzyme forms of (pseudo)-B_{12} (7), Lenhert and Hodgkin repeated the feat, and solved the structure of adenosylcobalamin in 1961 (55). For her efforts, Hodgkin was awarded in 1964 the second Nobel Prize related to vitamin B_{12} research in its relatively short history. In 1962, an alternative β-axial ligand was found to be present in biologically active forms of vitamin B_{12}. This methyl radical was demonstrated to be involved in methionine synthesis in bacteria (47).

Another important event in the history of vitamin B_{12} was its chemical synthesis. This test of persistence that dragged throughout the '60s and '70s was headed by Eschenmoser and by yet another Nobel Prize laureate, Woodward. It involved the participation of over one hundred scientists and culminated in a highly complicated chemical synthesis process involving around 70 steps (35). It became apparent that the

industrial production of vitamin B_{12} was not going to rely on chemical synthesis, since it was obviously far too technically challenging and economically unviable. At this stage, much attention turned to how this remarkable molecule was synthesized in Nature.

It was only in 1985 that the first gene sequence associated with B_{12} biosynthesis was identified (115). With the arise of molecular genetics in combination with well-established biochemistry and chemistry expertise, it took less than ten years for the groups of Blanche at Rhône-Poulenc Rorer, Battersby at Cambridge UK and Scott in Texas to elucidate most of the aerobic biosynthesis pathway (8). The recognition of a biosynthetic route towards B_{12} that did not depend on molecular oxygen by the group of Scott constituted a turning point in B_{12} history. It lead to the intense study of B_{12} biosynthesis in *Propionibacterium shermanii* by Scott himself and co-workers, and additionally to the study of the more genetically accessible anaerobic B_{12} producer, *Salmonella typhimurium*, in the groups of Roth, Warren, amongst others (115). Even though much progress has been made, the complete anaerobic route towards B_{12} biosynthesis remains to be elucidated.

Since the discovery of the two alternative upper ligands back in the early '60s, several adenosyl- and methyl-cobamide dependent enzymes have been identified. In general, these reactions are responsible for the catalysis of methyl transfers and carbon backbone rearrangements (115). Much progress has been achieved since then in the elucidation of their mechanisms and stereochemistry. In the last decade of the XX century, the three-dimensional structure of methionine synthase was solved (33, 34), followed shortly by that of methylmalonyl CoA mutase (60), the other B_{12}-dependent reaction known to be present in humans (5). In 1999, the structures of two other B_{12}-dependent enzymes in bacteria were solved, namely for glutamate mutase (76) and diol dehydratase (89).

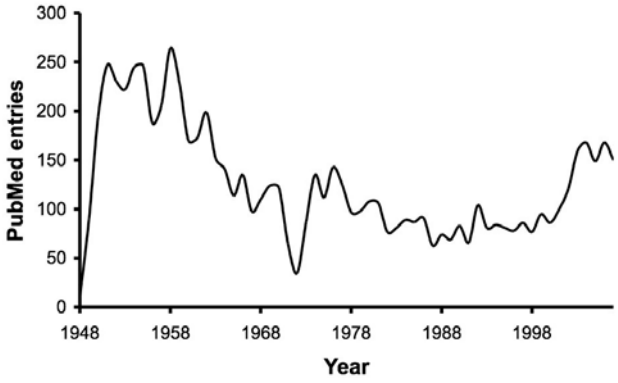

Figure 1.1. PubMed entries with B_{12} in the title from 1948 until 2007.

In 1948 the term vitamin B_{12} first appeared in the scientific records (78). Over the past 60 years, more than 7000 peer-reviewed papers have been published in relation to it (Fig. 1.1). This aided the improvement of technological issues surrounding its production, reduced the negative effects to human health caused by its deficiency, and last, but definitely not least, contributed to our better understanding of the world and the underlying principles that govern it.

Chemical structure of vitamin B_{12}

A vast variety of analogues of vitamin B_{12} have been described, and are generally considered the most complex of all small molecules known. Their shared molecular architecture consists of a ring-contracted porphinoid with a cobalt ion chelated at the center of a tetrapyrrole-derived macrocycle. The metal ion is covalently bond to an "upper" or β-ligand, and further coordinated with an axial "lower" or α-ligand (Fig. 1.2). The nature of these radicals determines the designation of the corrinoid. The term vitamin B_{12} refers strictly to cyanocobalamin, since this was the first form found after the compound was isolated from liver. However, in this thesis the term vitamin B_{12} refers to any compound with the described structure.

In naturally occurring analogues of vitamin B_{12}, it is either an adenosyl or a methyl group that is present in the β-ligand. The adenosyl group is found in B_{12} analogues that act as a co-factor in rearrangement or reductase reactions, while the methyl group is present in the upper ligand of the co-factor that is involved in B_{12}-dependent methionine synthesis. Soon after the isolation of vitamin B_{12}, it was observed at Organon in the Netherlands 1950, that a treatment with KCN greatly improved its stability (109, 118). Although this was not completely clear at that time, this treatment promoted the replacement of the upper ligand present in biologically active forms by a cyano moiety. The latter is only found in the β-ligand of synthetic preparations of vitamin B_{12} and not in naturally occurring analogues.

Cobalamin, the best-studied form of vitamin B_{12}, is a cobamide in which 5,6-dimethylbenzimidazole is the aglycon attached to the α-ligand bound in an atypical α-glycosidic linkage from its N-1 to the C-1 of ribose. Besides the different known radicals that can be found as β-ligands connected via a Co-C bond, different microorganisms have been described to synthesize B_{12} analogues that contain bases in the α-ligand other than 5,6-dimethylbenzimadazole. These are most commonly benzimidales, purines and phenolic compounds. The evolutionary significance of the existence of such a wide variety of natural forms of vitamin B_{12} is still under debate.

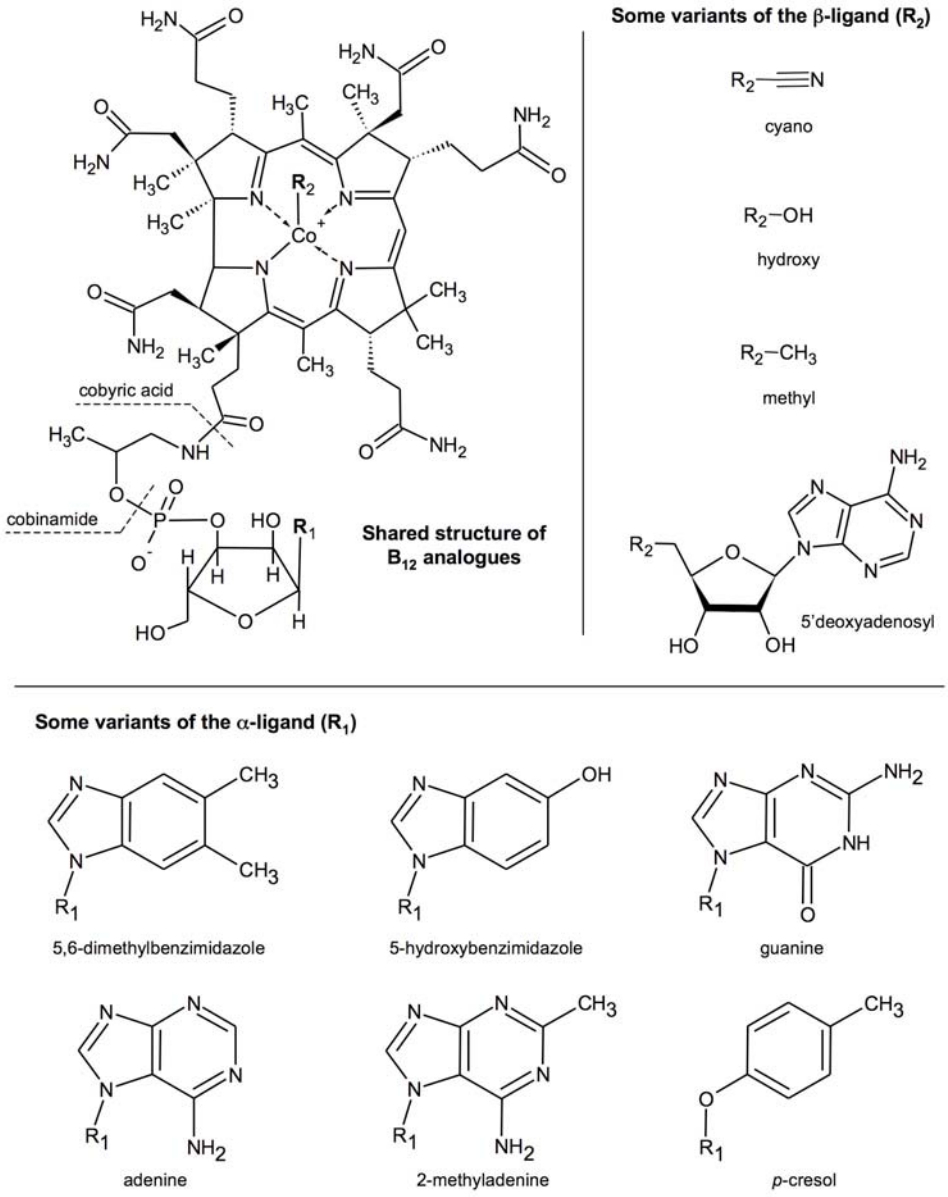

Figure 1.2. Shared structure of B_{12} analogues
along with possible alternatives found in the α- and β-ligands.

Vitamin B_{12}-dependent enzymes

Intriguingly, B_{12} biosynthesis is limited to a few representatives of bacteria and archaea (61) while B_{12}-dependent enzymes are widespread throughout all domains of life (80). In fact, only in plants and fungi have there not been any B_{12}-dependent processes identified yet (25). Generally, B_{12}-dependent reactions are described to catalyze methyl transfers and carbon backbone rearrangements (see Table 1.1).

Table 1.1. B_{12}-dependent reactions and their distribution

Enzyme	Description	Distribution
B_{12}-dependent methionine synthase	Involved in catalyzing the conversion of homocysteine to methionine using a methyl group donated by methyltetrahydrofolate (63).	Bacteria, animals
Methylmalonyl-CoA mutase	Involved in catalyzing the interconversion of (2R)-methylmalonyl-CoA to succinyl-CoA (6).	Bacteria, animals
Ribonucleotide reductases (class II)	Involved in catalyzing the reduction of ribonucleosides diphosphates (NDPs) and triphosphates (NTPs) to form the corresponding deoxyribonucleotides (38).	Bacteria, Archaea
Glutamate mutase	Involved in catalyzing the interconversion between (S)-glutamate and (2S,3S)-3-methylaspartate (18).	Bacteria
2-Methyleneglutarate mutase	Involved in catalysing the equilibration of 2-methyleneglutarate with (R)-3-methylitaconate (*idem*).	Bacteria
Diol and glycerol dehydratase	Involved in catalyzing the conversion of glycerol to β-hydroxypropionaldehyde, 1,2-propanediol to propionaldehyde, and 1,2-ethanediol to acetaldehyde (106).	Bacteria
Ethanolamine ammonia-lyase	Involved in catalyzing the conversion of ethanolamine and other short-chain vicinal amino alcohols to ammonia and the oxo product (4).	Bacteria
Aminomutases	Involved in catalysing the transferring of an amino group between two adjacent carbon atoms of an amino acid (40).	Bacteria
Isobutyryl-CoA mutase	Involved in catalyzing the interconversion of isobutyryl-CoA and n-butyryl-CoA (121).	Bacteria

Besides the reactions listed in Table 1.1, B_{12}-dependent reactions have also been described to be essential in reductive dehalogenases found in bacteria (92, 119). Additionally, corrinoids have also been found to play a crucial role in methanogenesis (84) and acetogensis (73).

Biosynthesis of vitamin B_{12}

The biosynthesis of coenzyme B_{12} is commonly divided in three parts: *(i)* the synthesis of uroporphyrinogen III from either glutamyl-tRNA or glycine and succinyl-CoA; *(ii)* the corrin ring synthesis, which differs between the anaerobic pathway starting with the insertion of cobalt into an early intermidiate, and the aerobic pathway where the cobalt chelation reaction occurs only after corrin ring synthesis; *(iii)* and the corrin ring adenosylation, attachment of the amino-propanol arm and assembly of the nucleotide loop bridging the lower ligand to the cobalt at the core of the corrin ring.

(i) Synthesis of uroporphyrinogen III (Fig. 1.3)

Two different pathways have been described to culminate in the synthesis of the first common precursor of all identified tetrapyrroles, 5-aminolevulinate. The C4-Way (Shemin-Way) derives from succinyl-CoA and glycine, which are condensed into 5-aminlevulinate in a single enzymatic reaction, catalyzed by 5-aminolevulinate synthase (E.C. 2.3.1.37) (52, 99, 113). The C5-Way is a two-step enzymatic conversion. First tRNA-bound glutamate is reduced by glutamyl-tRNA reductase (E.C. 1.2.1.-) to glutamate-1-semialdehyde (86), which is then rearranged to 5-aminolevulinate via an intramolecular shift of the amino group from the C-2 to the C-1, catalyzed by glutamate-1-semialdehyde 2,1-aminomutase (E.C. 5.4.3.8). In some microorganisms, 5-aminolevulinate was found to be transported into the cell from the environment (61).

The assembly of uroporphyrinogen III from 5-aminolevulinate requires three additional enzymatic reactions. First, the formation of the first pyrrole derivative, porphobilinogen, is catalyzed by porphobilinogen synthase (E.C. 4.2.1.24) through the combination of two 5-aminolevulinate molecules (44, 120). This is followed by the porphobilinogen deaminase (EC 2.5.1.61) mediated polymerization and rearrangement of four porphobilinogen molecules to form hydroxymethylbilane (66, 114). The latter is then finally cyclized by uroporphyrinogen III synthase (E.C. 4.2.1.75) (10). Uroporphyrinogen III is the first macrocyclic intermediate on the biosynthetic pathways of all tetrapyrroles, and its architectural blueprint is easily recognizable in their structure. Not surprisingly, the genes encoding these last two reactions are widely spread throughout the genomes of all domains of life (115). Uroporphyrinogen III is also the last common precursor shared by all tetrapyrroles. Its decarboxylation and oxidation leads to protoporphyrin IX, the precursor of

chlorophyll and haem. Its methylation forms precorrin-2, the branching point of the biosynthetic routes of sirohaem, coenzyme F_{430}, haem d_1 and coenzyme B_{12} (Fig. 1.3).

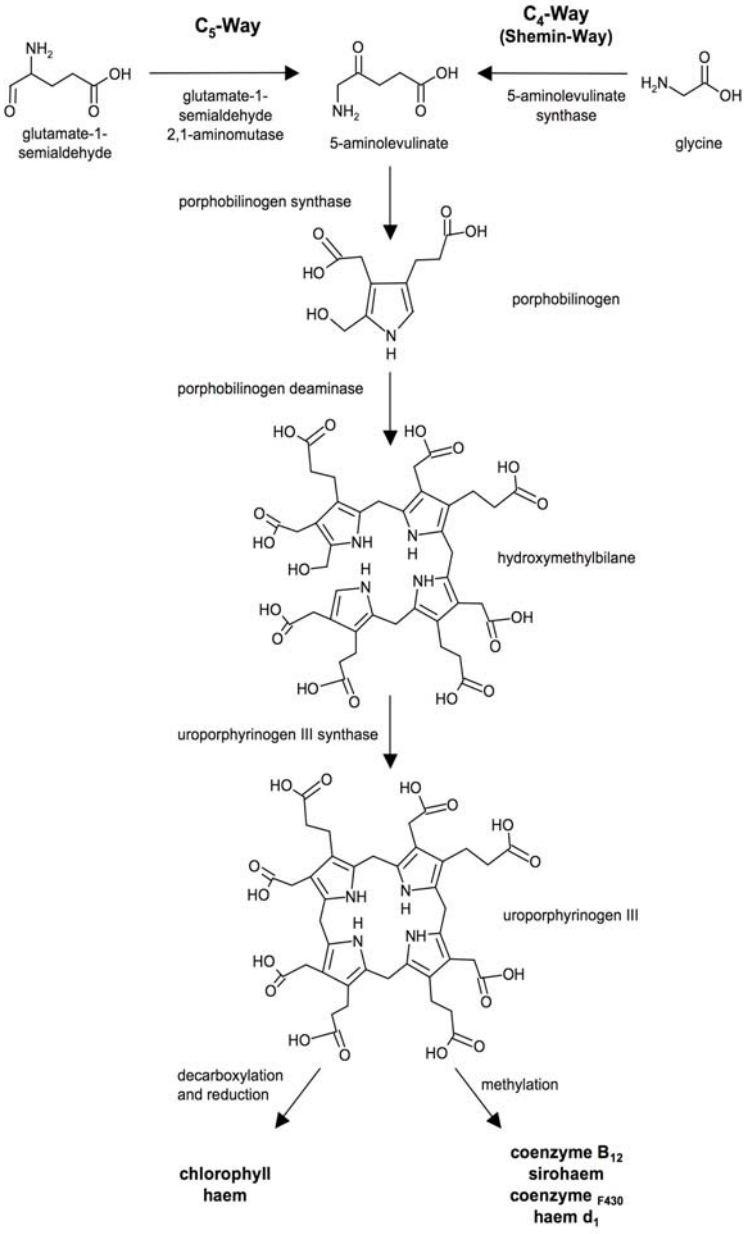

Figure 1.3. Synthesis of uroporphyrinogen III

(ii) Synthesis of the corrin ring (Fig. 1.4)

The methylation of uroporphyrinogen III to precorrin-2 is actually a two-step reaction that involves the donation of two S-adenosyl-L-methionine (SAM)-derived methyl groups. Both methylations are catalyzed by a single enzyme, uroporphyrinogen-III C-methyltransferase (EC 2.1.1.107). The first methylation occurs at carbon 2 forming precorrin-1, which is then released from the enzyme. The subsequent methylation occurs at position 7 of precorrin-1, leading to the formation of precorrin-2 (116). At this point coenzyme B_{12} biosynthesis splits in two different routes depending on oxygen dependency. The order of methylation and amidation appears to be the same in both of these pathways. However, the method used for ring contraction and the moment in which cobalt is inserted is different. As a consequence, even though several corrin ring biosynthesis genes are homologous between organisms that use the different routes, some genes are unique and can be used to predict which pathway a given organism uses.

The aerobic or late cobalt insertion pathway. This route starts immediately with the methylation of precorrin-2 at position 20 to form precorrin-3A. This reaction is catalyzed by precorrin-2 C20-methyltransferase (EC 2.1.1.130) using a SAM derived methyl group (31, 81, 82). The next step constitutes one of the crucial differences between the aerobic and anaerobic routes, as it is dependent on the availability of molecular oxygen. Precorrin-3A is oxidized by an iron-sulfur protein, precorrin-3B synthase (EC 1.14.13.83), which incorporates oxygen at position 20 of the macrocycle (31, 88). Precorrin-3B is then methylated at position 17 giving rise to precorrin-4, catalyzed by precorrin-3B C17-methyltransferase (EC 2.1.1.131) (31, 88). It is after this methylation that the ring contraction is thought to occur, since it requires the presence of the methyl group donor, SAM, for the reaction to take place. Precorrin-5 is then formed through the SAM dependent methylation of precorrin-4 at position 11 via precorrin-4 C11-methyltransferase (EC 2.1.1.133) (26). The SAM-dependent methylation of precorrin-5 at position 1 is catalyzed by precorrin-6A synthase (deacetylating) (EC 2.1.1.152) and associated with the release of an acetyl group as acetate (31). Precorrin-6A is transformed into precorrin-6B by the NADPH-dependent precorrin-6A reductase (EC 1.3.1.54), which catalyzes the reduction of the double bound between positions 18 and 19 (13, 115). Via precorrin-6Y C5,15-methyltransferase (decarboxylating) (EC 2.1.1.132) two SAM-derived methyl groups are then donated to precorrin-6B at positions 5 and 15, concomitantly with the decarboxylation of the acetate side chain at position 12, forming precorrin-8X. Unpublished studies suggest that there is no other intermediate between precorrin-8X and the subsequent step (Martin Warren, personal communication).

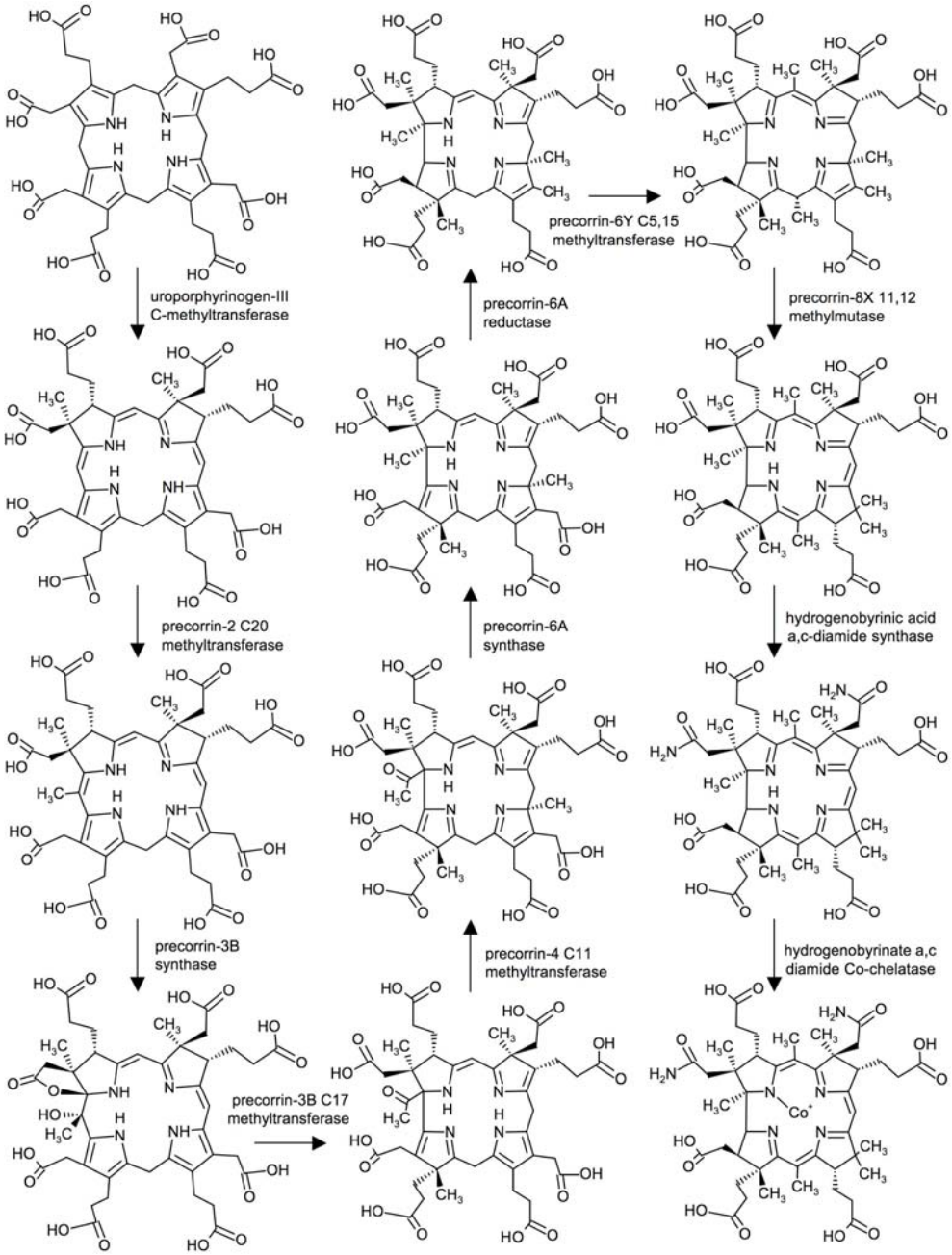

Figure 1.4. Aerobic route of corrin ring synthesis.

Hydrogenobyrinic acid is then formed as a result of the migration of the methyl group from position 11 to position 12 promoted by precorrin-8X 11,12-methylmutase (EC 5.4.1.2), and completing the synthesis of the corrin ring (26, 105). The next step is the amidation of the *c* and subsequently, *a* side chains by means of a Mg^{2+}, ATP and glutamine-dependent process catalyzed by hydrogenobyrinic acid a,c-diamide synthase (glutamine-hydrolysing) (EC 6.3.5.9) (115). Finally, at this stage cobalt is inserted via a heterotrimeric and ATP-dependent chelatase reaction catalyzed by hydrogenobyrinic acid a,c-diamide cobaltochelatase (EC 6.6.1.2), forming cob(II)yrinic acid a,c-diamide (32).

The anaerobic or early cobalt insertion pathway. This pathway starts with the conversion of precorrin-2 to sirohydrochlorin catalyzed by precorrin-2 dehydrogenase (EC 1.3.1.76) (87, 115). Sirohydrochlorin is where the anaerobic coenzyme B_{12} biosynthesis pathway definitely branches from the one to sirohaem, depending on whether it is cobalt or iron that is chelated (9). Unlike in the aerobic pathway, the chelatase reaction is here ATP-independent and catalyzed by sirohydrochlorin cobaltochelatase (EC 4.99.1.3) early in the synthesis of the corrin ring (17). Co-precorrin-2 is then methylated at position 20 by Co-precorrin-2 C20-methyltransferase (EC 2.1.1.151) forming Co-precorrin-3 (93). In contrast to the aerobic route, which requires the incorporation of molecular oxygen in position 20 of Co-precorrin-3 before the methylation at position 17, in the anaerobic route the latter is methylated directly via Co-precorrin-3B C17-methyltransferase (EC 2.1.1.131) using SAM as the methyl group donor (115). From Co-precorrin-4 onwards, there are some intermediates that have not been identified yet but deduced by analogy to the well-studied late cobalt insertion pathway. Such parallels should be drawn with a reasonable degree of caution, since the history of B_{12} biosynthesis is prosperous with surprises. There is evidence that Co-precorrin-4 is converted by Co-precorrin-4 C11-methyltransferase (EC 2.1.1.133) to Co-precorrin-5A resorting to SAM as the methyl donor (83). The formation of Co-Precorrin-5B is thought to be catalyzed by Co-precorrin-5A C20-acyltransferase (EC 2.3.1.-), concomitant with the release of acetaldehyde (in contrast with the aerobic route, where acetate was formed) (115). Co-precorrin-6A is then assumed to be formed by methylation at position 1 via Co-precorrin-5B C1-methyltransferase (EC 2.1.1.-) (61). Co-precorrin-6A is reduced to Co-precorrin-6B in a process catalyzed by Co-precorrin-6A reductase (EC 1.3.1.54) (115). At this stage, based on the presence of two subunits, often encoded by two separate genes, Co-precorrin-6B is thought to be methylated twice separately forming Co-precorrin-7, and subsequently, Co-precorrin-8X. This process is considered to be catalyzed by the SAM-dependent Co-precorrin-6Y C5,15-methyltransferase [decarboxylating] subunits CbiE and CbiT (EC 2.1.1.132) (*idem*). Cobyrinic acid is formed through the activity of Co-precorrin-8X methylmutase (EC 5.4.1.2) that promotes the migration of the methyl group from position 11 to postion 12 (82, 83). In resemblance to the late cobalt

insertion pathway, amidation of the corrin ring occurs first at side chains *c* and subsequently *a*, here catalyzed by the Mg^{2+}, ATP and glutamine dependent cobyrinic acid a,c-diamide synthase (EC 6.3.1.-), and resulting in the formation of cob(II)yrinic acid a,c-diamide (115). From cob(II)yrinic acid a,c-diamide onwards differences between the anaerobic and aerobic pathways are considerably attenuated and reside mostly on the genetic level.

(iii) Corrin ring adenosylation, attachment of the amino-propanol arm and assembly of the nucleotide loop bridging the lower ligand to the cobalt at the core of the corrin ring (Fig. 1.5).

Corrin ring adenosylation. Before adenosylation actually occurs, steps are taken to ensure that the cobalt is ligated securely. First cob(II)yrinic acid a,c-diamide is reduced to cob(I)yrinic acid a,c-diamide catalyzed by an enzyme that remained elusive until recently, cob(II)yrinic acid a,c-diamide reductase (EC 1.16.8.1) (54). Adenosylation is catalyzed by cob(I)yrinic acid a,c-diamide adenosyltransferase (EC 2.5.1.17) forming adenosyl cobyrinic acid a,c-diamide (11, 94, 97, 111), which is then the initial substrate of the final amidase, adenosylcobyric acid synthase (glutamine-hydrolysing) (EC 6.3.5.10). The latter will catalyze a four-step amidation sequence of the *b*, *d*, *e* and *g* side chains, culminating in adenosylcobyric acid hexaamide (12). Besides cob(I)yrinic acid a,c-diamide, adenosyltransferase (EC 2.5.1.17) can use other substrates, such as cobinamide or cobalamin (37).

Attachment of the amino-propanol arm. The aminopropanol group that is attached to the *f* side chain of coenzyme B_{12} is derived from either glycine or threonine metabolism. It can be attached as (R)-1-aminopropan-2-ol to adenosylcobyric acid hexaamide originating adenosylcobinamide, which is then phosphorilated by adenosylcobinamide kinase (EC 2.7.1.156) to form adenosylcobinamide phosphate using either ATP or GTP (68). Or in alternative, (R)-1-aminopropan-2-yl phosphate derived from L-threonine phosphate via threonine-phosphate decarboxylase (EC 4.1.1.81) can be attached to adenosylcobyric acid hexaamide and originate directly adenosylcobinamide phosphate, skipping the kinase step (20). Both the attachment of (R)-1-aminopropan-2-ol and (R)-1-aminopropan-2-yl phosphate is catalyzed by adenosylcobinamide (phosphate) synthase (EC 6.3.1.10) (115).

Assembly of the nucleotide loop. The nucleotide loop is an important structure bridging the α-axial ligand to the corrin ring. It contains two unusual features, namely the presence of a α-glycosidic bond (rather than β) joining the base to the ribose, and linking the (R)-1-aminopropan-2-ol to the nucleotide in a phosphodiester bond, in which the 3'-OH group (rather than 5'-) from the ribosyl moiety participates.

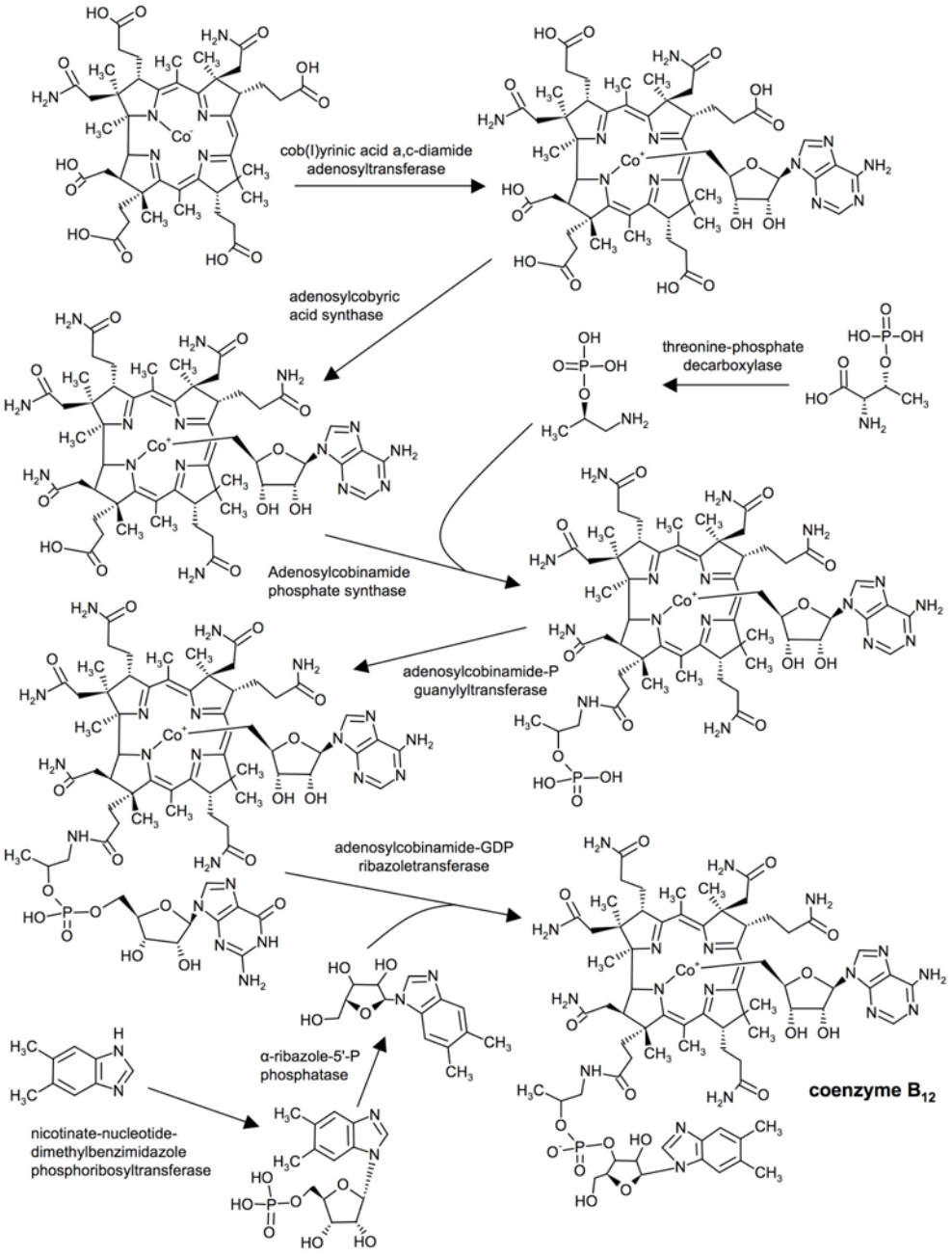

Figure 1.5. Corrin ring adenosylation, attachment of the amino-propanol arm and assembly of the nucleotide loop.

To achieve the assembly of such a structure, it is required that both cobinamide and the α-ligand base are activated separately before being joined. Adenosylcobinamide phosphate is activated by adenosylcobinamide-phosphate guanylyltransferase (EC 2.7.7.62) forming adenosylcobinamide-GDP (68). The activation of the base requires two enzymatic reactions. First the phosphoribosyl moiety of nicotinate mononucleotide is transferred to 5,6-dimethylbenzimidazole originating N^1-(5-phospho-α-D-ribosyl)-5,6-dimethylbenzimidazole (commonly known as α-ribazole-5'-P) (21, 41, 43). This step is mediated by nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase (EC 2.4.2.21), which has also been shown to function with substrates other than 5,6-dimethylbenzimidazol, such as adenine (22, 42). The second step is the removal of the phosphate group of α-ribazole-5'-P mediated by α-ribazole-5'-phosphate phosphatase (EC 3.1.3.73), yielding α-ribazole (69). The assembly of the activated cobinamide and α-ligand base is mediated by adenosylcobinamide-GDP ribazoletransferase (EC 2.7.8.26), finally yielding coenzyme B_{12} (19, 57).

Vitamin B_{12} and human nutrition

Humans have an auxotrophic requirement for vitamin B_{12} with a recommended nutrient intake (RNI) for healthy adults of 2.4 μg/day. The fact that B_{12}-dependent enzymes are present in neither plants nor fungi, has significant implications for the dietary sources and availability of vitamin B_{12}. The B_{12} synthesized by a few representatives of bacteria and archaea is the only source of this vitamin, which enters the human food chain mostly through incorporation into foods of animal origin. Dietary sources with a significant vitamin B_{12} content include meat (liver), poultry, milk and derivatives, eggs, amongst others.

Table 1.2. Recommended nutrient intake (RNI) for vitamin B_{12} in different age groups

Age (years)	RNI (μg/day)	Age (years)	RNI (μg/day)
0 – 0.5	0.4	10 – 18	2.4
0.5 – 1	0.7	19 – 65	2.4
1 – 3	0.9	> 65	2.4
4 – 6	1.2	Pregnant	2.6
7 – 9	1.8	Lactating	2.8

Source: FAO/WHO (12)

Vitamin B_{12} deficiency has been demonstrated to lead to pernicious anemia, and neurological dysfunction, amongst other complications (95). The onset of vitamin B_{12}

deficiency symptoms is often delayed by the increased intake of folate. This masking of B_{12} deficiency has resulted in the restriction of folate intake levels and prevented folate fortification in many countries (39). Strict vegetarian dietary regimes tend to be poor in vitamin B_{12}, which has boosted the popularity of fortifying vegetarian foodstuffs with B_{12} (1). Another risk group are the elderly, most often not because of a diet poor in B_{12}, but most often because of malabsorption phenomena.

The absorption of vitamin B_{12} in human is a complex process. Protein-bound B_{12} present in food is only released in the stomach by the action of a high concentration of hydrochloric acid. At this stage, free B_{12} is immediately bound to haptocorrin, a glycoprotein secreted in the mouth by the salivary glands and also to a certain extent in the stomach itself. Haptocorrin protects B_{12} from chemical denaturation by the acidic pH characteristic of the stomach. Also in the stomach, in the parietal cells (same that secrete hydrochloric acid), yet another glycoprotein involved in B_{12} absorption is made. The latter is known as the intrinsic factor and will eventually bind vitamin B_{12} enabling its active absorption. When entering the duodenum, haptocorrin is attacked by pancreatic proteases, releasing B_{12} once again. With the rise in pH in the transition from the stomach to the duodenum, intrinsic factor acquires an increasing binding affinity for vitamin B_{12}. Absorption of vitamin B_{12} will then finally occur by receptor-mediated endocytosis in the terminal ileum, where the specific receptor cubulin complexes with intrinsic factor bound to B_{12} (5). As a consequence, B_{12} produced by colonic bacterial fermentations is most likely not reachable to the host. However, it has been suggested that B_{12} produced by a microorganism capable of colonizing proximal to the ileum would potentially be host-accessible (2).

Lactic acid bacteria

Lactic acid bacteria (LAB) are Gram-positive rods or cocci with a low G+C content in their chromosome. They share the common feature of producing lactic acid as the major end product of carbohydrate metabolism. Taxonomically, LAB comprise a relatively large clade of the *Bacilli*, one of the three classes within the Firmicutes phylum. More specifically, the genera that make up LAB belong to the order of the *Lactobacillales*, and include *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weisella*.

LAB inhabit several ecological niches. Some of their representatives are autochonous of the gastro-intestinal tract (GI-tract) of humans and other animals (108), but by large they are found in decomposing plant material. The phenotypic traits of acidifying the environment and producing antimicrobial agents inhibit the growth of spoilage agents,

leading to the establishment of a fortuitous symbiotic relationship with humans. Historically, humans have domesticated LAB for thousands of years by incorporating them in our food fermentation processes. This is supported by anthropological evidence, but also by the genetic adaptation of LAB to these new environments as evidenced by their genomic features (15, 107). This long history of safe use contributed greatly to their prominent role in the food industry, being responsible for the manufacturing of several fermented foods and beverages. Their economic, industrial and nutritional value is clearly demonstrated by the wide variety of applications. For instance:

- LAB are the main components of dairy starter cultures, transforming milk into products such as yogurt or cheese, and extending its shelf-life by lowering the pH due to lactic acid production;
- LAB contribute to the wellbeing of human mucosal surfaces throughout most of the digestive tract (108);
- LAB may also produce bacteriocins (anti-microbial peptides or proteins) that improve the durability of fermented foods (30, 100);
- LAB may contribute to the organoleptic profile of fermented foods. For example, with the production of compounds such as diacetyl, acetaldehyde, alanine, amongst others (27, 50, 64, 98);
- LAB can improve the texture and rheological properties of food by the synthesis of exopolysaccharides (14, 74);
- More recently, LAB have been studied to produce novel foods with improved nutritional value (nutraceuticals) (51).

With the burst of the –omics revolution much of the focus of LAB research has been channeled to genome-wide studies. Since the release of the first complete LAB genome in 2001 (16), more than 50 genomic sequences of LAB have been deduced, including 27 species (Table 1.3). Only *L. brevis*, *L. casei*, *L. salivarius* and *Leuconostoc mesenteroides* have not yet had at least on of their sequenced strains assembled into a single contig. The genera *Aerococcus*, *Carnobacterium*, *Tetragenococcus*, *Vagococcus*, and *Weisella* have not yet had any of their representatives sequenced. However, as this thesis is being written there are sequencing projects running for *Carnobacterium* at the Marine Microbial Genome Sequencing Project (https://research.venterinstitute.org/moore/).

The analysis of all these genomes produced a wealth of information without precedent (71). Ever more often, one can find in literature related to LAB, accounts of genome-wide analysis instead of the separate study of individual fragments of it (96). The full extent of the impact of the genome-sequencing boom in the LAB field is yet hard to

determine, but expected to be large. The comparative genomics studies published so far indicate that the occurrence of lateral gene transfer events is more common than previously suspected (58, 59). This is in part attributed to the large number of phages, plasmids and mobile elements typically found in LAB (110).

Table 1.3. Overview of genome sequencing of LAB available at ERGO database (70) in the spring of 2008[a.]

Family	Organism Name	Genome sequences available	Average Genome size (kb)	Average number of ORF	Average number of contigs
Enterococcaceae	*Enterococcus faecalis*	2	2,763	2,747	83
Lactobacillaceae	*Lactobacillus brevis*[b.]	1	2,340	2,218	3
	Lactobacillus casei[b.]	1	2,924	2,771	2
	Lactobacillus delbrueckii	2	1,860	1,642	1
	Lactobacillus fermentum	1	2,098	1,843	1
	Lactobacillus gasseri	1	1,894	1,755	1
	Lactobacillus helveticus	1	2,080	1,610	1
	Lactobacillus johnsonii	1	1,992	1,821	1
	Lactobacillus plantarum	1	3,308	3,053	1
	Lactobacillus reuteri	2	2,087	2,011	52
	Lactobacillus sakei	1	1,884	1,884	1
	Lactobacillus salivarius[b.]	1	2,133	2,017	4
	Pediococcus pentosaceus	1	1,832	1,755	1
	Lactobacillus acidophilus	1	1,993	1,864	1
Leuconostocacaea	*Leuconostoc mesenteroides*[b.]	1	2,075	2,005	2
	Oenococcus oeni	1	1,780	1,691	1
Streptococcaceae	*Lactococcus lactis*	3	2,497	2,454	3
	Streptococcus agalactiae	3	2,166	2,071	1
	Streptococcus equi	1	2,253	2,232	1
	Streptococcus gordonii	1	2,196	2,051	1
	Streptococcus mutans	1	2,030	1,945	1
	Streptococcus pneumoniae	5	2,123	2,059	41
	Streptococcus pyogenes	12	1,880	1,897	1
	Streptococcus suis	4	1,915	2,019	142
	Streptococcus thermophilus	2	1,830	1,816	2
	Streptococcus uberis	1	1,852	1,849	1

[a.] Source: http://ergo.integratedgenomics.com/ERGO

[b.] Species for which there is no closed genome sequence

Lactobacillus reuteri

Lactobacillus reuteri is widely spread in the GI-tract of humans and other mammals (112) and is a predominant microbe in several food and feed fermentations, namely in sourdoughs of different cereals such as rye and wheat (65). Although it has been highlighted for this heterofermentative lactic acid bacterium that human intervention studies showing relevant benefits remain to be reported (85), potential probiotic effects such as reducing the incidence and gravity of diarrhea of multiple origins in humans (117), triggering anti-inflamatory activity in human cell lines (56), and reducing cholesterolemia in mice (102), amongst others, have been described for several strains of *L.reuteri.*

L. reuteri has the ability of producing and excreting reuterin (100), a mixture of monomeric, hydrated monomeric and cyclic dimeric forms of 3-hydroxypropionaldehyde (3-HPA) (101). 3-HPA inhibits several yeasts, moulds and protozoa, along with Gram-positive and Gram-negative bacteria (23). It has been proposed that the resilience of *L. reuteri* in a complex environment, such as the GI-tract, is linked to the significant growth advantage that is conferred by the production of this broad spectrum antimicrobial (23). The production of 3-HPA is catalyzed by a B_{12}-dependent enzyme, glycerol dehydratase (EC 4.2.1.30) (28). 3-HPA can be used to recycle NAD^+ through its reduction to 1,3-propanediol, by 1,3-propanediol dehydrogenase (E.C. 1.1.1.202). Under the current economical, political and environmental conditions, the production of 1,3-propanediol is of great biotechnological interest since it is a starting material for the production of plastics such as polyesters, polyethers and polyurethanes (28).

Not long before the work described in this thesis was initiated, a compound in its cyano form was isolated from *L. reuteri* CRL1098, which was capable of fulfilling the auxotrophic vitamin B_{12} requirements of three different indicator strains (103). In the same study, *L. reuteri*'s chromosomal DNA was found to contain sequences homologous to one complete, and two partial genes involved in the anaerobic biosynthesis of coenzyme B_{12}. One encoding CobA/HemD, a 464 amino-acid bifunctional protein with S-adenosyl-L-methionine-uroporphyrinogen III methyltansferase and uroporphyrinogen III synthase activities from *Selemonas ruminantium* (3) and *Listeria* sps. (45). And the other two, predicted to encode fragments homologous to the anaerobic cobalt chelatase CbiK and precorrin-6-reductase (*cbiJ*) from *Salmonella* sps (75). This finding was truly remarkable since it was the first account ever of a lactic acid bacterium producing cobalamin. Even to date, with all the recent genome sequencing efforts, *L. reuteri* remains the only LAB in which the presence of B_{12} biosynthetic genes was found. *L. coryniformis* has also been reported to produce cobalamin (62), but the authors do not provide any genetic evidence of the presence of a B_{12} biosynthesis gene cluster. Furthermore, the levels reported to be produced by *L. coryniformis* are very low (0.7 ng/ml) in comparison to the ones reported

for *L. reuteri* using the same bioassay to quantify vitamin B_{12} (over 700-fold difference) (103).

The sequencing of two *L. reuteri* strains is at the moment on going at the Joint Genome Institute (JGI) and the draft genome sequences have been made available. The genome of the type strain of *L. reuteri* (JCM1112) is now circularized. This strain is a human isolate that cannot persist in the GI-tract of mice. In contrast, strain 100-23, whose genome has not been assembled past 103 contigs, is rodent-specific. The comparison of the genome sequences of these two strains is expected to help in the elucidation of unique host-microbe interactions.

L. reuteri JCM1112 will be shown in this thesis to produce B_{12}. The fact that the whole genome sequence is available enables the development of genome-wide analysis tools. These can be used to understand the impact of producing this co-factor on different metabolic pathways and information transfer levels (46). Additionally, they can generate leads for potential metabolic engineering targets to be used in the increase of B_{12} production (104).

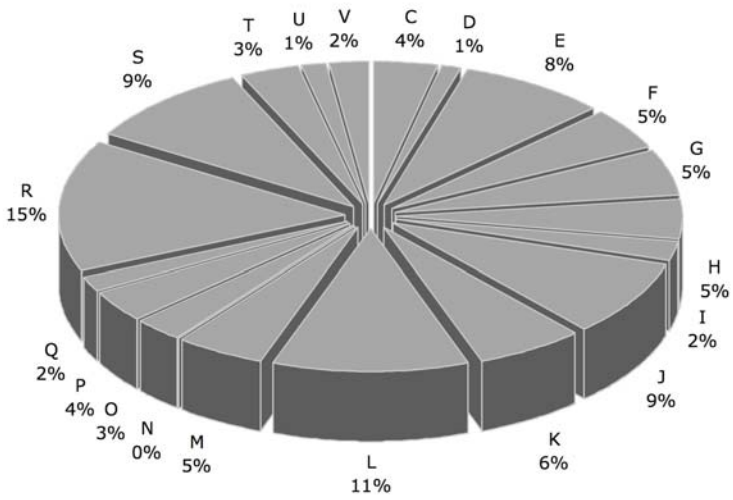

Figure 1.6. COG distribution of *Lactobacillus reuteri* JCM1112 predicted ORFs. C, energy production and conversion; D, cell division and chromosome partitioning; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H coenzyme metabolism; I, lipid metabolism; J, translation, ribosomal structure and biogenesis; K, transcription; L, DNA replication, recombination and repair; M, cell envelope biogenesis, outer membrane; N, cell motility and secretion; O, posttranslational modification, protein turnover, chaperones; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport and catabolism; R, general function prediction only; S, function unknown; T, signal transduction mechanisms; U, intracellular trafficking, secretion, and vesicular transport; V, defense mechanisms.

The draft genome sequence available at JGI evidences that *L. reuteri* JCM1112 has an average size LAB genome (2 Mbp). It has a G+C content of 38.8% and is predicted to harbor 1900 ORFs. The distribution of the predicted gene products of *L. reuteri* throughout the different categories of clusters of orthologous groups is present in Fig. 1.6. It is relevant here to highlight the elevated proportion of genes that are orthologuos to genes involved in amino acid transport and metabolism (E, 8%) and coenzyme biosynthesis (H, 5%) compared to *L. plantarum* (over two-fold increase) (53). *L. reuteri* has a genome approximately two thirds smaller than that of *L. plantarum*, and yet it contains more genes in absolute terms associated to categories E and H.

Modeling and –omics approaches

A genome sequence paves the way to the possibility of performing genome-scale analysis. It has been hypothesized that the transferring of sequence information between biopolymers (DNA, RNA, and proteins) obeys a set information flow scheme. This was enunciated by the Nobel Prize laureate Francis Crick shortly after the discovery of molecules encoding genetic information (24). Information encoded in DNA (genome) will be transferred to RNA (transcriptome), which will be transferred to proteins (proteome), which will subsequently determine metabolite concentrations (metabolome).

Omics technologies aim to measure these different information levels as exhaustively as possible. It is then the goal of systems biology to integrate these different levels of information to define a particular state of the biological system under examination. This is most often achieved by mathematical representations of the system with varying levels of complexity and detail. These mathematical representations are designated as models, and help challenge our understanding of a biological system. They enable to a certain extent the prediction of how the system will react to specific perturbations generating hypothesis that will then be challenged with experimentation.

Thesis overview

In this thesis, combining multiple scientific disciplines to target a central biological theme, we explore several aspects of B_{12} production in *L. reuteri*. We start by reporting on the chemical characterization of the corrinoid produced by *L. reuteri* (**Chapter 2**) and proceed to identify the gene cluster that encodes the machinery necessary for its synthesis (**Chapter 3**). Then the genome-scale metabolic network of *L. reuteri* is reconstructed and used to predict and interpret the transcriptional and physiological response to the presence of glycerol (**Chapter 4**). The leads acquired in this study are used to *(i)* characterize a regulon that includes vitamin B_{12} production, glycerol utilization and assembly of metabolosomes (**Chapter 5**), and *(ii)* increase vitamin B_{12} production in *L. reuteri* by changing the availability of amino acids in the medium (**Chapter 6**). The flexibility of *L. reuteri* for the combined production of vitamins is illustrated, and applied to the enrichment of substrates in vitamin content by fermentation (**Chapter 7**). We end by reviewing how our major findings fit with those of others, and discuss how they helped to shape our current understanding of vitamin B_{12}, particularly in relation to LAB (**Chapter 8**).

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CHAPTER 2

PSEUDOVITAMIN B_{12} IS THE CORRINOID PRODUCED BY *LACTOBACILLUS REUTERI* CRL1098 UNDER ANAEROBIC CONDITIONS

Abstract

Lactobacillus reuteri CRL1098 has been reported to produce a compound with vitamin B_{12} activity (Taranto *et al.*, Journal of Bacteriology, 185, 5643-5647). Here we report on the chemical characterization of this corrinoid-like molecule purified in its cyano form. High performance liquid chromatography coupled to an ultraviolet diode array detector, mass spectrometry and nuclear magnetic resonance spectroscopy has enabled us to identify the compound as Coα-[α-(7-adenyl)]-Coβ-cyanocobamide. Commonly known as pseudovitamin B_{12}, this molecule differs from cobalamin in the α-ligand, where it has adenine instead of 5,6-dimethylbenzimidazole bound in a α-glycosidic linkage to C-1 of ribose. *L. reuteri* is the first lactic acid bacterium in which the production of a cobalamin-like molecule has been identified and the first microorganism reported to produce exclusively pseudo-B_{12}.

In collaboration with José L. Vera, Pedro Lamosa, Graciela F. de Valdez, Willem M. de Vos, Helena Santos, Fernando Sesma and Jeroen Hugenholtz
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Introduction

Lactobacillus reuteri is a Gram-positive, heterofermentative lactic acid bacterium, frequently found in the gastrointestinal tracts of humans and other animals (14, 15). Relevant probiotic properties such as the lowering of blood cholesterol levels (21), and a direct anti-inflammatory activity (8, 9, 16) have been demonstrated for this microorganism. During growth on glucose and in the presence of glycerol, *L. reuteri* possesses the ability to produce and excrete reuterin (19) (Fig. 2.1). This broad spectrum antimicrobial is a mixture of monomeric, hydrated monomeric and cyclic dimeric forms of 3-hydroxypropionaldehyde (3-HPA) (20). The synthesis of reuterin is mediated by a B_{12}-dependent enzyme, glycerol dehydratase, which catalyses the conversion of glycerol to 3-HPA (3).

We have reported, previously, that a compound isolated in its cyano form from *L. reuteri* CRL1098 was capable of fulfilling the auxotrophic B_{12} requirements of three indicator strains (22). In the same study, DNA-sequences predicted to encode enzymes of the anaerobic B_{12} biosynthesis pathway were identified in the chromosome of *L. reuteri*.

A great variety of vitamin B_{12} analogues can be found in nature. They share a structural architecture consisting of a corrin ring with a cobalt ion chelated at the core. Cobalamin, the best studied corrinoid, is a cobamide in which 5,6 dimethylbenzimidazole (DMB) is the aglycon attached to the α-ligand bound in an α-glycosidic linkage from its N-1 to the C-1 of ribose. B_{12} biosynthesis is only found in a few prokaryotes (11). Some have been described to synthesize B_{12} analogues that contain bases in the α-ligand other than DMB, namely other benzimidazoles, purines and phenolic compounds (13).

Here we report on the chemical characterization of the corrinoid-like molecule isolated from *L. reuteri* in its cyanided form. Using high performance liquid chromatography (HPLC) coupled to an ultraviolet diode array detector (UV-DAD) followed by mass spectrometry and nuclear magnetic resonance spectroscopy, we have concluded that the corrinoid produced by *L. reuteri* CRL1098 cultured under anaerobic condition is pseudovitamin B_{12}. First reported in 1952 by Pfiffer et al. (4) this B_{12} analogue differs from cobalamin in the α-ligand, where DMB appears substituted by adenine. Because the majority of the structure is conserved, it has been suggested that this molecule could play a role in assessing the capability of B_{12}-dependent enzymes to utilize alternative cofactors (12), and in understanding the impact of B_{12} analogues in vitamin B_{12} metabolism (6, 7).

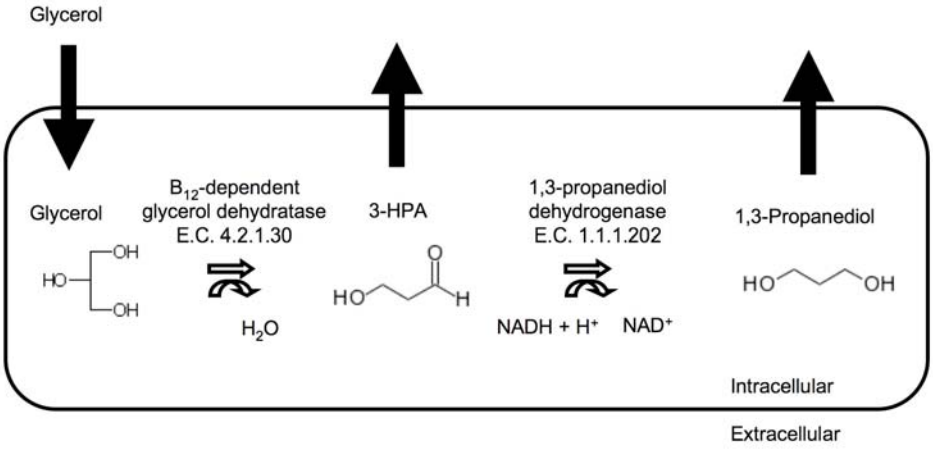

Figure 2.1. Schematic representation of glycerol metabolism in *L. reuteri*.

Materials and Methods

Preparation of cultures and cell-extracts. A culture of *L. reuteri* CRL1098 was inoculated in vitamin B_{12}-free assay medium (Difco), grown at 37°C for 16 h and transferred three times. Two different growth conditions, with and without adding DMB (100 mg/l), were used in these studies. Cell-extracts were prepared from a 10 L batch culture flushed with a mixture of 95% N_2 and 5% CO_2, containing approximately 50 μg/L of corrinoid. After harvesting, cells were washed twice in 0.1 M phosphate buffer, pH 7.0, resuspended in 10 ml of extraction buffer consisting of 0.1 M Na_2HPO_4, pH 4.5 (citric acid) and containing 0.005% KCN. The cell suspension was separated in 10 aliquots of 1 ml each, disrupted with 1 g of glass beads (0.1 mm diameter) in a FastPrep FP120 (Qbiogene, Carlsbad, Calif.) and again combined. Extraction buffer was then added up to a final volume of 20 ml and autoclaved (120°C for 15 min). The mixture was cleared by centrifugation (8,000 x g for 10 min), and the supernatant was passed over an Isolute solid-phase extraction (SPE) column (500 mg C18 end-capped column with a 3-ml reservoir volume) previously activated with 2 ml of acetonitrile. The column was washed twice with 2 volumes of distilled water to remove salts and other hydrophilic contaminants. Subsequently, the corrinoid was eluted with 1 volume of 50% acetonitrile and concentrated to dryness in vacuo at 30°C. The residue was dissolved in 5 ml of sterile distilled water and stored in the dark at -20°C until further use.

Corrinoid isolation. The corrinoid was purified from cell-extracts of *L. reuteri* by reverse-phase high performance liquid chromatography (RP-HPLC) with a Waters

(Milford, Mass.) 600E system automated gradient controller, a 250- by 3-mm Betasil phenyl column (Thermo Hypersil-Keystone, Waltham, Mass.), and a SPD-M10A VP Diode Array Detector (Shimadzu Corporation, Kyoto, Japan). The fraction showing a UV-DAD spectrum similar to that of cyanocobalamin was collected manually and lyophilised at 30°C.

Mass spectroscopy. The sample collected from the RP-HPLC was dissolved in 100 µl of an aqueous solution containing 30% acetonitrile and 0.1% formic acid. Mass spectrometric analyses of the corrinoid purified from *L. reuteri* were performed on a Quatro II triple-quadrupole mass spectrometer (Micromass, Inc., Altrincham, UK) equipped with an electrospray ionization (ESI) probe, operated in the positive ion mode. Experiments were carried out under the following conditions: capillary voltage 4.2 kV, cone voltage 36 V, extraction voltage 5V, source temperature 80°C, desolvation temperature 120°C.

Nuclear Magnetic Resonance spectroscopy. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were acquired on a Bruker DRX500 spectrometer (Bruker, Rheinstetten, Germany) usings a 5 mm inverse-detection probe head. The sample purified from the RP-HPLC was lyophilised at 30°C and dissolved in 10% D_2O, which allows the detection of amide resonances. Spectra were recorded at 303 K with pre-saturation of the water signal, using an 8.25 s pulse width corresponding to a 75^o flip angle and a repetition delay of 3 s.

Results

During purification, UV-DAD spectra data and retention times obtained by RP-HPLC analyses showed that *L. reuteri* CRL1098 grown under anaerobic conditions produces one major cobalamin-like molecule, in a ratio of ≥100:1 in relation to other minor corrinoid species. The identification and characterization of the major cobamide produced by this strain was based on the application of three different techniques: RP-HPLC isolation coupled to UV-DAD spectrum determination, mass spectrometry analysis and NMR spectrometry studies.

Corrinoid isolation. To isolate and purify the native corrinoid produced by *L. reuteri*, cell-extracts were eluted on a C18-SPE column followed by RP-HPLC. The cell-extracts from *L. reuteri* revealed a peak with a retention time of 36.31 min (Fig. 2.2A). Although the peak displayed similar UV-DAD spectra, this retention time did not agree with the one of the cyanocobalamin standard (37.83 min). The spectrum of the standard showed a peak of maximal absorbance at 350 nm and two other at 512 and 550 nm, respectively. The same pattern was observed when the isolated cobamide produced by *L. reuteri* was analyzed (Fig. 2.2B). Identical chromatography patterns and UV-spectra were recorded for the corrinoids isolated from *L. reuteri* CRL1098 grown in the presence or absence of DMB.

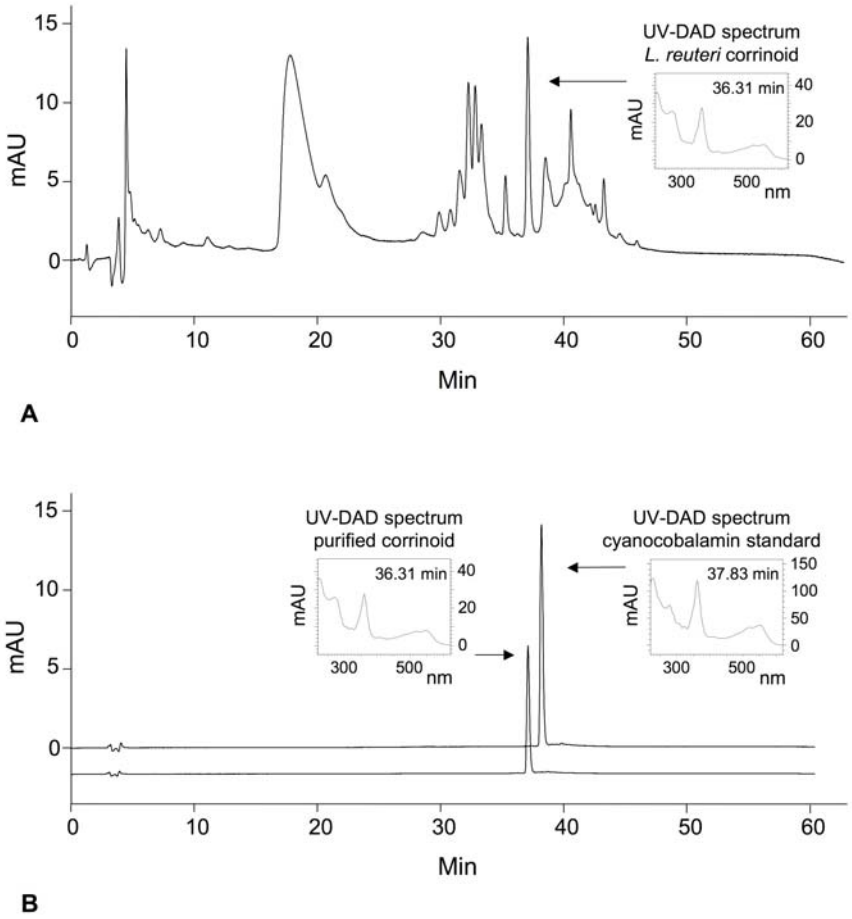

Figure 2.2A. HPLC chromatogram of the cell-extract and UV-DAD spectrum (inset) of the corrinoid produced by *L. reuteri* under anaerobic conditions in the presence of DMB. The same pattern was observed in the absence of DMB.

Figure 2.2B. HPLC chromatograms and UV-DAD spectra (inset) of the purified corrinoid produced from *L. reuteri* CRL1098 and the cyanocobalamin standard. Although their retention time does not coincide, both the spectra of the corrinoid purified from *L. reuteri* and the cyanocobalamin standard, display a peak of maximal absorbance at 350 nm and two other at 512 and 550 nm, respectively.

Mass spectroscopy. The mass spectrometry data (Fig. 2.3) obtained for the corrinoid produced by *L. reuteri* provided evidence that the complete molecule has a very similar m/z value to the one of methylcobalamin, 1344.6 and 1344.8 respectively. In both the mass spectra of the isolated compound and of the standard, the peaks corresponding to the displacement of both the β-ligand along with the ribosyl-bound aglycon (m/z 1183.6) and cobinamide (m/z 971.6) were identified. The presence of such peaks in the mass spectrum of our sample is highly suggestive that it is indeed a B_{12} analogue.

However, when all signals displayed on the mass spectra were considered in detail, the fractions in which only the upper ligand was not present, revealed a mass variation of 15.1 for the methylcobalamin standard (m/z 1329.7) and 26.0 for the isolated compound (m/z 1318.6), corresponding to the displacement of a methyl and a cyano radical, respectively. This implied that the α-ligand of methylcobalamin and the isolated compound differed approximately 11 mass units.

A peak with an m/z value of 359.3 was identified in the methylcobalamin standard, but not in the corrinoid purified from *L. reuteri*. This peak is derived from the lower ligand in which DMB is the aglycon attached by a glycosyl bound from its N-1 to the C-1 of ribofuranose 3-phosphate. Instead, another peak is present in the mass spectrum of the corrinoid purified from *L. reuteri* with an m/z value of 348.2, once again implying a mass variation of approximately 11 in the α-ligand. Furthermore, the peaks with an m/z value of 456.9 and 486.5, corresponding to different fractionation in which the corrin ring and the upper ligand are displaced (Fig. 2.4A), could be identified in the methylcobalamin standard but not in the corrinoid purified from *L. reuteri*. Hence, the possibility that the compound produced by *L. reuteri* had DMB in the lower ligand was ruled out.

The mass spectrum recorded for the isolated compound showed a peak corresponding to the sole displacement of the cyano radical (m/z 1318.6), which was in accordance with the substitution in the lower ligand of the DMB moiety by adenine. Also the observed peak with an m/z value of 348.2 corroborated this substitution, and thus, explained the difference of 11 mass units mentioned above. This observation implied that the corrinoid extracted from *L. reuteri* was actually Coα-[α-(7-adenyl)]-Coβ-cyanocobamide, commonly known as pseudo-B_{12}. Furthermore, the observed peaks in the spectrum with m/z values of 433.3 and 457.1 were consistent with this proposed structure and represent different fragmenting of the lower ligand containing adenine instead of DMB (Fig. 2.4B).

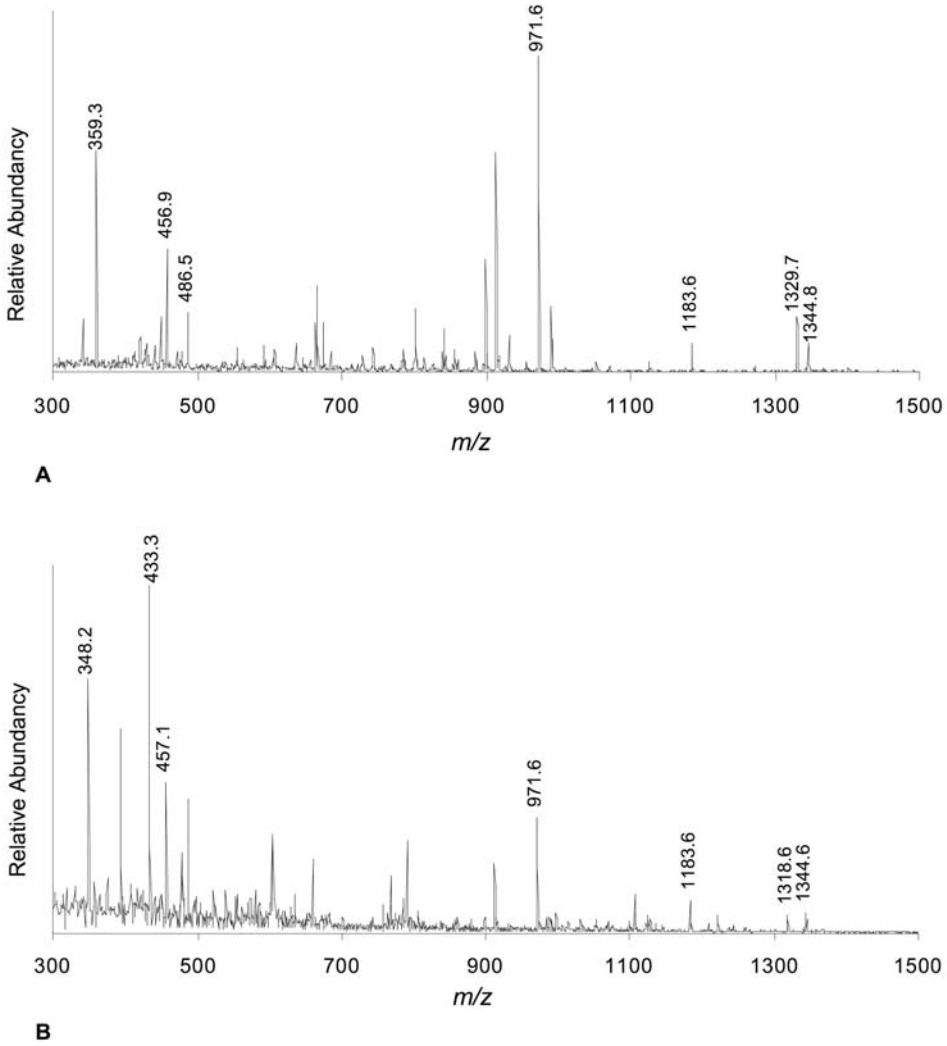

Figure 2.3A. Mass spectrometry spectrum of methylcobalamin. The peak with an m/z value of 1344.8 corresponds to the intact molecule; 1329.7 corresponds to the displacement of the upper ligand, the methyl radical; 1183.6 corresponds to the displacement of both the upper ligand and DMB; 971.6 corresponds to cobinamide; 359.3 corresponds to the lower ligand in which DMB is the aglycon attached to ribofuranose 3-phosphate. The peaks with an m/z value of 486.5 and 456.9 correspond to different fractioning in which both the corrin ring and the upper ligand have been displaced (see Fig. 2.4A).

Figure 2.3B. Mass spectrometry spectrum of the corrinoid purified from *Lactobacillus reuteri*. The peak with an m/z value of 1344.6 corresponds to the intact molecule; 1318.6 corresponds to the displacement of the upper ligand, a cyano radical; 1183.6 corresponds to the displacement of both the upper ligand and adenine; 971.6 corresponds to cobinamide; 348.2 corresponds to the lower ligand in which adenine is the aglycon attached to ribofuranose 3-phosphate. The peaks with an m/z value of 457.1 and 433.3 correspond to different fractioning in which both the corrin ring and the upper ligand have been displaced (see Fig. 2.4B).

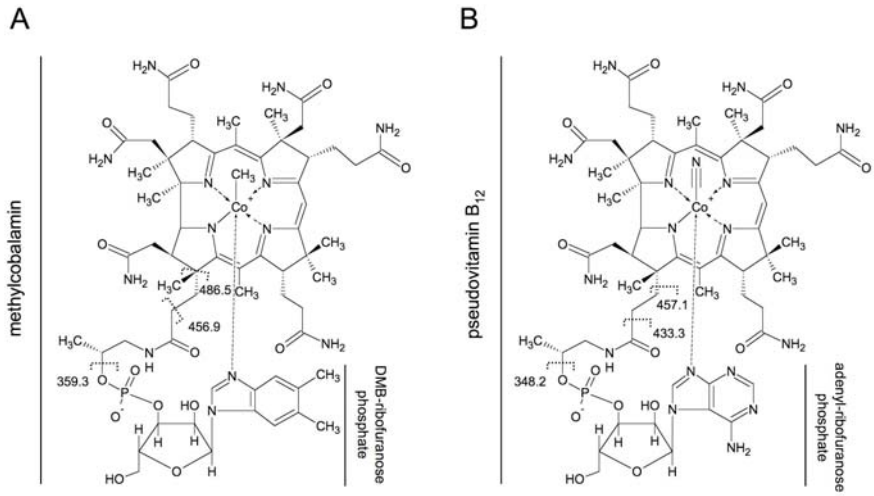

Figure 2.4. Chemical structure of (A) methylcobalamin and (B) pseudovitamin B_{12}: likely structures and measured m/z values of different fractions of the lower ligand.

Nuclear Magnetic Resonance spectroscopy. Additionally, a ^{1}H-NMR spectrum taken of the corrinoid purified from *L. reuteri* (Fig. 2.5) showed striking similarities with published NMR spectra of pseudovitamin B_{12}. In fact, a comparison between the acquired signals and those obtained for pseudovitamin B_{12} by Hoffman et al. (5) showed only six signals that were not present in the published spectrum. However, these signals are also not present in the spectra of other corrinoids, and therefore, we have assumed they are caused either by minor contaminants (peaks marked 2, 4 and 5 in Fig. 2.5), or by substances used during sample preparation (peaks marked 1, 3 and 6 in Fig. 2.5). Furthermore, the presence in the ^{1}H-NMR spectrum of a characteristic sharp signal around 8 ppm is indicative of a highly unshielded aromatic proton in its structure, which, by itself, rules out the identification of the corrinoid as cobalamin or factor A, both molecules lacking such a proton. Pseudovitamin B_{12}, on the other hand, has such a proton in its structure.

A ^{1}H homonuclear correlation spectrum was recorded and showed no visible correlations to the three signals at lower-field (8-9 ppm) (data not shown). This is in agreement with the proposed structure for the α-ligand of the native corrinoid of *L. reuteri*, and dismisses the possibility of the presence of [N8-methyl]-adeninyl-cobamide.

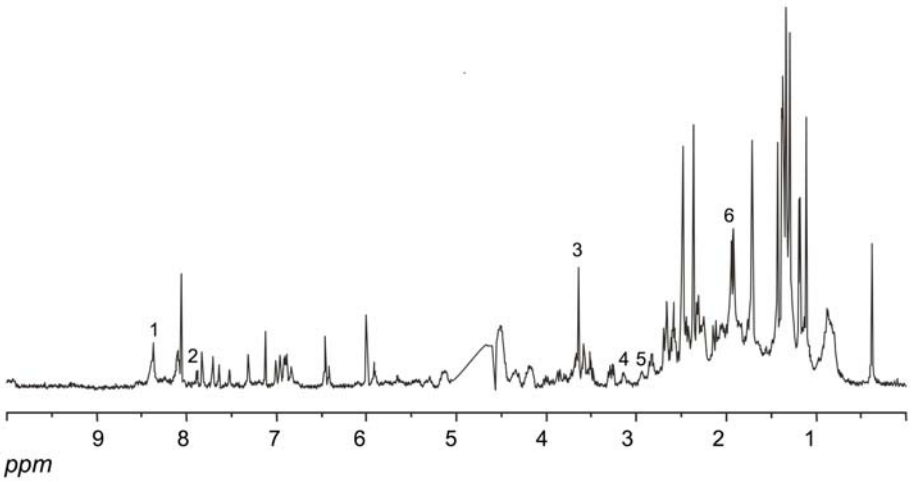

Figure 2.5. Proton nuclear magnetic resonance spectrum of the corrinoid purified from *L. reuteri*. ^{1}H-NMR spectrum was acquired on a Bruker DRX500 spectrometer using a 5 mm inverse-detection probe head and recorded with presaturation of the water signal, using an 8.25 s pulse width corresponding to a 75^{o} flip angle and a repetition delay of 3 s. The sample was dissolved in 10% D_2O. Peaks marked 1 through 6 have been assigned to contaminants.

Discussion

The corrinoid extracted by *L. reuteri* under anaerobic conditions has been identified as Coα-[α-(7-adenyl)]-Coβ-cyanocobamide, commonly known as pseudovitamin B_{12}. The results obtained performing mass spectrometry and nuclear magnetic resonance spectroscopy enabled us to identify the corrin ring with an atom of cobalt at the core, a cyano radical as the β-ligand, and adenine as the aglycon bound by a glycosyl link from its N-1 to the C-1 of the ribose forming the α-ligand.

During the purification of the corrinoid produced by *L. reuteri*, the difference in retention time between our sample and the cyanocobalamin standard was suggested to be due to a difference in the phosphorylation state of the molecule. This was supported by a previous report on the *in vitro* synthesis of the cobalamin nucleotide loop (10), in which, the compound was identified as cyanocobalamin-5'-phosphate using mass spectrometry. Based on this, we collected the purified molecule with the same UV-DAD spectrum as cyanocobalamin and performed mass spectrometry analysis for molecular characterisation. Considering all the cobalamin analogues known (13), pseudovitamin B_{12} was the only one with full spectral consistency based on the mass spectra collected. This analogue has a structure similar to that of cobalamin except for the lower ligand, in which, the DMB moiety is substituted by adenine. The mass spectrometry results reported here enabled the

identification of several peaks corresponding to the fractioning of the lower ligand containing an adenine base (Fig. 2.3B). Additional NMR studies were performed to confirm the identity of the corrinoid. The conjunction of all the spectral information acquired makes it clear that the corrinoid extracted from *L. reuteri* was indeed pseudo-B_{12}.

Recently, the gene *bluB* has been shown to be necessary for the aerobic biosynthesis of DMB in *Sinorhizobium meliloti* (18). It is involved in the fragmentation and contraction of flavin mononucleotide to form D-erythrose 4-phosphate and DMB in the presence of oxygen. The recently released genome sequence of *L. reuteri* JCM1112 (DOE Joint Genome Institute, GeneBank Accession nr. CP000705) lacks a homologue of *bluB* and the growth of this lactic acid bacterium is impaired in the presence of oxygen. As a consequence, we consider the presence of such pathway in *L. reuteri* to be unlikely. *Salmonella enterica* has been reported to be able to incorporate exogenous DMB into the lower ligand producing cobalamin (2). We could not detect a similar behaviour for *L. reuteri* CRL1098, which is not completely unexpected. Both phenotypically and phylogenetically, these two species are quite different, and thus, it is not surprising that they have different uptake capacities.

Some genera of cyanobacteria have been reported to contain pseudo-B_{12} amongst other corrinoids (23, 24). The same applies to *Clostridium cochlearium* (5), another Gram-positive bacterium with a low G+C content in its DNA. Interestingly, *L. reuteri* is the first microorganism reported to biosynthesize pseudovitamin B_{12} as its sole corrinoid product. Pseudo-B_{12} is not commercially available and until now its supply has solely relied on guided biosynthesis or chemical synthesis (1, 17, 25). Vitamin B_{12} and several of its analogues act as cofactors, catalyzing methyl-transfer and carbon-backbone rearrangement reactions. The comparison of the cofactor efficacy of the different analogues enables the elucidation of complex enzyme-cofactor interactions. Future studies of the capability of B_{12}-dependent enzymes to use pseudo-B_{12} as a cofactor, may rely on *L. reuteri* for its provision. Ultimately, this will help in the effort to elucidate the relevance of the lower ligand and Co-N coordination for enzymatic activity.

In humans, *in vitro* studies on B_{12} binding proteins have shown that only intrinsic factor can present a real bottleneck for the uptake of pseudo-B_{12} (17). However, comparative *in vivo* studies establishing the efficacy of B_{12} analogues with α-ligands other than DMB to correct B_{12} deficiency remain to be carried out. *L. reuteri* is the first lactic acid bacterium reported to produce a cobalamin-like molecule. Besides the system of producing pseudovitamin B_{12} characterised here, the possibility to engineer the production of other corrinoids is currently under investigation. *L. reuteri* possesses the GRAS status (Generally Regarded As Safe), and therefore, could eventually be used to increase the nutritional value of fermented food and feed products.

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CHAPTER 3

THE COMPLETE COENZYME B_{12} BIOSYNTHESIS GENE CLUSTER OF *LACTOBACILLUS REUTERI*

Abstract

The coenzyme B_{12} production pathway in *Lactobacillus reuteri* has been deduced using a combination of genetic, biochemical and bioinformatics approaches. The coenzyme B_{12} gene cluster of *L. reuteri* CRL1098 has the unique feature of clustering together the *cbi*, *cob* and *hem* genes. It consists of 29 open reading frames encoding the complete enzymatic machinery necessary for *de novo* biosynthesis. Transcriptional analysis showed it to be expressed as two tandem transcripts of approximately 22 and 4 kb, carrying *cobD, cbiABCDETFGHJ*, *cobA/hemD, cbiKLMNQOP*, *sirA*, *hemACBL*, and *cobUSC*, *hemD, cobT*, respectively. Both transcripts appear to be similarly regulated, and under the conditions assayed, are induced in the late-exponential growth phase. Evidence for a regulatory mechanism of negative feedback inhibition by vitamin B_{12} itself was observed. Comparative genomics analysis of the coding sequences showed them to be most similar to those coding for the anaerobic coenzyme B_{12} pathways previously characterised in a few representatives of the genera *Listeria* and *Salmonella*. This contrasts with the trusted species phylogeny and suggests horizontal gene transfer of the B_{12} biosynthesis genes. G+C content and codon adaptation index analysis is suggestive that the postulated transfer of these genes was not a recent event. Additional comparative genomics and transcriptional analysis of the sequences acquired during this study suggests a functional link between coenzyme B_{12} biosynthesis and reuterin production, which might be implicated in *L. reuteri*'s success colonizing the gastrointestinal tract. This information on gene organisation, gene transcription and gene acquisition is relevant for the development of (fermented) foods and probiotics enriched in vitamin B_{12}.

In collaboration with Jose L. Vera, René van der Heijden, Graciela Valdez, Willem M. de Vos, Fernando Sesma and Jeroen Hugenholtz
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Supplementary materials are also available with the online version of this paper at http://mic.sgmjournals.org/cgi/content/full/154/1/81/DC1

Introduction

Lactobacillus reuteri is a Gram-positive, heterofermentative, lactic acid bacterium, widely spread throughout the gastrointestinal tract (GI tract) of humans and other animals (46). Although presently marketed as a probiotic, human intervention studies showing relevant benefits remain to be reported (32). Nonetheless, potential probiotic effects have been demonstrated and include lowering blood cholesterol levels in mice (40), and stimulating anti-inflammatory activity in human cell lines (19).

L. reuteri possesses the unique ability of producing and excreting reuterin (38). This broad spectrum antimicrobial compound is a mixture of monomeric, hydrated monomeric and cyclic dimeric forms of 3-hydroxypropionaldehyde (3-HPA) (39). The synthesis of reuterin is mediated by glycerol dehydratase (EC 4.2.1.30), a B_{12}-dependent enzyme, which is involved in catalysing the conversion of glycerol to 3-HPA (12).

We have reported the isolation of a compound from *L. reuteri* CRL1098, capable of fulfilling the auxotrophic vitamin B_{12} requirements of three different indicator strains (41). In the same study, *L. reuteri* genomic DNA was found to contain sequences homologous to genes involved in the anaerobic coenzyme B_{12} biosynthesis pathway, including, *cysG*/*hemD* from *Selemonas ruminantium* (4), *Listeria innocua* and *Listeria monocytogenes* (15), and *cbiK* and *cbiJ* from *Salmonella typhimurium* (25).

Vitamin B_{12} consists of a tetrapyrrolic-derived corrin ring with a cobalt ion chelated at the core. Along with chlorophyll, coenzyme F_{430} and heme, amongst others, it constitutes one of the most structurally complex classes of cofactors. Various B_{12} derivatives with different upper axial ligands act as essential cofactors in many important enzymatic reactions responsible for the catalysis of methyl transfers and carbon backbone rearrangements (20). Coenzyme B_{12} biosynthesis is limited to a few representatives of bacteria and archaea (22). It appears that B_{12}-dependent enzymes are absent from plants and fungi, but widely spread in prokaryotes, protists and animals (11, 27).

In humans, vitamin B_{12} deficiency has been demonstrated to lead to pernicious anemia, and neurological dysfunction, amongst other complications (35). Three proteins are known to participate in the uptake and transport of vitamin B_{12}, namely haptocorrin, intrinsic factor and transcobalamin II. Absorption of vitamin B_{12} occurs by receptor-mediated endocytosis in the terminal ileum, where the specific receptor cubulin complexes with intrinsic factor bound to B_{12} (6). As a consequence, B_{12} produced by colonic bacteria is most likely inaccessible to the host. However, it has been suggested that B_{12} produced by a microorganism capable of colonising proximal to the ileum, such as *L. reuteri*, would potentially be host-accessible (2).

L. reuteri was the first lactic acid bacterium reported to possess the capability of producing B_{12} (41). Increasing our understanding on how this GRAS (Generally Regarded

as Safe) organism encodes, acquired and maintains a biosynthetic pathway of such complexity and magnitude is of great importance to the medical field and for the food and feed industries.

In this study, we extend the analysis of the presumed coenzyme B_{12} biosynthesis gene cluster of *L. reuteri* and describe the presence of a complete gene cluster encoding all the enzymatic machinery necessary for the *de novo* synthesis of this important cofactor. Additional comparative genomics and transcriptional analysis of the new sequences acquired during this study suggests a functional link between coenzyme B_{12} biosynthesis and reuterin production, which might be implicated in *L. reuteri*'s success colonizing the GI tract.

Materials and Methods

Strains, media and culture conditions. *L. reuteri* CRL1098, isolated from sourdough, was obtained from the CERELA stock culture collection. It was cultivated at 37ºC in MRS medium and in vitamin B_{12} assay medium (Sigma, Zwijndrecht, Netherlands) supplemented when mentioned with 1 mg/L of cyanocobalamin (Sigma-Aldrich, Zwijndrecht, Netherlands). The *Escherichia coli* strain XL-1 Blue MRA (P2) was obtained from Stratagene (La Jolla, USA) and cultivated at 37ºC under aerobic conditions in TY medium. *Salmonella enterica* serovar Typhimurium LT2 derivative strains TT25720 (*metE2119*::MudJ), and TT25722 [*metE2119*::MudJ, *cobS2621*::Frt(sw)] (5) were kindly provided by Prof. John R. Roth, and cultivated at 37ºC in TY, or in minimal E medium (21), supplemented with 100 nM cyanocobalamin when required.

Nucleotide sequence analysis. Sequence of the B_{12} biosynthesis gene cluster of *L. reuteri* was carried out by screening two genomic λ-phage libraries, and finalized by both inverted PCR and genomic primer walking. Total genomic DNA was isolated from *L. reuteri* according to standard molecular biology techniques (31).

A Southern blot analysis of a partial digestion of *L. reuteri*'s chromosomal DNA with the restriction enzymes *Bam*HI and *Bgl*II, using a *cysG*/*hemD* (41) probe amplified from the same strain, showed that the signals obtained correspond to DNA fragments larger than 15 kb for both restriction enzymes (data not shown). Based on this knowledge, two *L. reuteri* genomic λ-phage libraries were constructed by the separate ligation of *Bgl*II and *Bam*HI digested *L. reuteri* genomic DNA into Lambda-DASH II/BamHI vector and packaged with a Gigapack III Gold packaging extract (Stratagene, La Jolla, USA) according to the manufacturers recommendations. For the amplification of the Lambda-DASH II/BamHI libraries a lysogenic P2 strain, *E.coli* XL-1 Blue MRA (P2) was used. Titer determination of bacteriophages, blotting of plaques on nylon membranes, and λ-DNA isolation were all performed according to the manufacturer's recommendations.

Probes purified through the JETPURE PCR Product Purification Kit (GENOMED, Bad Oeynhausen, Germany), were amplified, radioactively labelled with α^{32}P-ATP (GE Healthcare Europe Gmbh, Belgium), and hybridized on membranes according to standard procedures (31). Membranes were exposed to BioMax MS or BioMax MR X-ray film manufactured by Kodak for at least 5 hours at -80ºC before developing. Sequencing of two ~15 kb non-overlapping inserts containing B_{12}-related DNA was carried out at Greenomics (Wageningen, The Netherlands).

For gap-closure between the two inserts, we resorted to inverted PCR. According to procedures previously described (31), *Hind*III digested genomic DNA of *L. reuteri* was ligated to pNZ8048 (37) digested with the same endonuclease. The ligation mixture was directly used as a template on a PCR reaction using a primer designed on the vector and another based on the 5' flanking region of the known sequence at the time. The resulting amplicon was isolated from an agarose gel and sequenced directly at Baseclear, The Netherlands.

Further sequencing efforts aimed at closing gaps and extending the flanking regions of the known sequence was done through genomic primer walking carried out at GATC Biotech, Germany.

The new sequence information obtained using the three different approaches described above, was analyzed and assembled resorting to in-house scripts, and online programs available from the Biology WorkBench of the San Diego Supercomputer Centre (http://workbench.sdsc.edu/). Standard RNA regulatory motif searches were performed in Rfam (16) and using Riboswitch finder (8). Predicted open reading frames were manually annotated based on homology searches using the Blast algorithm (3). Finally, all sequence information was deposited at GenBank database under accession n. º AY780645.

Complementation studies. A fragment containing *cobS* was amplified from *L. reuteri*'s genomic DNA using Herculase II DNA polymerase (Stratagene, La Jolla, USA), and primers LREf28196_28215 and LREr29724_29704 (Table 3.1). Additionally, a fragment containing the native *cob* operon promoter (23) was amplified from *Salmonella enterica* strain TT25720 using primers 5'-GACACCATTGTGGATGAGGTGGAG-3' and 5'-GATGATCGATCATACCGGCTCCTGATGT-3' (*Cla*I cleavage site underlined). 3'-A overhangs were added to both fragments by incubating the PCR reactions directly with 1 unit of *Taq* DNA polymerase for 3 min at 72ºC. The A-tailed fragments were then purified with the JETPURE PCR Product Purification Kit (GENOMED, Bad Oeynhausen, Germany) and digested with *Cla*I. The modified fragments were again purified with the same method and simultaneously cloned in pGEM-T Easy Vector (Promega Benelux BV, Leiden, The Netherlands), resulting in pNZ7749. The *Salmonella enterica* strain TT25722

was transformed with this vector as previously described (31), and its phenotype was characterized in minimal E medium (21).

Table 3.1. Oligonucleotide primers used in RT-PCR reactions

Primer	Nucleotide sequence (5' – 3')	Strand*	Location
LREf5899_5921	GCACCGTCGCAACAATATCCCAC	+	IS
LREf6317_6340	CGTTTTCTTTGATTTTAGTAGGTG	+	*cobD*
LREr7366_7343	CTGCCACTCGATAGTATTGTCGGC	-	*cobD*
LREf7343_7366	GCCGACAATACTATCGAGTGGCAG	+	*cobD*
LREr8862_8843	CACGAATGAGGGTCACCAAG	-	*cbiB*
LREf8843_8862	CTTGGTGACCCTCATTCGTG	+	*cbiB*
LREr10437_10416	GGTGTGACGGCCATACTCATCA	-	*cbiC* and *cbiD*
LREf10416_10437	TGATGAGTATGGCCGTCACACC	+	*cbiC* and *cbiD*
LREr11947_11927	GTTCGCCCATGACTACTTGTC	-	*cbiE*
LREf11927_11947	GACAAGTAGTCATGGGCGAAC	+	*cbiE*
LREr13430_13411	CACCTAAGAACTTACCAACC	-	*cbiF*
LREf13411_13430	GGTTGGTAAGTTCTTAGGTG	+	*cbiF*
LREr14920_14900	GAGCAGCAGCTGCAATACTTG	-	*cbiH*
LREf14900_14920	CAAGTATTGCAGCTGCTGCTC	+	*cbiH*
LREr16411_16391	CTAGCCCAGCAATTGCACTAG	-	*cysG/hemD*
LREf16391_16411	CTAGTGCAATTGCTGGGCTAG	+	*cysG/hemD*
LREr17886_17864	GTAAAAGCACTATGCGCTGTTCC	-	*cbiK*
LREf17864_17886	GGAACAGCGCATAGTGCTTTTAC	+	*cbiK*
LREr19409_19388	CGACTAACTTTCATTGCTCGAC	-	*cbiM*
LREf19388_19409	GTCGAGCAATGAAAGTTAGTCG	+	*cbiM*
LREr20906_20887	CGCCGTAAATTTCGAAGTCC	-	*cbiO*
LREf20887_20906	GGACTTCGAAATTTACGGCG	+	*cbiO*
LREr22471_22451	GCTACTAAATCTGCGTTCGTG	-	*cbiP*
LREf22451_22471	CACGAACGCAGATTTAGTAGC	+	*cbiP*
LREr24150_24130	CCTTGCTAAAGCCCATATTGC	-	*hemA*
LREf24130_24150	GCAATATGGGCTTTAGCAAGG	+	*hemA*
LREr25669_25648	CCTTAGCCAATAACTGATCAGC	-	*hemC*
LREf25648_25669	GCTGATCAGTTATTGGCTAAGG	+	*hemC*
LREr26711_26683	GGTGGGTTTGTTTTGAGTAAATTAGATAC	-	intergenic region
LREf26684_26718	GTGGGTTTGTTTTGAGTAAATTAGATACAACAAAG	+	intergenic region
LREr27526_27499	CTGGGAGTCCACCACCGATTACTTTGCC	-	*hemL*
LREf27499_27518	GGCAAAGTAATCGGTGGTGG	+	*hemL*
LREr27987_27967	CTTGGTTGCCGCATTAAATGC	-	*hemL*

Primer	Nucleotide sequence (5' – 3')	Strand*	Location
LREr28269_28250†	GTTCATCGACGTGCTGATAC	-	*cobU*
LREf28250_28269	GTATCAGCACGTCGATGAAC	+	*cobU*
LREr29724_29704‡	GGTATAGGTTAATGGAGCTGC	-	*cobC*
LREf29704_29724	GCAGCTCCATTAACCTATACC	+	*cobC*
LREr31148_31129	CTGCTATCGACATTGCTGGT	-	*cobT*
LREf31129_31148	ACCAGCAATGTCGATAGCAG	+	*cobT*
LREr31756_31737	GAAGTCCATCTCCTGCAATG	-	*cobT*
LREr32212_32193	CCTTGTGGCAACAGTCTTCT	-	hypothetical
LREf32193_32212	AGAAGACTGTTGCCACAAGG	+	hypothetical
LREf33354_33373	GGAATTCGCAACTCACGAAG	+	*rpsO*
LREr33545_33526	GCAGGTAAATCAGTCCGACG	-	*rpsO*
LREr33809_33790	TCGCGTACACCACCAAAAGG	-	metallo lactamase
LREf33790_33809	CCTTTTGGTGGTGTACGCGA	+	metallo lactamase
LREr35516_35496	CCACGACCACGATGATGTTCT	-	metallo lactamase
LREf35496_35516	AGAACATCATCGTGGTCGTGG	+	metallo lactamase
LREr36538_36518	CGCAATCAAAGCAGTTGAACG	-	hypothetical

* Primer were designed to the coding strand (+) or non-coding strand (-)
† Primer also used in Q-RT-PCR experiment
‡ Primer also used in complementation studies

Transcriptional analysis. The transcriptional organization of the vitamin B_{12} gene cluster of *L. reuteri* was determined by Northern blots, reverse transcriptase PCR (RT-PCR) and quantitative reverse transcriptase PCR (Q-RT-PCR). Cells were cultured in batch fermentations of MRS medium, vitamin B_{12} assay medium (commercial rich broth lacking B_{12}) and vitamin B_{12} assay medium supplemented with vitamin B_{12} to a final concentration of 1 mg/L. RNA was isolated according to standard procedures (31) from samples collected at different time points, namely, mid-log, late-log and stationary phases. The integrity and concentration of the RNA was analysed with a 2100 Bioanalyzer manufactured by Agilent Technologies (Amstelveen, The Netherlands). Northern blotting of RNA obtained from late-log cells cultivated in MRS was performed as previously described (17, 29). Probes were amplified from genomic DNA of *L. reuteri* by PCR using primer pairs designed to locate them throughout the cluster, namely on *cbiC*, *cbiP*, and *cobT*. Subsequent hybridization with radiolabelled probes was carried out according to standard molecular biology techniques described earlier (31). RT-PCR analysis of samples obtained from cells cultured in MRS was performed by systematically amplifying overlapping fragments

throughout the full extension of the B_{12} biosynthesis cluster and flanking regions. All RNA samples were diluted to the same concentration and an extra DNAse I (Invitrogen, Breda, The Netherlands) treatment was implemented to eliminate possible remaining chromosomal DNA contamination. First strand cDNA synthesis was carried out using Superscript III reverse transcriptase from Invitrogen according to the manufacture's recommendations. Primers were manually designed and are listed on Table 3.1. To quantify the differential expression of the two operons within the B_{12} biosynthesis gene cluster between late- and mid-log phases and in the presence or absence of B_{12}, we performed Q-RT-PCR. Amplification was carried out in 96-well plates in an ABI Prism 7700 from Applied Biosystems using the fluorescent agent SYBR Green for detection. Reactions were set up using the SYBR Green Master Mix from the same manufacturer following its recommendations. Specificity and product detection were checked after amplification by determining the temperature dependent melting curves. Primers were designed with the Primer Express software package (Applied Biosystems, The Netherlands) to have a Tm between 59 and 61ºC and an amplicon size of 100 ± 20 bp (Table 3.2.). Comparisons were established between the different growth phases and the different culture media.

Table 3.2. Oligonucleotide primers used in Q-RT-PCR reactions

Primer	Nucleotide sequence (5' – 3')	Strand*	Location
LREf65_84	CAATAACGCCAAGTGAAGCC	+	*pduU*
LREr211_192	CCACATGACGCAAAGCTGAT	-	*pduU*
LREf2543_2562	ATTCAATGTCGGCAGGGTCT	+	*pduV*
LREr2628_2609	GGCTGGCTTCTGTTCAATGT	-	*pduV*
LREf7315_7334	CGCCAATGTGATGATTACGC	+	*cobD*
LREr7431_7412	CAGCTCACGTCGTAACACTT	-	*cobD*
LREf8403_8422	GCAGAGTGTGGTGGCTTAAT	+	*cbiA*
LREr8505_8486	GCGGTGTCATCTCACTCATA	-	*cbiA*
LREf9418_9437	TCCAGCACGAATCACATGGT	+	*cbiB*
LREr9561_9542	CCTGCAACAACAGCTTCACT	-	*cbiB*
LREf22014_22033	GCTGATGCACCAGTAATCCT	+	*cbiP*
LREr22119_22100	TAATGCGTTGCTGGTCTTCG	-	*cbiP*
LREf26313_26332	CGTGATGCTGCTGATGGTTC	+	*hemB*
LREr26410_26391	GCTACTTCGCGCAATGCTTC	-	*hemB*
LREf27503_27522	AAGTAATCGGTGGTGGACTC	+	*hemL*
LREr27605_27586	GACAACGTTCCGGCATGATA	-	*hemL*
LREf28196_28215†	TCGAATTCAGCGTCACCAAG	+	*cobU*
LREf31622_31641	GCTCTCGGTCTTGATCCTTA	+	*cobT*
LREr31723_31704	GAGCATTGCCTTCACTCCAT	-	*cobT*
LREf33369_33388	CGAAGGAGACACTGGTTCTA	+	*rpsO*
LREr33511_33492	AGTTACGACGGTGACCAATC	-	*rpsO*

* Primer were designed to the coding strand (+) or noncoding strand (-)
† Primer also used in complementation studies

Phylogenetic analysis. Each individual B_{12}-related amino acid sequence reported in this study was entered as a string to search for distantly related homologues using the PSI-BLAST algorithm (3). Sequence entries found homologues were retrieved in 03/07 from ERGO (http://ergo.integratedgenomics.com/ERGO/) (24), and separately aligned using the MUSCLE algorithm (14). From the alignment of the proteins encoded by the coenzyme B_{12} biosynthesis cluster, a neighbour joining tree was obtained using ClustalW (42), analyzed in LOFT (43), and visualized in MEGA3 (18). An identical exercise was carried out for the predicted product of the *rpsO* gene that is located downstream of the vitamin B_{12} gene cluster of *L. reuteri*, and for the 16S RNA gene. Finally, the topology of all trees was compared.

G+C content and codon adaptation index. G+C content and codon adaptation index (33) was calculated using the geecee, cusp and cai scripts, part of EMBOSS: European Molecular Biology Open Software Suite (26). Comparisons were established between the coenzyme B_{12} biosynthesis gene cluster of *L. reuteri* presented here and the draft genome sequence of *L. reuteri* JCM1112 obtained by the DOE Joint Genome Institute and deposited at GeneBank under accession n.° CP000705. A similar exercise was performed for *Listeria innocua* Clip11262 (15) and *Salmonella enterica typhi* Ty2 (13), for which we compared the G+C content and codon usage of their vitamin B_{12} clusters to their published genomes.

Results

Operon organization. A sequence of approximately 43.4 kb was assembled from the *L. reuteri* genome through the combined effort of the different molecular biology techniques, and was found to harbour a coenzyme B_{12} gene cluster encoding the complete enzymatic machinery necessary for its biosynthesis. An overview of the organization of this gene cluster (Fig. 3.1) reveals that all predicted genes are in the same orientation with only few intergenic regions. Similar to what has been reported for *Salmonella typhimurium* (30), we observed that approximately half of the genes (46%) are overlapping and predicted to be translationally coupled.

The previously published sequence encoding the fusion protein homologous to CysG/HemD (41), is flanked by the large cluster of 17 *cbi* genes (Fig. 3.1). The *cbi* gene order is conserved amongst different B_{12} producers, notably representatives of *Listeria* and *Salmonella* (see Fig. 3.1). Quite unexpectedly, the *hem* genes are located directly downstream of the *cbi* genes. To our knowledge this genomic organisation has not yet been described. These genes are predicted to encode uroporphyrinogen III synthesis from 5-aminolevulinate, a derivative of glutamyl-tRNA. A cluster of five *cob* genes is located further downstream.

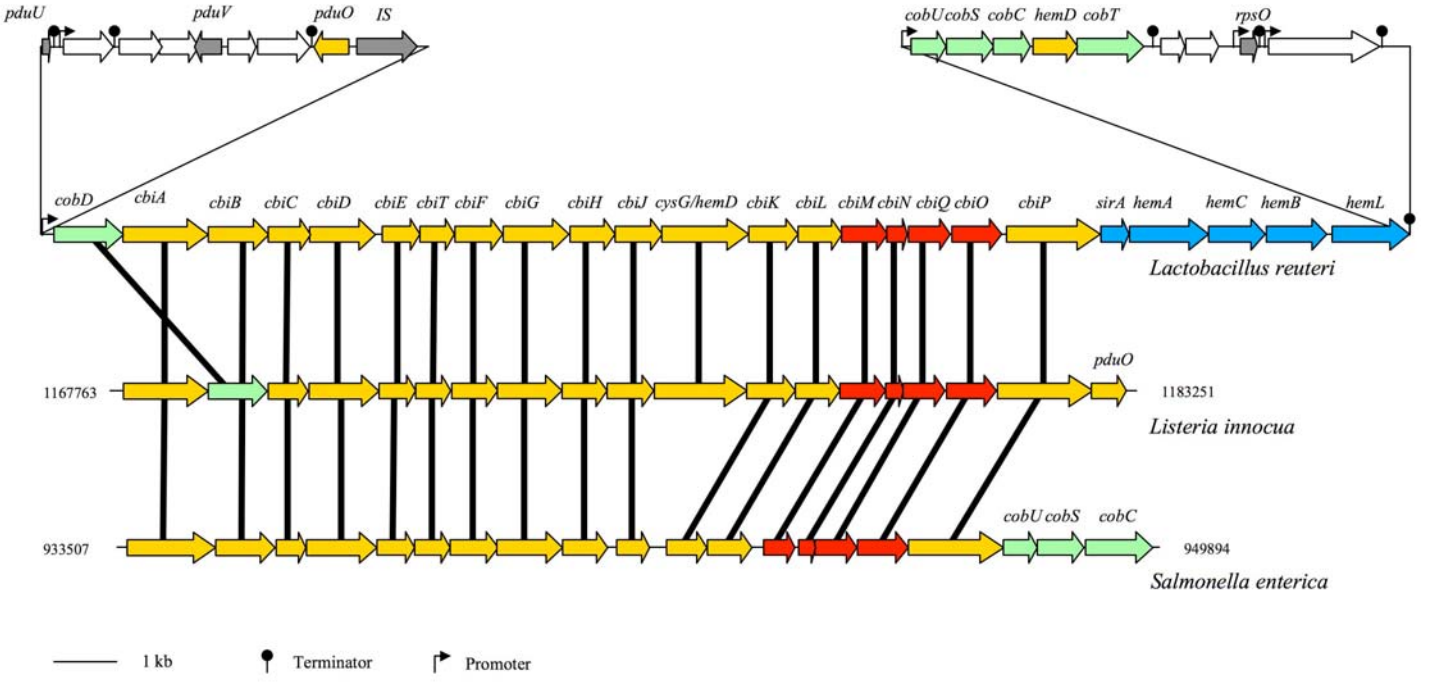

Figure 3.1. Schematic representation of the vitamin B_{12} gene cluster of *L. reuteri*: comparison of gene order with *Listeria innocua* and *Salmonella enterica*. The arrows represent genes that are involved in the synthesis of uroporphyrinogen-III if depicted in blue; involved in the synthesis of adenosylcobinamide if depicted in orange; involved in the synthesis of the lower ligand if depicted in green; involved in cobalt transport if depicted in red; not related to B_{12} biosynthesis if depicted in grey; not studied here if depicted in white. Functional annotation: *pduO*, ATP:Co(I)rrinoid adenosyltransferase (EC 2.5.1.17); *cobD*, Threonine-phosphate decarboxylase (EC 4.1.1.81); *cbiA*, Cobyrinic acid a,c-diamide synthase (EC 6.3.1.-); *cbiB*, Adenosylcobinamide-phosphate synthase (EC 6.3.1.10); *cbiC*, Precorrin-8X methylmutase (EC 5.4.1.2); *cbiD*, Precorrin-5B C1-methyltransferase (EC 2.1.1.-); *cbiE*, Precorrin-6Y C5,15-methyltransferase [decarboxylating] subunit CbiE (EC 2.1.1.132); *cbiT*, Precorrin-6Y C5,15-methyltransferase [decarboxylating] subunit CbiT (EC 2.1.1.132); *cbiF*, Precorrin-4 C11-methyltransferase (EC 2.1.1.133); *cbiG*, Precorrin-5A C20-acyltransferase (EC 2.3.1.-); *cbiH*, Precorrin-3B C17-methyltransferase (EC 2.1.1.131); *cbiJ*, Precorrin-6X reductase (EC 1.3.1.54); *cysG/hemD*, Uroporphyrin-III C-methyltransferase (EC 2.1.1.107) / Uroporphyrinogen-III synthase (EC 4.2.1.75); *cbiK*, Sirohydrochlorin cobaltochelatase (EC 4.99.1.3); *cbiL*, Precorrin-2 C20-methyltransferase (EC 2.1.1.130); *cbiM*, Cobalt transport protein; *cbiN*, Cobalt transport protein; *cbiQ*, Cobalt transport protein; *cbiO*, Cobalt transport ATP-binding protein; *cbiP*, Adenosylcobyric acid synthase (EC 6.3.5.10); *sirA*, Precorrin-2 dehydrogenase (EC 1.3.1.76); *hemA*, Glutamyl-tRNA reductase (EC 1.2.1.-); *hemC*, Porphobilinogen deaminase (EC 2.5.1.61); *hemB*, Delta-aminolevulinic acid dehydratase (EC 4.2.1.24); *hemL*, Glutamate-1-semialdehyde 2,1-aminomutase (EC 5.4.3.8); *cobU*, Adenosylcobinamide kinase (EC 2.7.1.156) / Adenosylcobinamide-phosphate guanylyltransferase (EC 2.7.7.62); *cobS*, Adenosylcobinamide-GDP ribazoletransferase (EC 2.7.8.26); *cobC*, Alpha-ribazole-5'-phosphate phosphatase (EC 3.1.3.73); *hemD*, Uroporphyrinogen-III synthase (EC 4.2.1.75); *cobT*, Nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase (EC 2.4.2.21).

This cluster is predicted to be involved in the attachment of the amino-propanol arm and assembly of the nucleotide loop, which connects the lower cobalt ligand to the corrin ring. Upstream of the B_{12} biosynthesis gene cluster are several genes predicted to be involved in the formation of polyhedral bodies, namely *pduU* and *pduV* (10).

The detailed comparison of the predicted coding sequences of *L. reuteri* CRL1098 and the draft genome sequence of *L. reuteri* JCM1112, recently released by the DOE Joint Genome Institute, demonstrates that they are mostly identical (Table 3.3). The few exceptions are due to minor changes in the N-terminus (CbiA and CbiB), or in the C-terminus (CbiD and CobD), or finally, due to the neutral replacement of residues with the same chemical properties (CbiC and CobU).

Table 3.3. ORFs of the coenzyme B_{12} biosynthesis gene cluster of *Lactobacillus reuteri* CRL1098: Comparison on amino acid level to *Lactobacillus reuteri* JCM1112, *Listeria monocytogenes* and *Salmonella typhimurium*. See legend of Fig. 3.1 for functional annotation of the genes.

***L. reuteri* CRL1098**		***L. reuteri* JCM1112**		***Listeria monocytogenes***		***Salmonella typhimurium***	
Name	**Length**	**Length**	**Identity**	**Length**	**Identity**	**Length**	**Identity**
cobD	369	362	99	361	41	364	34
cbiA	454	454	99	452	48	459	44
cbiB	319	319	99	315	52	319	44
cbiC	227	227	99	210	56	210	55
cbiD	349	383	97	373	51	379	46
cbiE	200	200	100	198	51	201	38
cbiT	184	184	100	189	49	192	42
cbiF	253	253	100	249	72	257	65
cbiG	351	351	100	343	41	351	32
cbiH	241	241	100	241	61	241	58
cbiJ	252	252	100	250	39	263	29
cysG/hemD	464	464	100	493	38	457	46
cbiK	259	259	100	261	46	264	45
cbiL	237	237	100	236	46	237	34
cbiM	248	248	100	244	61	245	54
cbiN	110	103	100	98	58	93	53
cbiQ	225	225	100	225	37	225	34
cbiO	267	267	100	268	49	271	48
cbiP	501	501	100	511	56	506	52
sirA	152	152	100	159	37	311	37
hemA	421	421	100	435	40	418	25
hemC	305	305	100	309	48	318	39
hemB	323	323	100	324	64	324	53
hemL	412	431	100	429	58	426	52
cobU	186	196	94	185	40	181	39
cobS	253	253	100	248	36	247	32
cobC	196	271	100	191	32	202	28
hemD	236	236	100	493	26	246	23
cobT	356	350	100	-	-	356	41

Complementation studies. To experimentally support our functional annotation of the newly sequenced coenzyme B_{12} biosynthesis gene cluster of *L. reuteri*, we performed complementation studies in *Salmonella enterica* mutants TT25720 (*metE2119*::MudJ) and TT25722 [*metE2119*::MudJ, *cobS2621*::Frt(sw)] (5). When cultured in minimal medium lacking methionine, both strains are dependent on the B_{12}-dependent methionine synthase (MetH), since they lack MetE activity. However, due to the additional *cobS* mutation, strain TT25722 has auxotrophic requirements for B_{12}, while strain TT25720 can rely on its own native production of this co-factor.

We transformed the double mutant TT25722 with pNZ7749, harbouring a fragment containing *cobS* amplified from *L. reuteri* under control of the native *cob* operon promoter from *Salmonella enterica* (23). Growth experiments were then performed in minimal E plates using strains TT25720 and TT25722 as a positive and negative control, respectively. When we complement the double mutant with *cobS* from *L. reuteri*, we reconstitute its ability to grow in minimal medium lacking methionine without the exogenous supplementation of vitamin B_{12}, and therefore, relying solely on its own native coenzyme B_{12} production (Fig. 3.2).

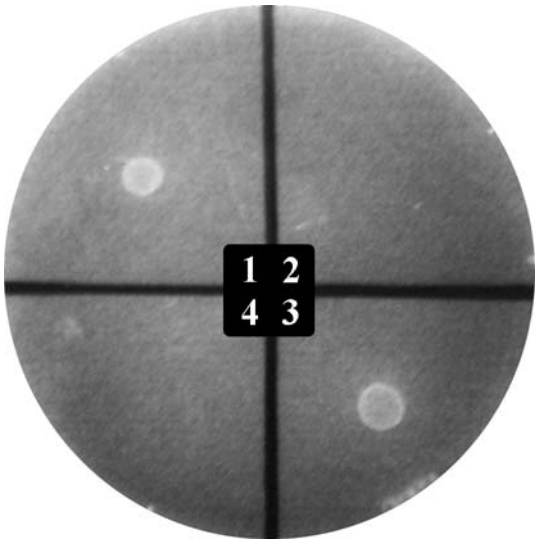

Figure 3.2. Phenotypic characterization of complemented *Salmonella* mutant. Minimal E agarose plate with (1) *Salmonella enterica* TT25720 (*metE2119*::MudJ); (2) *Salmonella enterica* TT25722 [*metE2119*::MudJ, *cobS2621*::Frt(sw)]; (3) *Salmonella enterica* TT25722 pNZ7749 (harbouring *cobS* from *L. reuteri*); (4) empty

Transcription analysis. In order to determine the transcriptional organization of the B_{12} biosynthesis gene cluster we performed Northern blot analysis included in the online version of this paper. As a consequence of the relative rarity of the transcripts encoding B_{12} biosynthesis enzymes and their remarkably large size, we could predict that technical difficulties with the Northern blots would not allow conclusive determination of the exact size of the different operons within this gene cluster. Nonetheless, probes were designed to be complementary to sequences from the beginning and end of the predicted operons and their use in Northern hybridizations revealed the presence of two transcripts, one with a size over 20 kb and another of 4 kb (see Supplementary Materials, Fig. S1.).

To further characterise the transcriptional organization of this gene cluster, a RT-PCR based strategy was implemented. It consisted of systematically amplifying overlapping RT-PCR fragments all throughout the full extension of the cluster and flanking regions. To validate the specificity of the designed primer pairs, all reactions were tested in parallel using chromosomal DNA of *L. reuteri* as a positive control. The absence of any chromosomal DNA contamination was established by carrying out all reactions using RT negative samples as a template, for a negative control. The results from the RT-PCR experiments (Table 3.4 and Fig. 3.3), confirmed that the B_{12} biosynthesis gene cluster is expressed in two separate, but tandem, operons approximately sized 22 and 4 kb. The large transcript includes the genes *cobD*, *cbiABCDETFGHJ*, *cobA/hemD*, *cbiKLMNQOP*, *sirA* and *hemACBL*. The 4 kb transcript derives from the *cobUSC*, *hemD* and *cobT* genes.

Table 3.4. Summary of RT-PCR reactions

Primer pair		Product*		Size (bp)	Location
		mid log	late log		
LREf5899_5921	LREr7366_7343	-	-	1467	IS, cobD
LREf6317_6340	LREr7366_7343	+	+++	1049	cobD
LREf7343_7366	LREr8862_8843	+	+++	1519	cobD, cbiAB
LREf8843_8862	LREr10437_10416	+	+++	1594	cbiBCD
LREf10416_10437	LREr11947_11927	+	+++	1531	cbiDE
LREf11927_11947	LREr13430_13411	+	+++	1503	cbiETF
LREf13411_13430	LREr14920_14900	+	+++	1509	cbiFGH
LREf14900_14920	LREr16411_16391	+	+++	1511	cbiHJ, cysG/hemD
LREf16391_16411	LREr17886_17864	+	+++	1495	cysG/hemD, cbiK
LREf17864_17886	LREr19409_19388	+	+++	1545	cbiKLM
LREf19388_19409	LREr20906_20887	+	+++	1518	cbiMNQO
LREf20887_20906	LREr22471_22451	+	+++	1584	cbiOP
LREf22451_22471	LREr24150_24130	+	+++	1699	cbiP, sirA, hemA
LREf24130_24150	LREr25669_25648	+	+++	1539	hemAC
LREf25648_25669	LREr26711_26683	+	+++	1063	hemCB
LREf26684_26718	LREr27526_27499	+	+++	842	hemBL
LREf27499_27518	LREr27987_27967	+	+++	488	hemL

Primer pair		Product* mid log	Product* late log	Size (bp)	Location
LREf27499_27518	LREr28269_28250	-	-	770	hemL, cobU
LREf28250_28269	LREr29724_29704	+	+++	1474	cobUSC
LREf29704_29724	LREr31148_31129	+	+++	1444	cobC, hemD, cobT
LREf31129_31148	LREr31756_31737	+	+++	627	cobT
LREf31129_31148	LREr32212_32193	-	-	1083	cobT, hypothetical
LREf32193_32212	LREr33545_33526	-	-	1352	Hypothetical
LREf33354_33373	LREr33545_33526	++	++	191	rpsO
LREf33354_33373	LREr33809_33790	-	-	455	rpsO, metalo β-lactamase
LREf33790_33809	LREr35516_35496	++	++	1726	metalo β-lactamase
LREf35496_35516	LREr36538_36518	-	-	1042	metalo β-lactamase, hypothetical

* Symbols refer to relative abundance of products on agarose gel. Increasing number of "+" corresponds to increasing intensity of band (see Fig. 3.3 for illustration); "-" corresponds to absence of expected band, indicating that both genes are not part of the same transcript.

The intensities of the RT-PCR amplicons were compared between samples collected from the same MRS culture at different time points (see Fig. 3.3 for illustration). This suggested that for cells cultured in MRS the expression of the B_{12} gene cluster is strengthened during late-exponential phase in comparison to mid-log phase. The *rpsO* gene, located immediately downstream from the B_{12} gene cluster, served as a control for the transcriptional analysis. RT-PCR samples of this gene collected from the same culture at different time points showed that it is expressed constitutively throughout the growth curve, in contrast to the neighbouring B_{12} genes.

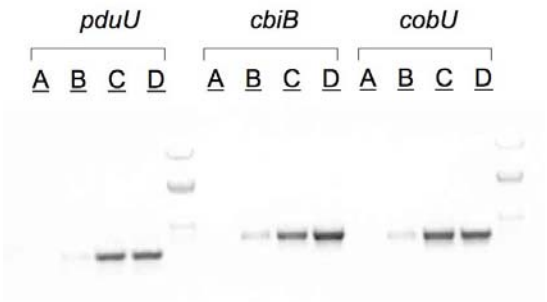

Figure 3.3. RT-PCR amplicons from different loci collected at different time points. Lane A, RT negative samples (negative control); Lane B, sample collected at mid-log phase; Lane C, sample collected at late-log phase; Lane D, *L. reuteri*'s chromosomal DNA (positive control).

To quantify the differential expression first evidenced by the RT-PCR experiments, quantitative RT-PCR (Q-RT-PCR) was carried out on different loci throughout the entire cluster, using a locus on the *rpsO* gene as a reference. The results are in accordance with the previous RT-PCR based observation, and confirm that for cells cultivated in MRS the cluster is indeed strongly induced during late-exponential growth (Fig. 3.4.A.). The operon carrying the *cbi* and *hem* genes is up-regulated 4.56 ± 0.92 fold during late-exponential growth when compared to mid-log phase. Similarly, the smaller operon carrying the *cob* and *hemD* genes is up-regulated by a factor of 5.03 ± 0.32, between late- and mid-log phases. The same approach was performed on two loci upstream of the B_{12} biosynthesis gene cluster predicted to encode PduU and PduV. We observed an average up-regulation of 5.91 ± 3.25 for these transcripts, similar to the one observed for the B_{12} gene cluster (Fig. 3.4.B.).

In order to confirm that the observed up-regulation of the B_{12} biosynthetic genes during late-log is not caused by the exhaustion of the vitamin B_{12} present in MRS, further experimentation was carried out. We analysed by Q-RT-PCR samples obtained from cells grown in B_{12} assay medium, which is B_{12} free, and in B_{12} assay medium supplemented with cyanocobalamin (Fig. 3.4.B.). In the absence of B_{12} we observed that during late-log phase the *cbi* and *hem* operon was up-regulated 6.89 ± 0.93, slightly more than what was observed for MRS. In the presence of an excess of exogenous B_{12} there was a 3.47 ± 0.90 fold change between late- and mid-log growth phases for this same operon. Even though this up-regulation is diminished in comparison to the one in the absence of exogenous B_{12}, it is still quite considerable. Similar results were observed for the *cob* operon, up-regulated 6.35 ± 0.73 and 3.31 ± 0.56 in the absence and presence of exogenous B_{12}, respectively. The up-regulation in MRS of the transcript levels of the *pdu* loci was also observed in the absence of B_{12} from the medium. For these we observed a fold-change of 3.76 ± 2.2 in the absence of exogenous B_{12} and 1.98 ± 0.68 when there was an excess of B_{12}.

To characterise in greater detail the specific impact of B_{12} supplementation for each growth phase, additional comparisons were established between the cultures lacking exogenous B_{12} and the ones with an excess of cyanocobalamin (Fig. 3.4.C.). During exponential phase, expression of the B_{12} biosynthesis genes does not vary significantly with the absence of exogenous vitamin B_{12} (average fold-change of 0.96 ± 0.08 for the *cbi* and *hem* operon, and 0.92 ± 0.08 for the *cob* operon). During late-log phase, even though in both conditions the abundance of B_{12} biosynthesis transcripts is increased relative to mid-log, in the absence of vitamin B_{12} supplementation, their induction is stronger. For late-log, when we compared the levels for cells cultured in the absence of exogenous B_{12} in relation to the ones cultivated in its presence, we determined an average fold-change of 1.90 ± 0.22

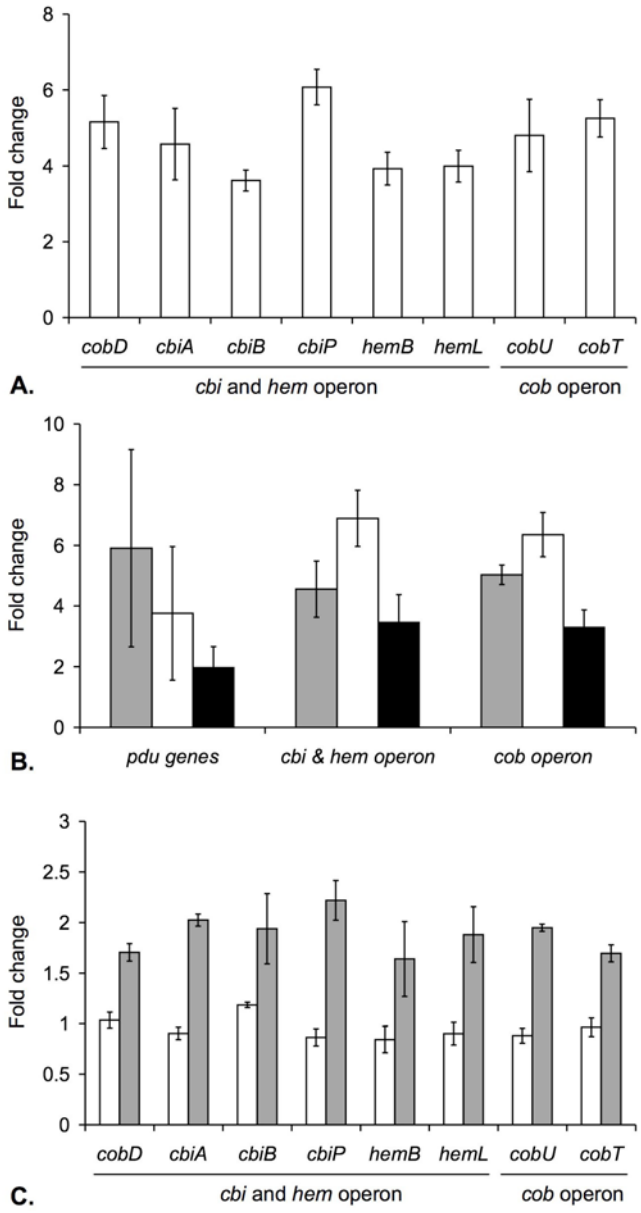

Figure 3.4. Differential gene expression as determined by Q-RT-PCR. **A.** Differential expression of the coenzyme B_{12} biosynthesis genes in late-log relative to mid-log in cells cultivated in MRS. **B.** Average fold change between late-log and mid-log growth phases of *pdu* genes, *cbi* and *hem* operon and *cob* operon from *L. reuteri* cells cultured in MRS (grey bars), B_{12}-free medium (white bars) and B_{12}-free medium supplemented with 1 mg/ml cyanocobalamin (black bars). **C.** Differential expression of B_{12} biosynthesis genes between cells cultivated in B_{12}-free medium and in B_{12}-free medium supplemented with 1 mg/ml cyanocobalamin during mid-log (white bars) and late-log (grey bars) growth phases.

for the *cbi* and *hem* operon, and 1.82 ± 0.08 for the *cob* operon.

In silico analysis and comparative genomics. We determined the phylogeny of each predicted individual amino acid sequence encoded by the B_{12} gene cluster. We then compared them amongst each other, and with the deduced protein sequence of a control gene, *rpsO*, for which we performed the same exercise. The RpsO protein tree resembled the canonical phylogenetic topology deduced from 16S rRNA sequences (see Supplementary Material, Fig. S2.). In contrast, the predicted B_{12} proteins of *L. reuteri* were found to repeatedly cluster together with those of the genus *Listeria*, and closely neighboured by those of the genus *Salmonella* and other closely related γ-Proteobacteria. (see Fig. 3.5 for illustration). This is suggestive of a common origin for the coenzyme B_{12} production pathway in these organisms. Variations to the mentioned tree topology were observed for *sirA*, *hemACBL* and *cobT*, and are addressed later in the discussion.

Both the G+C content and codon adaptation index (33) of the B_{12} cluster were compared with the draft genome sequence of *L. reuteri* JCM1112. The average G+C content of the coenzyme B_{12} biosynthesis gene cluster (36%) does not differ significantly from the average of the draft genome sequence of *L. reuteri* available at the date of analysis (39%). Concerning codon usage, again we did not observe any significant differences between the coenzyme B_{12} gene cluster of *L. reuteri* and other *L. reuteri* sequences. The average codon adaptation index for the genes of this cluster was calculated to be 0.69 ± 0.026, and we did not detect the usage of any rare codon. Additionally, we also compared the G+C content and codon usage of the B_{12} biosynthesis clusters of *Listeria innocua* Clip11262 (15), 39%, and *Salmonella enterica typhi* Ty2 (13), 56%, with their published genomes, 38% and 52% respectively.

Discussion

The biosynthesis of coenzyme B_{12} from uroporphyrinogen III, the last shared metabolic precursor of the various tetrapyrrolic cofactors, requires about 25 enzymes, and has two different routes described: *(i)* the aerobic pathway studied in *Pseudomonas denitrificans* (7); *(ii)* and the anaerobic pathway partially resolved in *Salmonella enterica*, *Bacillus megaterium* and *Propionibacterium shermanii* (28). This biosynthetic pathway is commonly divided in three parts: *(i)* the synthesis of uroporphyrinogen III from either glutamyl-tRNA or glycine and succinyl-CoA; *(ii)* the corrin ring synthesis, which differs between the anaerobic pathway starting with the insertion of cobalt into precorrin-2, and the aerobic pathway where the cobalt chelation reaction occurs only after corrin ring synthesis; *(iii)* and the corrin ring adenosylation, attachment of the amino-propanol arm and assembly of the nucleotide loop bridging the lower ligand to the cobalt at the core of the corrin ring.

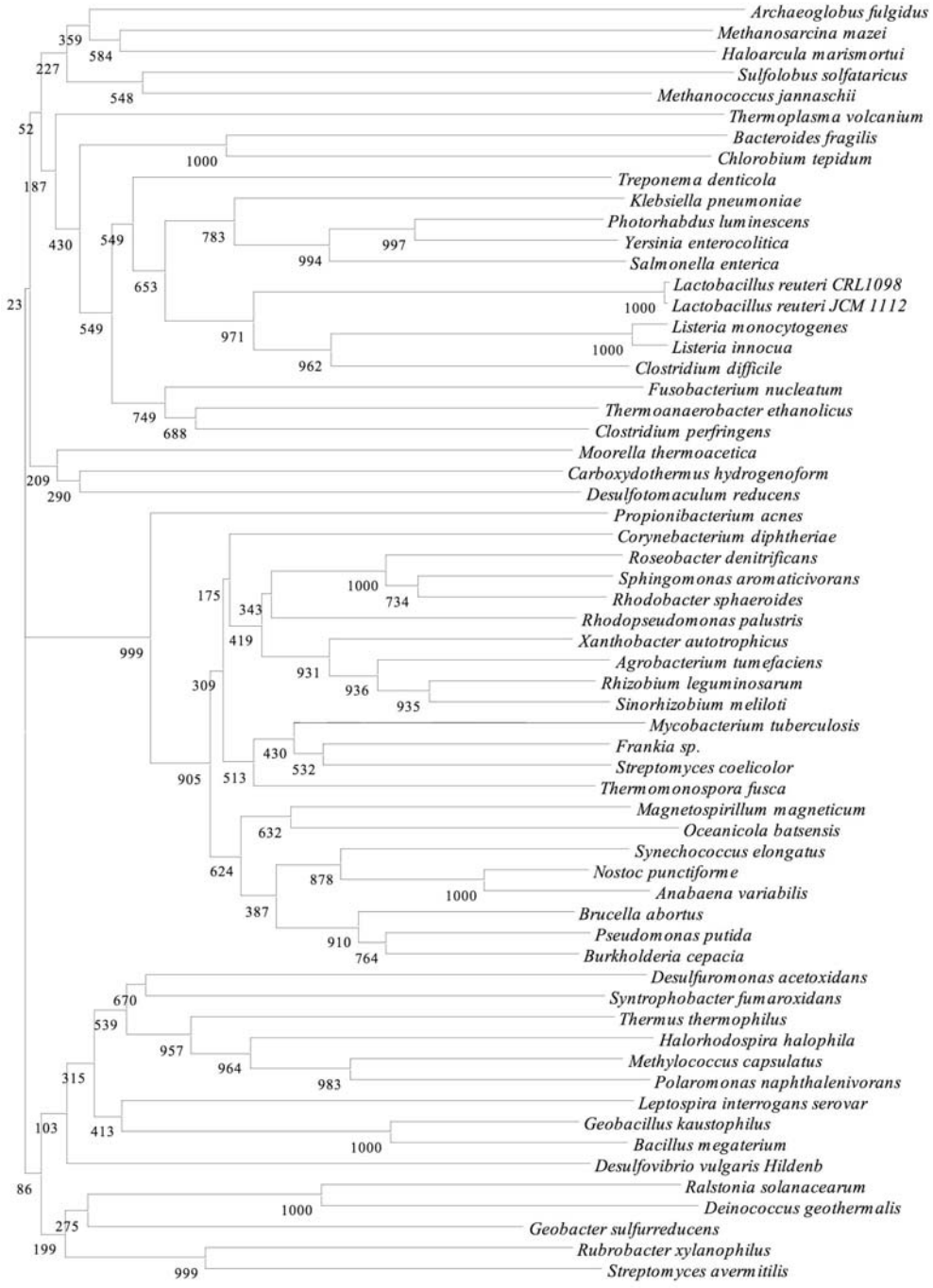

Figure 3.5. Bootstrapped neighbour-joining phylogenetic tree of the CbiC protein.

In *L. reuteri* we have found all genes necessary to encode the complete anaerobic biosynthesis pathway of coenzyme B_{12}. Remarkably, and unlike other B_{12}-producing prokaryotes, all the three different parts of B_{12} biosynthesis are clustered together in one continuous stretch of the chromosome. This presents a great advantage if considering metabolic engineering strategies aiming at transferring B_{12} production capability, as has been done before for other complex B vitamins (36, 47).

Based on the homology paradigm, our functional annotation of the newly sequenced coenzyme B_{12} biosynthesis gene cluster of *L. reuteri*, was experimentally verified for *cobS* by the complementation of *Salmonella* mutant TT25722 (5) lacking MetE and CobS activity. If cultured in minimal medium lacking methionine, this strain relies on the B_{12}-dependent methionine synthase (MetH), and has auxotrophic requirements for this co-factor. When we transformed TT25722 with pNZ7749, harbouring a fragment containing *cobS* amplified from *L. reuteri*, we reconstituted its ability to grow in minimal medium depending on its own native coenzyme B_{12} production, and indirectly showed the functionality of *cobS* from *L. reuteri* (Fig. 3.2). Another example of functional evidence can be found in the recent report of the crystal structure of the PduO-type ATP:Co(I)rrinoid adenosyltransferase (34) also sequenced within the course of this study.

Northern blotting and RT-PCR have shown that both the *cbi* genes, responsible for corrin ring synthesis, and the *hem* genes, responsible for the synthesis of uroporphyrinogen-III, are transcribed together as part of a nearly 22-kb multicistronic operon. Although remarkably large, similar sized transcripts have been detected in other lactic acid bacteria (44). The *cob* genes are clustered in the same orientation, but expressed in a different operon of approximately 4 kb, situated just downstream of the previously mentioned *cbi* and *hem* transcript (Fig. 3.1).

The results from the Q-RT-PCR experiment, corrobated the hypothesis emergent from the RT-PCR studies, that the B_{12} biosynthesis gene cluster is strongly induced during late-exponential growth phase (Fig. 3.4A). Both operons are approximately five times up-regulated in late-exponential growth when compared to mid-log, as determined by Q-RT-PCR for cells cultured in MRS broth. To ensure that the observed induction of the B_{12} biosynthesis genes in late-log is not due to the depletion of B_{12} pools in MRS, we carried out additional experiments in B_{12} free medium. We compared the induction of these genes between late-log and mid-log, for cultures in the absence or presence of excessive exogenous B_{12}. Although there was some variation in the levels of induction, it was clear that in all conditions assayed the B_{12} biosynthesis transcripts are more abundant in late-log in comparison with mid-log (Fig. 3.4.B.).

The lower induction of the B_{12} biosynthesis genes during late-log in the medium supplemented with B_{12} (Fig. 3.4.C) suggests the presence of a regulatory feedback

mechanism, which inhibits the biosynthesis of this costly co-factor whenever it is available from the environment. Vitamin B_{12} metabolism has been shown to be often regulated by a conserved RNA structural element, known as riboswitch (45). We searched the coenzyme B_{12} biosynthesis gene cluster of *L. reuteri* for such conserved motifs using Rfam (16) and Riboswitch finder (8), but none could be found. The presence of a transposase immediately upstream of the first gene of the B_{12} cluster might have disturbed the riboswitch. The regulatory gene *pocR* (9), which is often in between the B_{12} biosynthesis and *pdu* clusters is not in such location in the chromosome of *L. reuteri*. In fact, this common regulator can be found at the far end of the adjacent *pdu* operon in the recently released genome of *L. reuteri* JCM1112. Its presence is in agreement with the experimental evidence gathered during this study suggesting co-regulation between the B_{12} cluster and the *pdu* genes located immediately upstream. PocR has been shown to be an activator of the coenzyme B_{12} biosynthesis cluster (9), and is likely to be involved in the observed negative feedback phenomena. Furthermore, PocR itself has been shown to be activated under carbon and redox control (1), which explains why we observed in all conditions assayed an induction of the B_{12} biosynthesis cluster during late-log in comparison to mid-log.

The topology of the phylogenetic tree obtained for the predicted product of the *rpsO* gene (data not shown), is similar to the canonical phylogenetic trees deduced from 16S rRNA sequences (see Supplementary material, Fig. S2.). In contrast, the phylogenetic comparison of all predicted amino acid sequences related to B_{12} biosynthesis showed that *L. reuteri* systematically clusters together with members of the genus *Listeria*, and closely neighbours the genus *Salmonella* and closely related Enterobacteriaceae. An illustration of a B_{12} biosynthesis protein phylogenetic tree is here depicted for CbiC, which was found to follow this topological pattern (Fig. 3.5). Exceptions of this topology include the products of *sirA* and *hemABCL*, for which *L. reuteri* clusters with *Listeria* and related genera of Gram-positive bacteria, while the Enterobacteriaceae are now clustering with other γ-Proteobacteria, likely because their *hem* genes are properly adapted to aerobic conditions as well. In addition the *CobT* protein is not encoded by the *Listeria* genomes, which may have suffered gene loss, while *L. reuteri* still clusters with *Salmonella* and closely related genera.

L. reuteri was the first lactic acid bacterium reported to produce coenzyme B_{12}, and the recently released genome sequences of a dozen lactic acid bacteria show no traces of genes related to B_{12} production. This observation, combined with the great differences in topology of the B_{12} related trees and the canonical phylogenetic tree, suggest the acquisition of this capability through the occurrence of horizontal gene transfer. This promiscuity related to B_{12} metabolism between some genera of the Firmicutes and γ-Proteobacteria has

been noted before when the phylogeny of the B_{12} regulatory motifs was being investigated (45).

The G+C content of *L. reuteri*'s B_{12} biosynthesis gene cluster does not clearly differ from the rest of its available genomic sequences, and the average codon adaptation index of this cluster is elevated, indicating that it is well suited to *L. reuteri*'s translational machinery. The same holds true for the B_{12} homologues *Listeria* and *Salmonella*, indicating that the postulated horizontal gene transfer is not a recent event.

Associated with its survival strategy, *L. reuteri* is capable of producing and excreting reuterin, a broad spectrum antimicrobial (38, 39). The production of this key component for its competitiveness is mediated by a B_{12}-dependent enzyme, glycerol dehydratase, responsible for catalysing the conversion of glycerol to 3-HPA, an intermediate of 1,3-propanediol on the glycerol catabolism pathway. The hypothesis that the acquisition of reuterin production and production of coenzyme B_{12} was a single event is supported by the following observations: *(i)* the genes involved in reuterin production are located just upstream of the B_{12} biosynthesis gene cluster; *(ii)* both sets of genes show similar phylogeny; and *(iii)* both sets of genes have similar expression patterns and seem to be part of the same regulon. This evolutionary event has presumably resulted in the speciation of *L. reuteri* from the other *Lactobacillae* species, and possibly, might have had a great importance in its evolution to colonize the GI tract.

L. reuteri possesses the GRAS status and is an industrially relevant microrganism. From a biotechnological point of view, the findings reported in this study, can be applied for natural enrichment of (fermented) foods with B_{12}. Furthermore, they shed light on *L. reuteri* as a good candidate to investigate the possibility of in situ delivery of vitamin B_{12} in the GI tract.

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CHAPTER 4

THE IMPACT OF GLYCEROL ON THE METABOLISM OF *LACTOBACILLUS REUTERI* STUDIED BY FUNCTIONAL GENOMICS AND GENOME-SCALE MODELING

Abstract

Lactobacillus reuteri is a heterofermentative lactic acid bacterium best known for its ability to co-ferment glucose and glycerol. Its genome sequence has recently been deduced enabling the implementation of genome-wide analysis. In this study, we developed a dedicated cDNA microarray platform, and a genome-scale metabolic network model of *L. reuteri*, and use them to revisit the co-fermentation of glucose and glycerol. The model was used to simulate experimental conditions and to visualize and integrate experimental data, in particular the global transcriptional response of *L. reuteri* to the presence of glycerol. We show how the presence of glycerol affects cell physiology and triggers specific regulatory mechanisms, allowing simultaneously a better yield and more efficient biomass formation. Furthermore, we were able to predict and demonstrate for this well-studied condition, the involvement of previously unsuspected metabolic pathways, for instance, related to amino acids and vitamins. These could be used as leads in future studies aiming at the increased production of industrially relevant compounds, such as vitamin B_{12} or 1,3-propanediol.

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Supplementary materials are available with the electronic version of this thesis or with the online version of this paper.

Introduction

Lactobacillus reuteri is an heterofermentative, Gram-positive lactic acid bacterium ubiquitous in the gastrointestinal tract (GI-tract) of humans and other mammals (49). In addition, *L. reuteri* is a predominant microbe in several food and feed fermentations, notably in sourdoughs of different cereals (21). *L. reuteri* is best known for its ability to co-ferment glucose and glycerol. In the presence of the latter, it has the ability of producing and excreting reuterin (41), a mixture of monomeric, hydrated monomeric and cyclic dimeric forms of 3-hydroxypropionaldehyde (3-HPA) (42). This broad spectrum antimicrobial is active against not only Gram-positive and Gram-negative bacteria, but also has an inhibitory effect over several yeasts, moulds and protozoa (7). As a consequence, reuterin is assumed to confer significant growth advantage to *L. reuteri* in complex environments, such as the GI-tract (7).

Reuterin synthesis is mediated by a B_{12}-dependent glycerol dehydratase (EC 4.2.1.30) (9), and *L. reuteri* is capable of synthesizing this co-factor *de novo* (32, 33). 3-HPA can be further reduced by 1,3-propanediol dehydrogenase (E.C. 1.1.1.202) to 1,3-propanediol, a compound with biotechnological interest (9). As *L. reuteri* has a predominantly anaerobic lifestyle, production of 1,3-propanediol contributes to the recycling of NAD^+ in this organism.

Recently the genome sequence of the type strain of *L. reuteri* was sequenced (ref. and website), enabling the development of dedicated genome-wide analysis tools. Amongst these, cDNA microarrays are particularly useful to probe the transcriptional response of microbial cells to particular changes in environment (13). Typically, these experiments generate large data sets that are difficult to process, integrate, visualize and finally, interpret (15). Stoichiometric genome scale models have been shown to at least partially resolve such issues, whilst generating new insights in the physiology of the modeled organism (8, 45). Recently, tools have been made available to accelerate the time-consuming task of developing genome scale models based on genomic sequences (22, 29, 40). These are particularly useful when models of closely related species are available. Genome-scale metabolic models have been reported for both *Lactococcus lactis* (25) and *L. plantarum* WCFS1 (47), a member of the same genus as *L. reuteri* that shares many ecological niches (16). More recently, the metabolic network of yet another Firmicute, *Bacillus subtilis*, has been modeled (24).

Here we report the development of a genome-scale metabolic network model of *L. reuteri* to simulate experimental conditions and to visualize experimental data, in particular the global transcriptional response to the presence of glycerol. We show how the presence of glycerol affects cell physiology and triggers specific regulatory mechanisms, allowing simultaneously a better yield and more efficient biomass formation.

Materials and Methods

Construction of the genome scale metabolic model. The *in silico* reconstruction of the genome-scale metabolic network of *L. reuteri* was initiated by implementing AUTOGRAPH (22). This semi-automatic method combines orthology with existing manually curated metabolic networks to predict gene-reaction associations. In contrast to other tools available to automate network reconstruction (29, 40), this method has the advantage of exploiting the efforts that have been invested into curating other metabolic networks. In addition, it has been benchmarked with another lactic acid bacteria with the encouraging recovery of 74-85% of the gene-reaction associations in the manually curated network (22). The method consists of (i) establishing pairwise orthologous relationships between a query species (*L. reuteri* in this case) and species for which curated metabolic networks are available, (ii) transfer the original gene-reaction association of the orthologous genes to the query species, (iii) and finally establish a reconciled list of gene-reaction associations for the query species.

Amongst the increasing number of manually curated genome-scale metabolic networks available, we chose the one of *L. plantarum* (47), *Lactococcus lactis* (22, 25) and *B. subtillis* (24) based on phylogenetic and phenotypic proximity. Additionally, the model of *E. coli* (11, 31) was also used because it is the most comprehensive compilation of prokaryotic pathways experimentally validated. We established orthologous relationships between the draft genome sequence of *L. reuteri* JCM1112 released by JGI in 03/2006 and those of *E. coli* (4), *B. subtilis* (18), *Lactococcus lactis* (6) and *L. plantarum* (16) using Inparanoid with default settings (23). An in-house PERL script was implemented to produce the initial metabolic network of gene-reaction associations based on phylogenetic proximity followed by level of homology between hits. The fully automated version of the model was implemented and further developed within the SimPheny™ software platform (Genomatica, Inc., San Diego, CA). It was also within this computational environment that we implemented constraint-based modeling techniques, in particular Flux Balance Analysis (FBA) (30, 48) and Flux Variability Analysis (FVA) (20), based on linear programming techniques as described elsewhere (20, 48). The manual curation of the *L. reuteri* model began with the comparison of the automated gene-reaction associations with the annotation available within the ERGO bioinformatics suite (http://ergo.integratedgenomics.com/ERGO/) (26). Network gaps were analyzed and identified as previously described (47), by performing *in silico* growth experiments using FBA and by visual inspection of the model projected on metabolic maps. In order to validate our *in silico* reconstruction of the metabolic network of *L. reuteri*, we implemented a similar approach as carried out for *E. coli* (12) and *L. plantarum* (46). We compared the amino acid and vitamin requirements of *L. reuteri* determined from single omission growth

experiments in chemically defined medium (CDM), with those predicted by our constructed model.

Strains, media and culture conditions. The type-strain of *L. reuteri* (ATCC 23272, DSM 20016, JCM1112 or F275) was obtained from the Japanese Collection of Microorganisms (Riken, Japan). This human isolate was cultured at 37ªC in CDM similar to the one described previously (46), but without vitamin B_{12}. When mentioned, the medium was supplemented with 0.5% glycerol (v/v).

Single omission growth experiments. Single omission growth experiments were performed by cultivating *L. reuteri* in single amino acid- or vitamin-deficient CDM. Overnight batch cutures of *L. reuteri* inoculated with cells washed twice in 0.85 % NaCl (w/v) were propagated with an initial optical densitity at 600 nm (OD_{600}) of 0.05 during five consecutive serial transfers. Only then, was the final biomass formation taken into account to avoid interference of the carryover of undesired compounds. If the final OD_{600} was lower than 10% of that obtained with complete CDM, *L. reuteri* JCM1112 was considered to have an auxotrophic requirement for the omitted amino acid or vitamin.

Fermentation conditions. Fermentations were carried out in a batch set-up consisting of four vessels with a reaction volume of 400 ml. Temperature was kept constant at 37°C throughout all fermentations, and along with pH, it was continuously monitored. The cultures were continually stirred and the media vessels were gassed with O_2-free N_2 (15 ml/min) for 1 hour prior to inoculation. Fermentors were inoculated to an initial OD_{600} of 0.05 from an exponentially growing culture of CDM without glycerol. Biomass formation was monitored by periodic measurements of OD_{600}, and if mentioned, pH was fixed to 5.8 by titration with 5 M NaOH. Throughout the course of the fermentations, supernatant samples were harvested by rapidly forcing 5 ml of culture through a 22 μm filter and storing immediately at -20°C until further analysis. Samples for B_{12} analysis prepared as described below were also harvested through out different time-points of the growth curve. Transcriptomics samples were harvested at two time points, namely at OD_{600} 1 (corresponding to mid-logarithmic phase), and 15 minutes after exponential growth ceased (corresponding to early-stationary phase), and prepared as described below.

End-product and amino acid analysis. We determined the extracellular concentration of acetate, 1,2-butanediol, butyrate, citrate, ethanol, formate, glucose, glycerol, 3-hydroxybutanone (acetoin), 3-hydroxypropionaldehyde, 1,2- and 1,3-propanediol, propionate, pyruvate and succinate in several time points through out the course of the fermentation by HPLC as previously described (7, 39). The extracellular concentrations at different time-points of all standard amino acids were determined by RP-

HPLC as dabsyl derivatives (17), with a detection limit of 50 μM except for asparagine and glutamine (0.1 and 0.2 mM, respectively).

Coenzyme B_{12} analysis. The production of B_{12} by *L. reuteri* under the different fermentation conditions was determined according to the Official Methods of Analysis of AOAC International, using the *L. delbrueckii* subsp. *lactis* ATCC 7830 vitamin B_{12} assay (14). Cell extracts for B_{12} analysis were prepared as previously described for *L. reuteri* CRL1098 (32).

Growth rate determination. L. reuteri JCM1112 and derivative strain were cultivated in CDM in the presence or absence of glycerol, in 96 well or 384 well microplates (Greiner, Alphen a/d Rijn, The Netherlands) using a checkerboard format distribution (10). Plates were incubated at 37°C in a Genios microplate reader (Tecan, Zurich, Switzerland) and growth was monitored by measuring optical density at 595 nm every 15 min. All measurements were independently performed at least twice in at least 40 biological replicates. Maximum specific growth rate (μ_{max}) was determined in a high-throughput fashion by in-house scripts that calculate μ for every five consecutive time points through out the growth curve and return its maximum value per well.

Microarray design. Microarrays were spotted on the Agilent 11K platform (Agilent Technologies, Santa Clara, CA, USA). The custom probe design was based on the draft genome sequence of *L. reuteri* JCM1112 released by JGI (retrieved in 03/2006). A total of 9551 unique 60-mers with a theoretical melting temperature of approximately 82ºC were selected, probing 1700 (out of 1900) predicted coding regions (~90% coverage). The melting temperature was estimated using the nearest neighbor calculations (27), for a Na^+ and oligonucleotide concentration of 1 M and 10^{-12} M, respectively. Probes were designed all throughout the sequence of the coding regions, representing them with 6 unique probes or more in 82.9% of the cases, and leaving only 3.9% to be represented by less than 3. The full details of this microarray platform are available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GPL6856.

RNA isolation. Cultures were sampled at different time points from different conditions by rapid quenching using a cold methanol method (28). Total RNA was extracted and purified as previously described (35) introducing the following minor modifications: (i) liquid nitrogen was used to cool the cell pellet, instead of cooled 60% (vol/vol) ethylene glycol; (ii) three treatments of 40 seconds intercalated with 1 minute on ice were implemented to disrupt the cells; (iii) further purification using the High Pure RNA Isolation kit (Roche Diagnostics, Germany) was carried out following the manufacturer's recommendations, except for the on column DNAse I treatment time, which we extended to 60 min. RNA samples were aliquoted and stored at -80°C. Concentration

was determined with a ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA), and its integrity was analyzed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples in which the 23S/16S RNA ratio was superior to 1.6 were considered satisfactory and used for further labeling.

cDNA synthesis and labeling. First strand cDNA synthesis from 10 μg of RNA per sample was carried out using Superscript III reverse transcriptase (Invitrogen, Breda, The Netherlands) according to the manufacture's recommendations. Newly synthesized cDNA was purified and labeled with cyanine 3 and cyanine 5 for all samples. Indirect labeling was performed with the CyScribe first-strand cDNA labeling kit (Amersham, United Kingdom) according to the recommendations of the manufacturer. Labeling efficacy and cDNA concentration was determined using the ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA).

Microarray hybridization. Hybridizations were carried out using 0.5 μg of labeled cDNA per dye, and all samples were hybridized at least once with each label to enable the identification of potential dye effects. A total of twelve microarrays (Agilent Technologies) were used in this study in samples originated from two different fermentation set-ups. An initial exploratory experiment consisting of a simple dye swap with samples from early-stationary growth phase harvested from batch fermentations in the presence or absence of glycerol was performed requiring one slide (two microarrays). Subsequently a hybridization scheme consisting of a loop design comprised of ten microarrays was carried out to analyze the transcriptome of mid-logarithmic and early-stationary growth phase cells from pH-controlled batch fermentations in the presence or absence of glycerol. The following samples were hybridized per array labeled with cyanine3 and cyanine5, respectively: exp-F1 and sta-F1, sta-F1 and sta-F2, sta-F2 and exp-F2, exp-F2 and exp-F1, exp-F2 and exp-F3, exp-F3 and exp-F4, exp-F4 and sta-F4, sta-F4 and sta-F1, sta-F2 and exp-F4, exp-F4 and exp-F3. Here, F1 and F3 represent completely independent biological duplicates cultured in the absence of glycerol, and F2 and F4 represent duplicates cultured in the presence of glycerol. The prefix exp- and sta- stand for cells harvested at mid logarithmic and early stationary growth phases, respectively. Hybridizations were carried out for 17 h at 60°C and slides were subsequently washed according to the manufacturer's procedures and dried as described elsewhere (35), before proceeding immediately with scanning.

Scanning and microarray data analysis. Slides were scanned with a ScanArray Express scanner (Perkin-Elmer), using a resolution of 10 μm. Image analysis, spot quantification and data extraction were done using ImaGene (BioDiscovery) version 5.6. Microarrays were analyzed as previously described (35), after normalization by local fitting of an M-A plot applying the loess algorithm (52) using the Limma package (36) in R

(http://www.r-project.org). Statistical significance was determined from variation in biological duplicates by implementation of the eBayes function included in Limma (cross-probe variance estimation) and false discovery rate adjustment of the *p*-values (37). In order to characterize the response of *L. reuteri* to glycerol, comparisons were established (i) during exponential growth phase (mid-logarithmic phase response), (ii) during early stationary growth phase (early-stationary phase response), and (iii) by comparing the differences between early-stationary and mid-logarithmic cells cultured with and without glycerol (phase shift response). Only transcripts with an average absolute $\log_2$ (ratio) greater than 0.585 and a *p*-value smaller than 0.05 were considered for analysis. The three lists of differentially regulated genes were projected on metabolic maps of the genome-scale model developed in this study.

Microarray accession numbers. The microarray platform developed in this study is available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GPL6856. The microarray data were deposited in the same repository under accession number pending GSE11885.

Motif searches. The upstream regions of differentially regulated genes were analyzed for the presence of regulatory motifs using MEME (2). Transcriptional Units (TUs) were predicted for *L. reuteri* as described previously (51). We used for motif detection the 300 bp sequence directly upstream of TUs found to harbor differentially regulated genes. MEME was run with default settings except for the motif length (-minw 8 and –maxw 40), the total number of motifs to be found (-nmotifs 10) and the number of motif occurances (-minsites 5). All motifs were subsequently used to search the complete genome of *L. reuteri* using MAST with default settings (3).

Results

Genome-scale metabolic network model. A first fully automated version of the metabolic model of *L. reuteri* obtained with AUTOGRAPH (22) included 645 genes (~35%) associated to 457 reactions, involving 552 metabolites and contained a total of 270 network gaps. This list of many-to-many gene-reaction associations was compared with the genome annotation available within the ERGO bioinformatics suite (26) and inconsistencies were manually corrected. About 17% of the initial gene-reaction associations were deleted and 47% permutated. A total of 299 new reactions were entered, bestowing the model with the idiosyncrasies of *L. reuteri*, including B_{12} biosynthesis (33) and the B_{12}-dependent usage of glycerol as an H^+ acceptor (9). The improved version of the model (supplementary material 4.S1) used here to simulate the impact of glycerol on the metabolic network of *L. reuteri* includes 563 genes (30% of total number predicted from closed genome) and 623 reactions, involving 659 metabolites and containing a total of 187

network gaps. 523 reactions (16%) are not associated with a gene, but inferred from biochemical and/or physiological data available or acquired during the course of this study. These include (i) key enzymes such as phosphofructokinase (PFK), for which no unambiguous encoding gene is found, but activity has been reported (1); (ii) vitamin transport systems, such as *p*-aminobenzoate uptake, which has been demonstrated recently for this strain but is not associated to a gene (34); (iii) amino acid uptake systems, as amino acids were demonstrated here to be consumed by *L. reuteri* and yet no specific transported is associated to them; (iv) spontaneous reactions, such as oxidative decarboxylation of acetolactate or dissociation of NH_4^+, which were derived from literature or inferred by similarity to other genome scale models.

Under the conditions assayed, we have found *L. reuteri* to have an auxotrophic requirement for ten amino acids, namely arginine, glutamate, histidine, leucine, methionine, phenylalanine, threonine, tryptophan, valine and tyrosine. Overall, the amino acid single omission experiments corroborated *in silico* predictions in 18 out of 20 cases. The two exceptions are arginine and isoleucine. Arginine can theoretically be synthesized from aspartate and glutamine, and it was not predicted to be essential. However, *L. reuteri* and several other lactobacilli can not rely on *de novo* synthesis of arginine countering *in silico* predictions (46). A possible explanation could be that these lactic acid bacteria contain the arginine-ornithine pathway that is one of the few non-sugar degradation pathways generating metabolic energy. Concerning isoleucine, no route towards its biosynthesis could be predicted. The growth of *L. reuteri* was significantly (80 %) reduced in the absence of isoleucine but was not completely abolished. This can most likely be explained by the presence of trace amounts of isoleucine in the stock of one of the many components of CDM like it has been shown previously for *L. plantarum* (46). Therefore, we did not impose any additional constraints to the model because of these minor incongruences. Concerning vitamin and co-factor requirements, *L. reuteri* has a surprisingly small amount of auxotrophies. In fact, only three vitamins were found to be essential in the conditions assayed, namely nicotinate, panthothenate and thiamin.

Model simulations. Constraint-based modeling techniques were implemented to explore and predict the impact of glycerol on the metabolic capabilities and growth rate of *L. reuteri*. First, fine-tuning of the ATP-related parameters was performed as previously described (24, 47). Briefly, the ATP-related maintenance factor was adjusted in the model until the *in silico* growth rate, using all experimentally determined fluxes as boundaries of the system, was equal to the observed growth rate. The dominance of the phosphoketolase pathway (PKP) over the Embden-Meyerhof (EMP) glycolytic route has been clearly demonstrated for *L. reuteri* (1). This was soon realized to be an important constraint, since in its absence, FBA would always predict EMP to be optimal, with

important consequences for the growth rate. The ratio between the EMP and the PKP pathways was only allowed to fluctuate between 0.19 and 0.36 as determined previously (*idem*), and this constraint was implemented in all simulations presented hereafter. Using the measured consumption rates of CDM components to constrain the solution space and setting biomass formation as the objective function, product formation was predicted. The optimal solution predicted by FBA was compatible with the production of either lactate and a proton or ethanol and carbon dioxide (Table 4.1, simulation A). Flux variability analysis showed that the metabolic network can freely distribute fluxes between these stoichiometrically equivalent routes, with the exception of a minimum flux to ethanol as a result of the imposed split ratio between the PKP and EMP.

Subsequently, we proceeded to simulate *in silico* the effect of glycerol in the metabolism of *L. reuteri* cultivated in CDM. This was realized by performing a robustness analysis of the exchange reaction for glycerol, *i.e.* titrating glycerol into the system and assessing what is its impact on the maximal growth rate, using the constraints of simulation A (Fig. 4.1). The model predicts that glycerol will have a prominent effect on the growth rate of *L. reuteri* cultured in CDM with a predicted increase as high as 30%. At glycerol uptake rates higher than 19.54 $mmol.h^{-1}.gDW^{-1}$, the model predicts a growth rate limitation other than energetic.

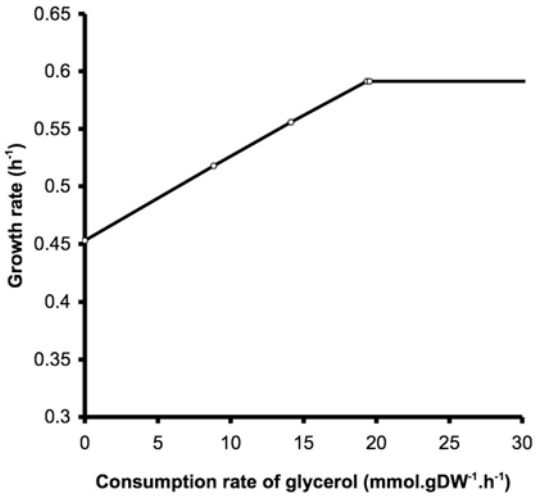


Figure 4.1. *In silico* simulation (robustness analysis) of the effect of an increasing glycerol consumption rate on the set objective function (growth rate).

Table 4.1. Flux variability analysis of model simulations under different constraints setting maximized biomass formation as objective function. The full simulation details are available in Table 4.S2.1 (supplementary material 4.S2)

	Reaction	Simulation A		Simulation B		Simulation C	
		Median	Span	Median	Span	Median	Span
Exchange reactions	EX_etoh(e)	31.3864	30.4692	23.8455	46.3075	12.6146	23.8457
	EX_lac-L(e)	15.2342	30.4684	17.4053	34.8105	11.7898	23.5796
	EX_h(e)	14.4984	30.4684	16.0263	34.8105	21.6417	23.5796
	EX_co2(e)	33.0311	30.4684	31.2728	34.8105	25.6573	23.5796
	EX_ac(e)	0.2844	0.0008	5.7485	11.4970	11.3639	0.2661
	EX_13ppd(e)	0.0004	0.0008	16.4424	11.4970	22.0579	0.2661
Network reactions	ACALD	-31.3864	30.4692	-23.8455	46.3075	-12.6146	23.8457
	ALCD2x	-31.3864	30.4692	-23.8455	46.3075	-12.6146	23.8457
	PDH	15.2342	30.4684	17.4053	34.8105	11.7898	23.5796
	PTAr	-17.1069	0.0008	-7.5254	11.4970	-1.9100	0.2661
	GLYK	0.0004	0.0008	5.7485	11.4970	0.1330	0.2661
	DHAPT	0.0004	0.0008	5.7485	11.4970	0.0000	0.2661
	TPI	6.2921	0.0008	10.4971	11.4970	4.8817	0.2661
	GLYCDx	0.0004	0.0008	5.7485	11.4970	0.0000	0.2661
	GDHYD	0.0004	0.0008	16.4424	11.4970	22.0579	0.2661
	PPN13D	-0.0004	0.0008	-16.4424	11.4970	-22.0579	0.2661
	ACKr	-0.2182	0.0008	-5.6732	11.4970	-11.2887	0.2661
	PYK	5.3667	0.0008	9.4452	11.4970	3.8297	0.2661
	ENO	30.1429	0.0008	28.7105	11.4970	23.0950	0.2661
	PGM	30.1429	0.0008	28.7105	11.4970	23.0950	0.2661
	GAPD	30.1429	0.0008	28.7105	11.4970	23.0950	0.2661
	PGK	30.1429	0.0008	28.7105	11.4970	23.0950	0.2661

Note: In simulation A the measured uptake fluxes of CDM components were used as constraints; in simulation B the measured uptake fluxes of components of CDM supplemented with glycerol were entered as constraints; simulation C is similar to simulation B, except that now the fluxes GLYCDx and GDHYD were made irreversible in resemblance to what is suggested by the expression data.; Median and Span are expressed as $mmol.h^{-1}.gDW^{-1}$; EX_etoh(e), exchange of ethanol; EX_lac-L(e), exchange of lactate; EX_h(e), exchange of protons; EX_co2(e), exchange of carbon dioxide; EX_ac(e), exchange of acetate; EX_13ppd(e), exchange of 1,3-propanediol; ACALD, acetaldehyde dehydrogenase (acetylating); ALCD2x, alcohol dehydrogenase; PDH, pyruvate dehydrogenase; PTAr, phosphotransacetylase; GLYK, glycerate kinase; DHAPT, dihydroxyacetone phosphotransferase; TPI, triosephosphate isomerase; GLYCDx, glycerol dehydrogenase; GDHYD, glycerol dehydratase; PPN13D, 1,3-propanediol dehydrogenase; ACKr, acetate kinase; PYK, pyruvate kinase; ENO, enolase; PGM, phosphoglycerate mutase; GAPD, glyceraldehyde 3-phosphate dehydrogenase; PGK, phosphoglycerate kinase.

We experimentally determined the consumption rate of glycerol under these conditions and found it to be close to, but somewhat higher than, the *in silico* predicted optimal uptake rate (22.19 $mmol.h^{-1}.gDW^{-1}$). Consequently, when using this flux to constrain the glycerol exchange reaction in a new simulation (data not shown), glycerol was not totally consumed. A very significant reduced cost (*i .e.* impact on the objective function if the flux to a certain metabolite slightly increases) was observed for several amino acids. This reduced cost, most prominently observed for aspartate, indicated that either these amino acids limit growth during glucose/glycerol co-fermentation, or amino acid fluxes are affected by the presence of glycerol (note that we used the fluxes of the glucose fermentation to constrain these fluxes).

At this stage, we used data on the consumption rate of all amino acids as boundary conditions to the network in a new simulation (Table 4.1, simulation B). We found that also in the glycerol/glucose co-fermentation growth is most likely energy-limited. FVA of this simulation reflects a second degree of freedom, besides the one found for simulation A. Even though most glycerol is going to 1,3-propanediol, a significant amount is free within the FBA-predicted optimum to be channeled to pyruvate yielding ATP, and subsequently be converted to acetate and ethanol generating more ATP and NAD^+, respectively. The span of several reactions of lower glycolysis confirms this interpretation of the network behavior.

To challenge our interpretation of the previous simulation, we constrained the reversibility of glycerol dehydrogenase and glycerol dehydratase (so that no glycerol could be channeled into glycolysis whilst still allowing biosynthesis of glycerol 3-phosphate) and ran a new simulation (Table 4.1, simulation C). FVA of this new metabolic network state confirmed that under these conditions glycerol is indeed mostly used as an electron acceptor, enabling the re-routing of acetyl phosphate adjuvant of the phosphoketolase pathway to acetate instead of ethanol, leading to the production of extra ATP. Again, the flexibility between lactate and proton or ethanol and carbon dioxide production remains as two stoichiometrically equivalent routes.

Characterization of growth in the presence or absence of glycerol. In the presence of glycerol, the differences in the metabolism of *L. reuteri* result in a shift in end-product formation and a faster acidification rate (data not shown). In an attempt to distinguish the effect of glycerol from other potential responses evoked by a varying pH, we chose to carry out further experimentation in a fixed pH environment. Under such conditions, *L. reuteri* reaches a final cell density in CDM complemented with glycerol approximately 30% higher than in CDM alone (Fig. 4.2).

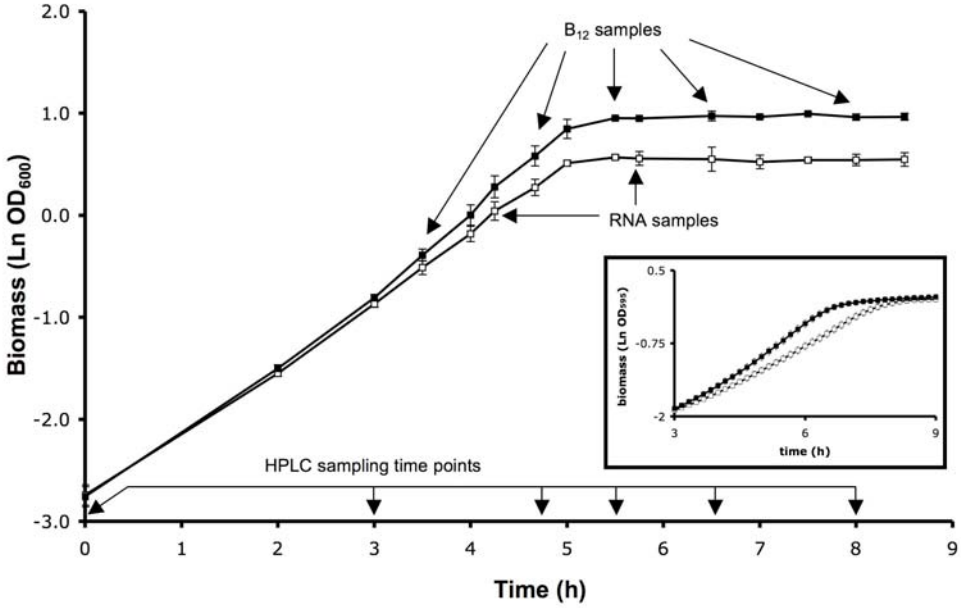


Figure 4.2. *L. reuteri* biomass formation and sampling scheme in pH-controlled batch fermentations of CDM in the presence (black squares) or absence (white squares) of glycerol and growth rate determination in 96-well microtiterplates (inset).

In CDM without glycerol, one molecule of glucose yields one molecule of lactate and 0.9 molecules of ethanol (Fig. 4.3a.). While in CDM supplemented with glycerol, one molecule of glucose is converted to one molecule of lactate, 0.3 molecules of ethanol and 0.6 molecules of acetate, along with the formation of 1.2 molecules of 1,3-propanediol derived from glycerol (Fig. 4.3b.).

The utilization of glycerol as an electron acceptor is initiated with a B_{12}-dependent reaction. We did not supply any exogenous source of B_{12}, and therefore, to run this pathway *L. reuteri* relied on its own native production. We measured B_{12} production under both conditions and indeed in CDM supplemented with glycerol it is five fold higher compared with CDM without (Fig. 4.4).

The examination of the growth curves (Fig. 4.2) indicates that the increase in biomass yield in the presence of glycerol is accompanied by an increase in μ_{max} as well. We observed a 22% increase of μ_{max} in batch fermentations regardless of whether pH was fixed or not, and this was confirmed by the accurate determination of μ_{max} (inset Fig. 4.2).

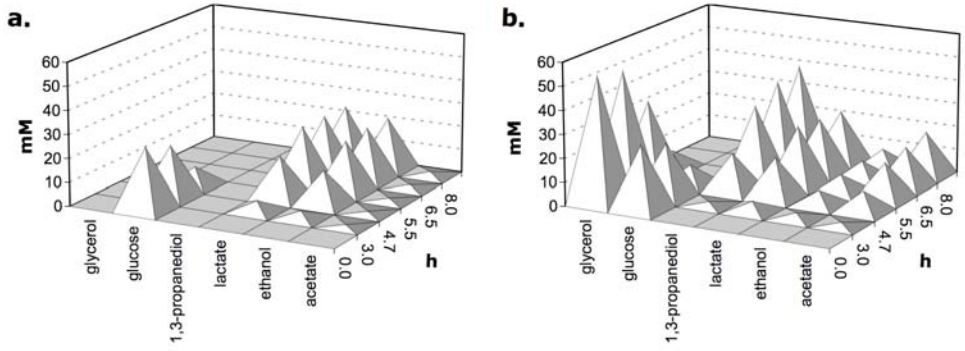


Figure 4.3. Substrate consumption and product formation by L. reuteri during pH-controlled batch fermentations of CDM (a.) in comparison to CDM supplemented with glycerol (b.)

Finally, in order to challenge previously unsuspected model predictions, we measured the consumption rate of all twenty standard amino acids in pH-controlled batch fermentations of CDM in the presence or absence of glycerol. Twelve were found to have their consumption rate affected by the presence of glycerol (Table 4.2). Only the consumption rate of arginine, histidine and tyrosine was decreased, while the consumption rate of alanine, aspartate, cysteine, glutamate, glycine, isoleucine, leucine, methionine and serine was increased.

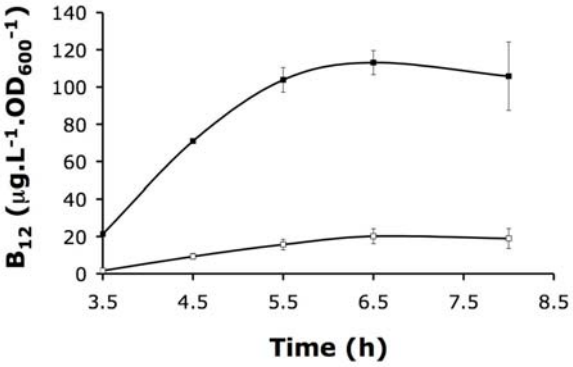


Figure 4.4. Vitamin B_{12} production by *L. reuteri* in CDM supplemented with glycerol (black squares) or not (white squares).

Table 4.2. Consumption rate of amino acids that are affected by the presence of glycerol

	Consumption rate ($mmol.h^{-1}.gDW^{-1}$) in the presence of:	
	Glucose	Glucose and Glycerol
Alanine	0.27 ± 0.09	0.36 ± 0.05
Arginine	0.57 ± 0.06	0.39 ± 0.09
Aspartate	0.40 ± 0.02	0.56 ± 0.05
Cysteine	0.42 ± 0.03	0.58 ± 0.17
Glutamate	0.22 ± 0.01	0.30 ± 0.06
Glycine	0.37 ± 0.06	0.65 ± 0.02
Histidine	0.07 ± 0.03	0.04 ± 0.01
Isoleucine	0.17 ± 0.08	0.25 ± 0.08
leucine	0.26 ± 0.08	0.38 ± 0.02
Methionine	0.07 ± 0.00	0.13 ± 0.04
Serine	0.55 ± 0.02	0.60 ± 0.03
Tyrosine	0.20 ± 0.13	0.13 ± 0.01

Global transcriptional response of L. reuteri to the presence of glycerol. To gain a broader perspective on the effects of cultivating *L. reuteri* in the presence of glycerol, we developed and applied cDNA microarrays to study its genome-wide transcriptional response. To assist in experimental design, and simultaneously develop and validate our transcriptomic platform and procedures, we carried out an exploratory set of experiments. Worth mentioning is the comparison of the transcriptional response of *L. reuteri* during late-logarithmic phase of batch fermentations of CDM supplemented, or not, with glycerol. These microarray results suggested a change in flux distribution leading to different end products, and as expected, glycerol-utilizing genes were differentially expressed, along with the B_{12}-biosynthesis cluster. For reasons already explained above, to isolate as much as possible the specific glycerol response from other indirect effects, the main focus of the transcriptome experiments was directed to the pH-controlled batch fermentations. Three comparisons were carried out to study the effect of glycerol specifically in mid-exponential phase (exp) and in early–stationary phase (sta), and to identify changes caused by the presence of glycerol in the transcriptional response between mid-log and early-stationary growth phases (phase shift, exp-sta). Defining differential as an absolute Log_2 (ratio) > 0.585 and a *p*-value < 0.05, we found 228 differentially regulated genes (93 up- and 135

down-) in the mid-logarithmic phase response, 643 in the early-stationary phase response (325 up- and 318 down-), and 479 in the phase shift response (231 up- and 248 down-) data sets (Fig. 4.5). Respectively, 98, 186 and 48 genes are unique to exponential, stationary and phase shift comparisons and only 92 are found in all. A detailed list of all genes found to be differentially expressed is available as supplementary material 4.S3 (Table 4.S3.1).

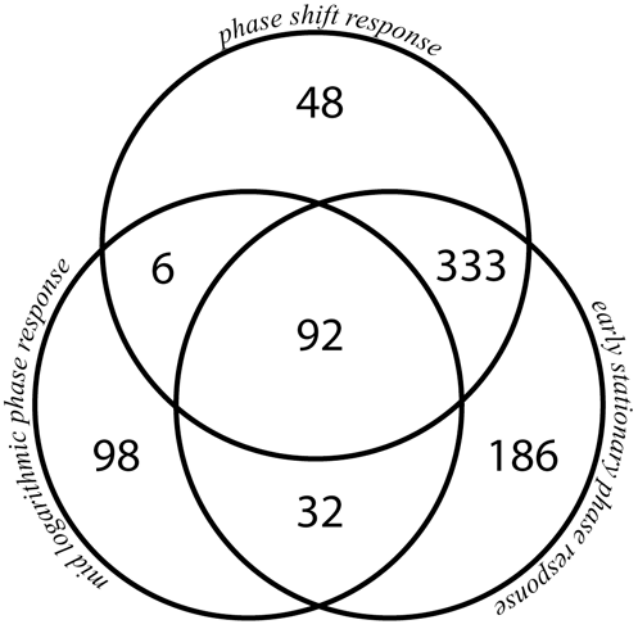


Figure 4.5. Overlap between comparisons established to identify mid-log and late-log specific glycerol responses, and to detect the effect of glycerol in the response to the shift between mid-logarithmic and early-stationary growth phases.

In order to facilitate the analysis of the expression data, we clustered the genes that were differentially expressed according to the category of their assigned COG (44). The full distribution is available in Table 4.S3.2 (supplementary material 4.S3) and a small selection is present in Fig. 4.6. Considering the model predictions and the measured flux distribution already presented here, it is not surprising that a large proportion of the genes found to be differentially expressed by glycerol is involved in energy production and conversion (34% in exp and exp-sta, and 47% in sta), and strikingly overrepresented as up-regulated in the COG category comprising genes involved in secondary metabolites biosynthesis, transport and catabolism. We found key genes regulated in the presence of glycerol that can account for the observed shift from ethanol to acetate production relying on the formation of 1,3-propanediol to regenerate NAD^+ (Fig. 4.2). Amongst others, alcohol dehydrogenase (EC

1.1.1.1), phosphate acetyltransferase (EC 2.3.1.8) and pyruvate dehydrogenase α- and β-subunits were down-regulated in the presence of glycerol, while acetate kinase, 1,3-propanediol dehydrogenase (EC 1.1.1.202) and all the subunits of the B_{12}-dependent glycerol dehydratase (EC 4.2.1.30) were up-regulated.

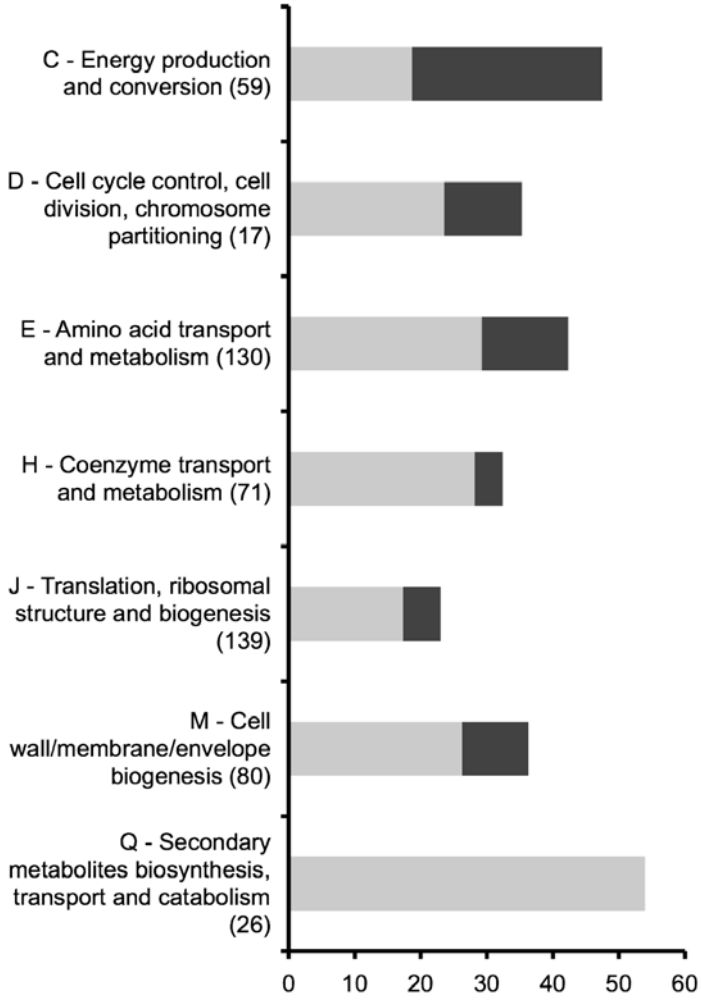


Figure 4.6. Distribution of transcripts whose expression was affected by glycerol during early-stationary growth phase throughout a selection of COGs (see Supplementary Material 4.S3, Table 4.S3.2 for full distribution). Transcripts were considered to be differentially regulated for $M > |0.585|$ and p-value < 0.05. Between brackets is the total number of genes from the genome of *L. reuteri* included in the corresponding COG. Light and dark grey bars represent proportion of up-regulated transcripts in the presence or absence of glycerol, respectively.

There is another obvious overrepresentation of up-regulated genes by glycerol in the coenzyme transport and metabolism category (Fig. 4.6). This can be accounted for by the up-regulation of the full length of the ~26 kb B_{12}-biosynthesis gene cluster, causing the increased production of this co-factor.

The response to glycerol also involved the differential regulation of genes assigned to categories such as D – cell cycle control, cell division, chromosome partitioning; J – translation, ribosomal structure and biogenesis; and M – cell wall biogenesis. These are most likely related to the increased μ_{max} in the presence of glycerol, which was predicted by constraint-based modeling tools and experimentally verified (Fig. 4.2).

The response of *L. reuteri* to glycerol had a conspicuous effect in genes assigned to amino acid transport and metabolism. Almost 50% of the 130 genes included in this category were differentially regulated, of which 60-70% emerged as being up-regulated in response to glycerol (Fig. 4.6). This previously unsuspected observation can be partially attributed to the increased metabolic activity of cells cultivated in CDM supplemented with glycerol, since this puts an increased demand on protein synthesis. However, this cannot fully explain the drop in consumption rate measured for three amino acids and the large amount of genes involved in amino acid metabolism that are up-regulated in the presence of glycerol. This issue will be further addressed later.

Motif searches. We searched the upstream region of the transcriptional units harboring genes that were differentially regulated for the presence of conserved motifs. The mid-logarithmic phase response data set, proved to be the most useful for this purpose. It contains the least number of transcriptional units (Fig. 4.5), increasing the chances that a particular motif is overrepresented. We analyzed all motifs that had a lower E-value than the Shine-Dalgarno sequence and found three to be relevant (Fig. 4.7).

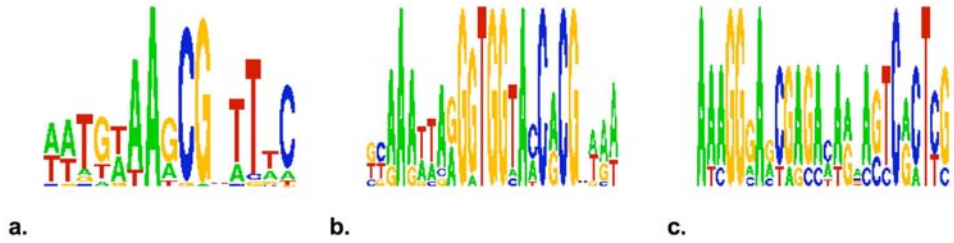


Figure 4.7. Regulatory motifs overrepresented in the upstream regions of TUs found to harbor differently regulated genes in the mid-logarithmic phase response data set. From left to right, a. CRE-box; b. T-box; c. motif X (putatively associated with lipid metabolism).

Two of them (Fig 7a. and b.) were easily recognizable as a carbon responsive element (CRE-box) and a regulatory motif involved in amino acid metabolism (T-box). The motif present in Fig. 4.7c. remains to the best of our knowledge uncharacterized. It displays a possible inverted repeat structure, and its distribution throughout the genome of *L. reuteri* suggests it to be most likely associated with lipid metabolism (Table 4.3).

Table 4.3. Downstream genes of the motif X (Fig. 4.7c.)

ORF	Function	Position	E-value
Lreu_0025	phosphatidylglycerophosphatase A	-67	1.0×10^{-15}
Lreu_0932	Fibronectin-binding A domain protein	-132	2.4×10^{-14}
Lreu_1841	unknown function DUF915, hydrolase family protein	-3	2.4×10^{-14}
Lreu_1865	type I phosphodiesterase/nucleotide pyrophosphatase	-1244	2.4×10^{-14}
Lreu_1887	diguanylate phosphodiesterase	-30	2.4×10^{-14}
Lreu_0942	2,5-didehydrogluconate reductase	-26	3.5×10^{-14}

Discussion

Glycerol has an effect on the growth behavior of *L. reuteri*, which extends far beyond what was previously thought, improving greatly the growth performance of this humane isolate. Here we integrate *in silico* predictions with information from the several experimental approaches, discussing from a systems biology perspective, the effect of glycerol in the several branches of metabolism and different levels of information transfer.

Effect of glycerol on the redox balance of central carbon metabolism. Maximization of biomass formation in model simulations of *L. reuteri* cultured in CDM supplemented with glycerol predicted a shift from ethanol to acetate formation leading to additional ATP production. This shift was indeed observed experimentally (Fig. 4.3). However, the production of acetate leads to a redox unbalance since it does not permit the regeneration of any $NAD(P)^+$ moiety. This metabolic strategy is only viable because glycerol is used here as an electron acceptor, regenerating one NAD^+ per 1,3-propanediol molecule formed. As stated above, *L. reuteri* has been shown to possess two distinctive glycolytic routes (1), the PKP being clearly dominant over the EMP. Via the PKP two additional NAD(P)H molecules are formed, which are balanced by the conversion of acetyl

phosphate to ethanol. When glycerol is present, acetyl phosphate can be converted to acetate yielding one ATP with a theoretical acetate:1,3-propanediol ratio of 2:1. This ratio was observed experimentally with the synthesis of 1.2 molecules of 1,3-propanediol per 0.6 molecules of acetate deriving from 1 molecule of glucose.

The synthesis of 1,3-propanediol from glycerol is a two-step enzymatic conversion, relying on the B_{12}-dependent glycerol dehydratase and 1,3-propanediol dehydrogenase (9). Recently, the presence of microcompartments (metabolosomes) has been shown in the *L. reuteri* strain used, along with their connection with glycerol and diol dehydratase activity (38). The genes encoding the metabolosomes and the several subunits of the dehydratase are clearly up-regulated in the presence of glycerol, corroborating model simulations and the measured flux distribution (Fig. 4.3). Since B_{12} is not present in CDM, under these conditions *L. reuteri* must synthesize it *de novo*. The B_{12} biosynthesis gene cluster was found to be upregulated in the presence of glycerol. The increased production of this industrially relevant compound was confirmed and is a valuable asset of the glucose/glycerol co-fermentation (Fig. 4.4). The model was not useful for quantitative predictions related to B_{12}, which can be easily understood as it represents only a minor elemental flux. For instance, the flux to B_{12} is at least five orders of magnitude smaller than the one to lactate. Another feature of B_{12} production is the clear increase during the later stages of growth. This has been reported for a different strain in a medium without glycerol (33), and was here observed both in the presence and absence of glycerol.

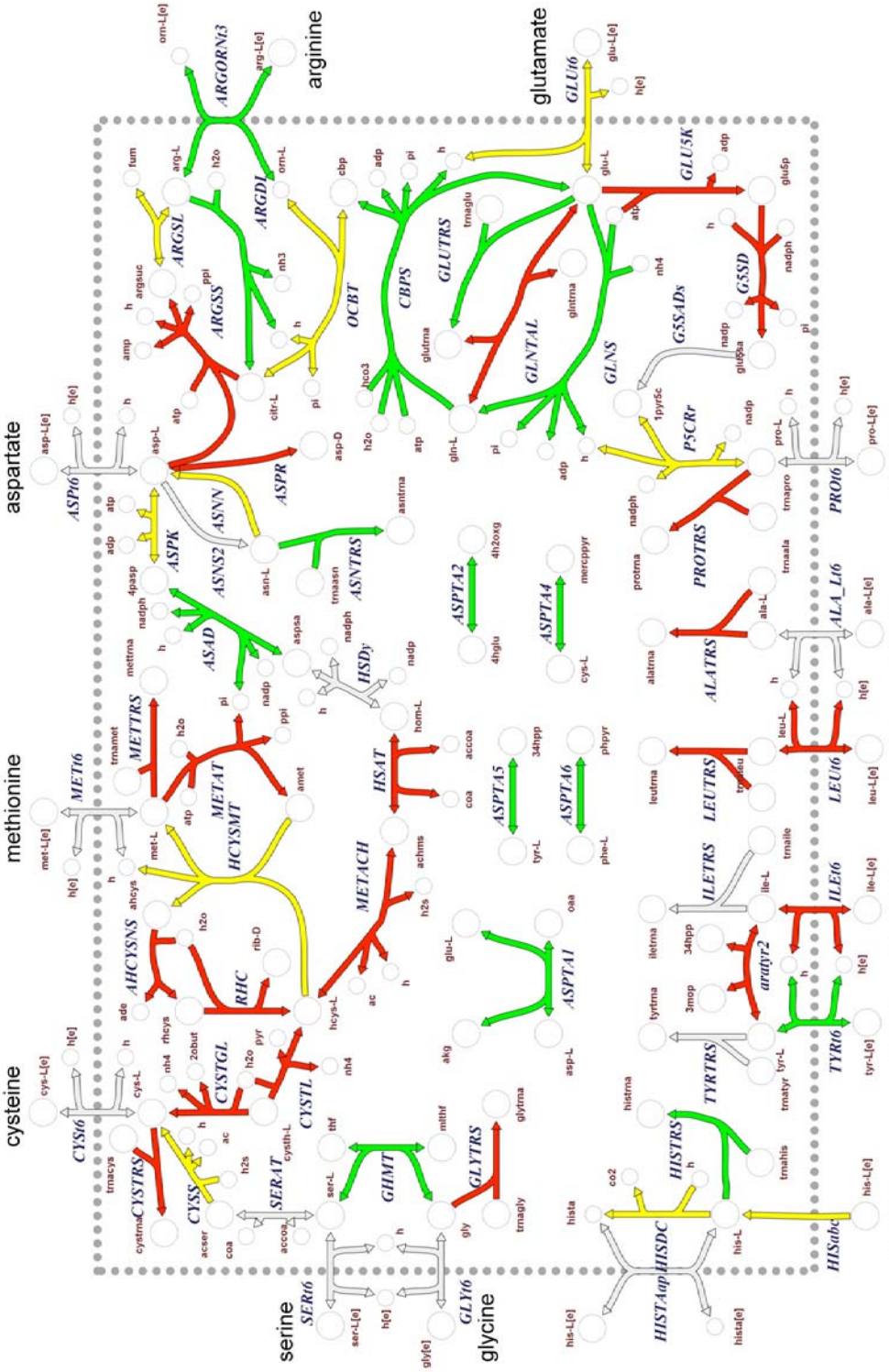
FVA of the model simulation in which, the measured uptake fluxes of components of CDM supplemented with glycerol were entered as constraints, permitted a span between 1,3-propanediol and acetate (Table 4.1, simulation B). Interestingly, the fermentation of glycerol through a dismutation process involving an oxidative pathway and another for the regeneration of reduced equivalents has been extensively characterized in *Klebsiella pneumoniae* and *Clostridium freundii* (9). However, under these conditions lactobacilli have not been reported to be able to grow on glycerol as their sole carbon source (38). To the best of our knowledge, the reason for this remains elusive. The transcriptome results shed some light on this issue, by evidencing a sharp downregulation of several reactions crucial for the channeling of glycerol to the lower part of glycolysis, namely glycerol dehydrogenase, glycerol kinase, dihydroxyacetone phosphate synthetase, amongst others. Interestingly, through an arduous process of adaptation, *L. plantarum* has been evolved to grow in the presence of heme and oxygen (for regeneration of reduced equivalents) with glycerol as its sole carbon source. Enzymes that we have found to be tightly repressed in the presence of glycerol are, in the adapted strain, severely deregulated by the presence of a single point mutation in a promoter region (Teusink, et al. personal communication).

Effect of glycerol on growth rate. We have reported that the synthesis of B_{12}, together with the activation of the flanking cluster that encodes the assembly of metabolosomes and glycerol dehydratase subunits, requires the expression of ~2% of the total genome of *L. reuteri* (33). Yet, model simulations indicate an increase in μ_{max} as high as 30% in CDM supplemented with glycerol (Fig. 4.1). For all conditions tested, in the presence of glycerol *L. reuteri* has a higher μ_{max} when compared to the same situation without glycerol. The increase in μ_{max} when an external electron acceptor is available has also been observed previously (1). They report an increase of 46% when fructose is supplied in addition to glucose.

Considering all the changes in primary metabolism that we have described above, it was not surprising to find a CRE-box as being overrepresented in the systematic analysis of all *cis* regions of genes that were differentially regulated with glycerol (Fig. 4.7a.). This is the binding site of the general carbon catabolite control protein, CcpA, that regulates several PTS systems and other genes involved in sugar metabolism. Even though it can act as an activator in special circumstances, it is most often a repressor (53). The CRE-box is mostly found upstream of genes that are down-regulated with glycerol. This is indicative of a decreased carbon catabolite repression when glycerol is available due to which glycolysis can operate faster, a situation that is compatible with the observed increase in μ_{max}.

Effect of glycerol on amino acid metabolism. A model simulation in which the measured glycerol uptake rate was added as a constraint to simulation A (Table 4.1), first suggested the effect of glycerol on amino acid metabolism. The incongruence between measured and predicted growth rate indicated that either the limitation was no longer energetic, or that the consumption rates of amino acids were significantly altered. The latter turned out to be correct, as evidenced by the measured fluxes and transcriptome response. In accordance, we found a regulatory motif with structure typical of a T-box clearly overrepresented in the upstream region of TUs that harbor genes that were up-regulated with glycerol. T-boxes are associated with regulation of amino acid availability and are usually located upstream of aminoacyl tRNA synthetases and amino acid transport and biosynthesis genes (51), which was confirmed by the output of the MAST search (data not shown).

Even though lactic acid bacteria have amino acid auxotrophies, they still conserve a considerable, yet varying, degree of flexibility to interconvert the amino acids that they scavenge from the environment. This interconnectivity is complex and hard to navigate through unaided with visualization tools. Illustrating the potential of the genome scale metabolic model to visualize experimental data, we have projected the expression profiles of the differentially expressed genes in the stationary-phase response data set, on a


cysteine
methionine
aspartate
arginine
glutamate
serine
glycine
histidine
tyrosine
isoleucine
leucine
alanine
proline
CYSt6
CYSTRS
CYSS
SERAT
CYSTL
CYSTGL
RHC
AHCYSNS
METAT
HCYSMT
METTRS
METt6
SERt6
GHMT
GLYTRS
GLYt6
METACH
HSAT
ASPt6
ASPK
ASAD
ASNS2
ASNN
ASPR
ASNTRS
HSDy
ASPTA1
ASPTA2
ASPTA4
ASPTA5
ASPTA6
ARGORNt3
ARGSL
ARGSS
ARGDL
OCBT
CBPS
GLUTRS
GLNTAL
GLNS
GLUt6
GLU5K
G5SD
G5SADs
P5CRr
PROTRS
PROt6
ALATRS
ALA_Lt6
LEUTRS
LEUt6
ILETRS
ILEt6
aratyr2
TYRTRS
TYRt6
HISTRS
HISDC
HISTAap
HISabc
cys-L[e]
h[e]
met-L[e]
ser-L[e]
gly[e]
his-L[e]
tyr-L[e]
ile-L[e]
leu-L[e]
ala-L[e]
pro-L[e]
glu-L[e]
asp-L[e]
arg-L[e]
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hista[e]

metabolic map depicting the interconnectivity of the amino acids found to have a different consumption rate in the presence or absence of glycerol (Fig. 4.8). This exercise enabled us to identify some previously unsuspected differences between the amino acid metabolism of cells cultured in the presence or absence of glycerol described below.

The consumption rates of alanine, leucine and glycine are increased in the presence of glycerol. This is in accordance with the microarray results, in which their cognate aminoacyl tRNA synthetases are up-regulated. For proline, the corresponding aminoacyl tRNA synthetase was also found to be up-regulated in the presence of glycerol, but we could not see a repercussion of this on the consumption rate of this amino acid. The additional source of proline became obvious when the transcriptomics data were projected on the metabolic map. Glutamate has an increased consumption rate and yet most reactions known to use it as a substrate, except for two, are either not differentially expressed or down-regulated. One upregulated reaction leads to glutamine, which can be easily understood by the fact that the latter is not present in CDM. Whereas the other, is the first-step in an amino acid interconversion pathway that culminates in proline, and for which at least one more reaction surpasses the stringency of our criteria to be considered up-regulated.

Several transcripts associated to reactions related to sulfur amino acids were up-regulated in the presence of glycerol. This was corroborated by the measured consumption rates, however the underlying reason remains elusive. The consumption rate of serine is also slightly increased and since its conversion to glycine is clearly down-regulated, it is most likely being channeled to cysteine biosynthesis. This is supported by the fact that the expression of Lreu_1553 encoding cysteine synthase (EC 2.5.1.47) tends to be upregulated more than two-fold (p-value < 0.1). We speculate that this increased uptake and biosynthesis of cysteine might be related to either metal ion binding and/or its involvement in the reduction of proteins by thiol-disulfide exchange. The fact that Lreu_0539, encoding thioredoxin, is also up-regulated tentatively supports the latter conjecture.

Figure 4.8. (opposite page) Projection of the expression profiles of the differentially regulated genes in the stationary-phase response data set, on a metabolic map depicting the interconnectivity of the amino acids found to have a different consumption rate in the presence or absence of glycerol.

From the three amino acids whose consumption rates were lower in the presence of glycerol, only histidine also shows a down-regulation of the related aminoacyl tRNA synthetase. We could not find any significant change in amino acid composition of differentially regulated proteins that could eventually account for this observation. Nonetheless, the transcriptomic data is in full accordance with measured fluxes.

As for arginine, the lower expression of the arginine/ornithine antiporter is in agreement with the decrease in consumption rate in the presence of glycerol. However, since the esterification of arginine to its cognate tRNA does not appear down-regulated, it is not unreasonable to hypothesize that there might be an additional source of this amino acid. The projection of the microarray data on the metabolic maps evidences aspartate as the most likely candidate. The uptake rate of aspartate is 40% higher in the presence of glycerol, and in resemblance with glutamate, most reactions using it as a substrate do not appear to be up-regulated. Besides aspartate racemase (EC 5.1.1.13), only Lreu_0731 encoding argininosuccinate synthetase (EC 6.3.4.5) appeared up-regulated in the presence of glycerol. Argininosuccinate can be cleaved into fumarate and arginine by argininosuccinate lyase (EC 4.3.2.1), whose encoding gene, Lreu_0732 showed a tendency to be up-regulated (1.5-fold change, p-value < 0.2).

Finally, the decreased uptake of tyrosine seems to be intimately related to the increased uptake of isoleucine. For a reason not completely understood, in the presence of glycerol, *L. reuteri* reduces its consumption rate of tyrosine proportionally to its increase in that of isoleucine (0.07 *versus* 0.08 $\mu mol.l^{-1}.h^{-1}.gDW^{-1}$). The latter is then converted to tyrosine via tyrosine aminotransferase, of which the enconding gene, Lreu_1200, is up-regulated in the presence of glycerol.

Final remarks. In this study, we have constructed a genome-scale metabolic network model of *L. reuteri* and used it to deepen our understanding of the physiological and transcriptional responses during glucose and glycerol co-fermentation. We were able to predict and demonstrate for this well-studied condition, the involvement of previously unsuspected metabolic pathways, for instance, related to amino acids. Briefly, the genome-scale model proved to be very useful *(i)* for the identification of stoichiomterically equivalent metabolic routes which are compatible with maximization of the objective function; *(ii)* for the visualization of large experimental data sets; and *(iii)* for the integration of model predictions with experimental data.

Part of the predictive power of FBA in *L. reuteri* resides in the absence of pyruvate formate lyase (PFL). In the genome-scale models of other lactic acid bacteria, FBA stubbornly predicts acetate in detriment of lactate to be produced, since this route yields extra free energy (47). This is in contrast with *L. reuteri* where we have shown to be a reasonable agreement between model-simulated and experimentally determined fluxes.

FBA has revealed that the heterofermentative behavior of *L. reuteri* appears to be justified by the dominance of PKP over EMP. A finding that can most likely be extrapolated to other biological systems. The fact that the primary metabolism of *L. reuteri* is so much dependent on external electron acceptors instigates us to hypothesize about the surplus of such compounds in its environmental niche. This has also been noted previously while studying another human isolate of *L. reuteri*, strain ATCC 55730 (1).

The multiple comparisons established for the measurements of the global transcriptome response were important to gain new insight into potential regulatory mechanisms. Additionally, they provided us with new leads for metabolic engineering that could potentially assist the development/improvement of industrially relevant processes, as foreseen previously (45). The modeling exercise demonstrated here of 1,3-propanediol production independent of exogenous vitamin B_{12} supply, is particularly relevant in the current economical and environmental context. 1,3-propanediol can be used as a starting material for the production of plastics, providing an alternative to oil-based non-renewable sources (9).

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Content of supplementary materials to Chapter 4

- Supplementary material S1 to Chapter 4: **Genome-scale metabolic network model**. This document presents the full details of all reactions and their many-to-many associations with genes. Abbreviations of metabolites and reactions are provided, along with EC codes if they exist. Page 114.
- Supplementary material S2 to Chapter 4: **Model simulations**. Here one can find the full details of the several *in silico* simulations carried out in this study. Both the given constraints and the flux variability analysis output are listed for all model reactions. Maximal biomass formation (*i.e.* maximize growth rate) was set as the objective function. Page 143.
- Supplementary material S3 to Chapter 4: **cDNA microarrays experiments**. This file consists of two tables, one listing the complete array results and one depicting their relative abundance through out the several categories of clusters of orthologous groups (COG). Page 179.

Content of supplementary material S1 of Chapter 4:
Genome-scale metabolic network model

Table 4.S1.1. Complete list of model reactions and their many-to-many associations with genes. EC numbers are provided when available.

Abbrev.	Name	Equation	Gene[a.]	EC number
G6PI	Glucose-6-phosphate isomerase	[c] : g6p <==> g6p-B	RLRE01355	EC-5.3.1.9
MNTD	D-mannitol dehydrogenase	[c] : mnl + nad <==> fru + h + nadh	RLRE00076	EC-1.1.1.67
MNTD2	mannitol 2-dehydrogenase (NADP+ as acceptor)	[c] : mnl + nadp <==> fru + h + nadph	RLRE00076	
MCMAT2	Butyryl-[acyl-carrier protein]:malonyl-[acyl-carrier-protein]C-acyltransferase (decarboxylating)	[c] : butacp + h + malacp --> 3oxhacp + acp + co2	RLRE01405	EC-2.3.1.41
MCMAT3	Hexanoyl-[acyl-carrier protein]:malonyl-[acyl-carrier-protein] C-acyltransferase	[c] : h + hexacp + malacp --> 3oxocacp + acp + co2	RLRE01405	EC-2.3.1.41
MCMAT4	Octanoyl-[acyl-carrier protein]:malonyl-[acyl-carrier-protein] C-acyltransferase (decarboxylating)	[c] : h + malacp + octacp --> 3oxdeacp + acp + co2	RLRE01405	EC-2.3.1.41
MCMAT5	Decanoyl-[acyl-carrier protein]:malonyl-[acyl-carrier-protein] C-acyltransferase	[c] : decacp + h + malacp --> 3oxddacp + acp + co2	RLRE01405	EC-2.3.1.41
MCMAT6	Dodecanoyl-[acyl-carrier-protein]:malonyl-[acyl-carrier-protein] C-acyltransferase	[c] : ddeacp + h + malacp --> 3oxtdacp + acp + co2	RLRE01405	EC-2.3.1.41
MCMAT7	Tetradecanoyl-[acyl-carrier-protein]:malonyl-[acyl-carrier-protein] C-acyltransferase	[c] : h + malacp + tdeacp --> 3oxhdacp + acp + co2	RLRE01405	EC-2.3.1.41
MCMAT8	Hexadecanoyl-[acyl-carrier-protein]:malonyl-[acyl-carrier-protein] C-acyltransferase	[c] : h + hdeacp + malacp --> 3oxocdacp + acp + co2	RLRE01405	EC-2.3.1.41
G1SAT	glutamate-1-semialdehyde aminotransferase	[c] : glu1sa <==> 5aop	RLRE00281	EC-5.4.3.8
HMBS	hydroxymethylbilane synthase	[c] : h2o + (4) ppbng --> hmbil + (4) nh4	RLRE00279	EC-4.3.1.8
PPBNGS	porphobilinogen synthase	[c] : (2) 5aop --> h + (2) h2o + ppbng	RLRE00280	EC-4.2.1.24
R05218	Cob(II)yrinate a,c diamide <=> Cob(I)yrinate a,c diamide	[c] : (2) co2dam + nadh --> (2) co1dam + h + nad	RLRE00255	
SHCHD	sirohydrochlorin dehydrogenase	[c] : nadp + shcl --> (2) h + nadph + srch	RLRE00277	
SRCHCOC	sirohydrochlorin cobaltochelatase	[c] : cobalt2 + srch --> coprec2 + (2) h	RLRE00270	
UPP3MT	uroporphyrinogen methyltransferase	[c] : (2) amet + uppg3 --> (2) ahcys + h + shcl	RLRE00269	EC-2.1.1.107
UPP3S	uroporphyrinogen-III synthase	[c] : hmbil --> h2o + uppg3	RLRE00269	EC-4.2.1.75
2HXICDH	L-2-hydroxyisocaproate dehydrogenase	[c] : 4mop + h + nadh <==> 2hxic-L + nad	RLRE00966, RLRE00183, RLRE00434	
2MPDH	2-methylpropanol dehydrogenase	[c] : 2mpal + h + nadh <==> 2mpol + nad	RLRE00247	
AALDH	aryl-alcohol dehydrogenase	[c] : h + nadh + pacald <==> nad + pea	RLRE00720, RLRE01618	EC-1.1.1.90
ABTA	4-aminobutyrate transaminase	[c] : 4abut + akg --> glu-L + sucsal	RLRE01065	EC-2.6.1.19
ACACT1	acetyl-CoA C-acetyltransferase	[c] : (2) accoa --> aacoa + coa		EC-2.3.1.9
ACALD	acetaldehyde dehydrogenase (acetylating)	[c] : acald + coa + nad <==> accoa + h + nadh	RLRE00555	EC-1.2.1.10
ACALDt	acetaldehyde reversible transport	acald[e] <==> acald[c]		
ACBIPGT	Adenosyl cobainamide GTP transferase	[c] : adcobap + gtp + h --> adgcoba + ppi	RLRE00282	
ACCOAC	acetyl-CoA carboxylase	[c] : accoa + atp + hco3 --> adp + h + malcoa + pi	RLRE00830, RLRE01404, RLRE01400, RLRE01401, RLRE01402	EC-6.4.1.2
ACGALpts	N-acetylgalactosamine PTS	acgala[e] + pep[c] --> acgal6p[c] + pyr[c]	RLRE00126, RLRE00764	TC-4.A.6
ACGAMT	UDP-N-acetylglucosamine:undecaprenylphosphate N-acetylglucosamine -1-phosphate transferase	[c] : uacgam + udcpp --> ump + unaga		
ACGApts	N-Acetyl-D-glucosamine transport via PEP:Pyr PTS	acgam[e] + pep[c] --> acgam6p[c] + pyr[c]	RLRE00126, RLRE00764	
ACHBS	2-aceto-2-hydroxybutanoate synthase	[c] : 2obut + h + pyr --> 2ahbut + co2	RLRE01210	
ACKr	acetate kinase	[c] : ac + atp <==> actp + adp	RLRE00506, RLRE00248	EC-2.7.2.1
ACLDC	acetolactate decarboxylase	[c] : alac-S + h --> actn-R + co2	RLRE01209	EC-4.1.1.5
ACLS	acetolactate synthase (Also catalyzes ACHBS)	[c] : h + (2) pyr --> alac-S + co2	RLRE01210	EC-4.1.3.18
ACPS2	acyl-carrier protein synthase	[c] : apoACP + coa --> acp + h + pap	RLRE00613, RLRE01408	EC-2.7.8.7
ACt6	acetate transport in/out via proton symport	ac[e] + h[e] <==> ac[c] + h[c]		TC-2.A.1.13
ACTD	acetoin dehydrogenase	[c] : actn-R + nad <==> diact + h + nadh	RLRE01049	EC-1.1.1.5
ACTNdiff	(R)-acetoin diffusion	actn-R[e] <==> actn-R[c]		
ACTPASE	acylphosphatase	[c] : actp + h2o --> ac + h + pi	RLRE01467	EC-3.6.1.7

Abbrev.	Name	Equation	Gene[a.]	EC number
ADA	Adenosine deaminase	[c] : adn + h + h2o --> ins + nh4	RLRE00559	EC-3.5.4.4
ADCL	4-aminobenzoate synthase	[c] : 4adcho --> 4abz + h + pyr		
ADCOBAK	Adenosyl cobinamide kinase	[c] : adcoba + atp --> adcobap + adp + h	RLRE00282	
ADCOBAPS	Adenosylcobinamide-phosphate synthase	[c] : 1ap2olp + adcobhex + atp --> adcobap + adp + pi	RLRE00260	
ADEt2	adenine transport via proton symport (reversible)	ade[e] + h[e] <==> ade[c] + h[c]		
ADK1	adenylate kinase	[c] : amp + atp <==> (2) adp		EC-2.7.4.3
ADK2	adenylate kinase (Inorganic triphosphate)	[c] : amp + pppi <==> adp + ppi		EC-2.7.4.3
ADPDS	acetyl-diaminopimelate deacetylase	[c] : al26da + h + h2o --> 26dap-LL + ac	RLRE00627	EC-3.5.1.47
ADPRDP	ADPribose diphosphatase	[c] : adprib + h2o --> amp + (2) h + r5p	RLRE00641	EC-3.6.1.13
ADPT	adenine phosphoribosyltransferase	[c] : ade + prpp --> amp + ppi	RLRE01305, RLRE00452	EC-2.4.2.7
ADPTA	acetyldiaminopimelate transaminase	[c] : akg + al26da + h <==> acamoxm + glu-L	RLRE00624	
ADSL1	adenylosuccinate lyase	[c] : dcamp --> amp + fum	RLRE01206	EC-4.3.2.2
ADSL2	adenylosuccinate lyase	[c] : 25aics --> aicar + fum	RLRE01206	EC-4.3.2.2
ADSS	adenylosuccinate synthetase	[c] : asp-L + gtp + imp --> dcamp + gdp + (2) h + pi		EC-6.3.4.4
AGAT_LRE	1-Acyl-glycerol-3-phosphate acyltransferase (Lb plantarum specific)	[c] : (0.12) 2chdeacp + (0.32) 2cocdacp + (0.01) agly3p_LRE + (0.25) cpocdacp + (0.26) hdeacp + (0.02) ocdacp + (0.03) tdeacp --> acp + (0.01) pa_LRE	RLRE00916	EC-2.3.1.51
AGDC	N-acetylglucosamine-6-phosphate deacetylase	[c] : acgam6p + h2o --> ac + gam6p		EC-3.5.1.25
AHCYSNS	adenosylhomocysteine nucleosidase	[c] : ahcys + h2o --> ade + rhcys	RLRE00639	EC-3.2.2.9
AHMMPS	4-amino-5-hydroxymethyl-2-methylpyrimidine synthetase	[c] : air + h2o --> 4ahmmp + gcald + (0.5) o2 + pi	RLRE01633	
AICART	phosphoribosylaminoimidazolecarboxamide formyltransferase	[c] : 10fthf + aicar <==> fprica + thf	RLRE01420	EC-2.1.2.3
AIRC	phosphoribosylaminoimidazole carboxylase	[c] : air + co2 --> 5aizc + h	RLRE01208, RLRE01052, RLRE01207, RLRE01208	EC-4.1.1.21
ALA_Lt6	L-alanine transport in/out via proton symport	ala-L[e] + h[e] <==> ala-L[c] + h[c]		TC-2.A.25
ALALAC	D-alanine-D-lactate ligase (reversible)	[c] : ala-D + atp + lac-D <==> adp + alalac + pi	RLRE01706	
ALAR	alanine racemase	[c] : ala-L <==> ala-D	RLRE00614	EC-5.1.1.1
ALATA_L	L-alanine transaminase	[c] : akg + ala-L <==> glu-L + pyr		EC-2.6.1.2
ALATA_Lr	alanine transaminase	[c] : ala-L + pydx <==> h + pydam + pyr		EC-2.6.1.30
ALATRS	Alanyl-tRNA synthetase	[c] : ala-L + atp + trnaala --> alatrna + amp + h + ppi	RLRE00478, RLRE00479	EC-6.1.1.7
ALCD19	alcohol dehydrogenase (glycerol)	[c] : glyald + h + nadh <==> glyc + nad	RLRE00955, RLRE01297	EC-1.1.1.1
ALCD19_D	alcohol dehydrogenase (D-glyceraldehyde to glycerol, NADP)	[c] : glyald + h + nadph <==> glyc + nadp	RLRE00460	EC-1.1.1.21
ALCD19_L	alcohol dehydrogenase (L-glyceraldehyde to glycerol, NADP)	[c] : Lglyald + h + nadph <==> glyc + nadp	RLRE00460	
ALCD2x	alcohol dehydrogenase (ethanol: NAD)	[c] : etoh + nad <==> acald + h + nadh	RLRE00955, RLRE01297, RLRE00555	EC-1.1.1.1
ALDD2x	aldehyde dehydrogenase (acetaldehyde, NAD)	[c] : acald + h2o + nad --> ac + (2) h + nadh	RLRE00555	EC-1.2.1.3
ALDD8x	aldehyde dehydrogenase (D-glyceraldehyde, NAD)	[c] : glyald + h2o + nad --> glyc-R + (2) h + nadh	RLRE00555	EC-1.2.1.3
ALKP	alkaline phosphatase	[c] : dhap + h2o --> dha + pi		EC-3.1.3.1
ALOX	oxidative decarboxylation of acetolacate (chemical)	[c] : alac-S + h + (0.5) o2 --> co2 + diact + h2o		
AMAA	N-acetylmuramoyl-L-alanine amidase	[c] : acmama + h2o --> acmam + ala-L	RLRE00591, RLRE00174	EC-3.5.1.28
AMANAPE	N-acetylmannosamine 6-phoshpate epimerase	[c] : acmanap --> acgam6p	RLRE00546, RLRE00188	
AMANK	N-acetyl-D-mannosamine kinase	[c] : acmana + atp --> acmanap + adp + h	RLRE00301	EC-2.7.1.60
AMMQT7	S-adenosylmethione:2-demthylmenaquinone methyltransferase (menaquinone 7)	[c] : 2dmmq7 + amet + nadph --> ahcys + mql7 + nadp	RLRE01381	
AMMQT8_2	S-adenosylmethione:2-demethylmenaquinone methyltransferase	[c] : 2dmmq8 + amet --> ahcys + h + mqn8	RLRE01381	
ANTIMt1	antimonite transporter via uniport	antim[c] --> antim[e]	RLRE01250	TC-2.A.59
AP4AH	bis(5'-nucleosyl)-tetraphosphatase	[c] : ap4a + h2o --> (2) adp + (2) h	RLRE01379	EC-3.6.1.41
APAT	apolipoprotein N-acyl transferase	[c] : accoa + h2o + thdp <==> acamoxm + coa	RLRE00628	EC-2.3.1.89

Abbrev.	Name	Equation	Gene[a.]	EC number
APRAUR	5-amino-6-(5-phosphoribosylamino)uracil reductase	[c] : 5apru + h + nadph --> 5aprbu + nadp	RLRE01152	EC-1.1.1.193
ARABRr	arabinose reductase	[c] : arab-L + h + nadph <==> abt-L + nadp	RLRE00460	EC-1.1.1.21
ARAI	L-arabinose isomerase	[c] : arab-L <==> rbl-L	RLRE01513	EC-5.3.1.4
araphe2	aromatic amino acid aminotransferase - phenylalanine	[c] : 3mop + phe-L <==> ile-L + phpyr	RLRE00855	
aratry2	aromatic amino acid aminotransferase - tryptophan	[c] : 3mop + trp-L <==> ile-L + indpyr	RLRE01324	
aratyr2	aromatic amino acid aminotransferase - tyrosine	[c] : 3mop + tyr-L <==> 34hpp + ile-L	RLRE00840	
aratyr4	aromatic amino acid aminotransferase - tyrosine	[c] : indpyr + tyr-L <==> 34hpp + trp-L	RLRE00840	
ARBt2	L-arabinose transport via proton symport	arab-L[e] + h[e] <==> arab-L[c] + h[c]	RLRE01516	
ARGabc	L-arginine transport via ABC system	arg-L[e] + atp[c] + h2o[c] --> adp[c] + arg-L[c] + h[c] + pi[c]	RLRE01664, RLRE01665	TC-3.A.1.3
ARGDI	arginine deiminase	[c] : arg-L + h2o --> citr-L + h + nh3	RLRE01446	EC-3.5.3.6
ARGORNt3	Arginine/ornithine antiporter	arg-L[e] + orn-L[c] <==> arg-L[c] + orn-L[e]	RLRE00773, RLRE00710, RLRE00195, RLRE00052, RLRE00774	TC-2.A.3.2
ARGSL	argininosuccinate lyase	[c] : argsuc <==> arg-L + fum	RLRE00828	EC-4.3.2.1
ARGSS	argininosuccinate synthase	[c] : asp-L + atp + citr-L --> amp + argsuc + h + ppi	RLRE00827	EC-6.3.4.5
ARGt2	L-arginine transport in via proton symport	arg-L[e] + h[e] --> arg-L[c] + h[c]	RLRE00773	
ARGTRS	Arginyl-tRNA synthetase	[c] : arg-L + atp + trnaarg --> amp + argtrna + h + ppi	RLRE01694	EC-6.1.1.19
ARSNAt1	Arsenate transporter	arsna[c] --> arsna[e]	RLRE01250	
ARSt1	arsenite transporter via uniport	arsni2[c] --> arsni2[e]	RLRE01250	
ASAD	aspartate-semialdehyde dehydrogenase	[c] : aspsa + nadp + pi <==> 4pasp + h + nadph	RLRE00623	EC-1.2.1.11
ASNN	L-asparaginase	[c] : asn-L + h2o --> asp-L + nh4	RLRE00431	EC-3.5.1.1
ASNS1	asparagine synthase (glutamine-hydrolysing)	[c] : asp-L + atp + gln-L + h2o --> amp + asn-L + glu-L + h + ppi	RLRE00465	EC-6.3.5.4
ASNS2	asparagine synthetase	[c] : asp-L + atp + nh4 --> amp + asn-L + h + ppi		EC-6.3.1.1
ASNt6	L-asparagine transport in/out via proton symport	asn-L[e] + h[e] <==> asn-L[c] + h[c]		TC-2.A.3
ASNTRS	Asparaginyl-tRNA synthetase	[c] : asn-L + atp + trnaasn --> amp + asntrna + h + ppi	RLRE00417	EC-6.1.1.22
ASPCT	aspartate carbamoyltransferase	[c] : asp-L + cbp --> cbasp + h + pi	RLRE01219	EC-2.1.3.2
ASPK	aspartate kinase	[c] : asp-L + atp <==> 4pasp + adp	RLRE00630	EC-2.7.2.4
ASPR	aspartase racemase	[c] : asp-D <==> asp-L	RLRE01548	EC-5.1.1.13
ASPt6	L-aspartate transport in via proton symport	asp-L[e] + h[e] <==> asp-L[c] + h[c]		
ASPTA1	aspartate transaminase	[c] : akg + asp-L <==> glu-L + oaa	RLRE00583	EC-2.6.1.1
ASPTA2	aspartate transaminase	[c] : 4hglu + akg <==> 4h2oxg + glu-L	RLRE00583	EC-2.6.1.1
ASPTA4	aspartate transaminase	[c] : akg + cys-L + h <==> glu-L + mercppyr	RLRE00583	EC-2.6.1.1
ASPTA5	aspartate transaminase	[c] : akg + tyr-L <==> 34hpp + glu-L	RLRE00583	EC-2.6.1.1
ASPTA6	aspartate transaminase	[c] : akg + phe-L <==> glu-L + phpyr	RLRE00583	EC-2.6.1.1
ASPTRS	Aspartyl-tRNA synthetase	[c] : asp-L + atp + trnaasp --> amp + asptrna + h + ppi	RLRE00589	EC-6.1.1.12
ATPM	ATP maintenance requirment	[c] : atp + h2o --> adp + h + pi		
ATPS3r	ATP synthase (three protons for one ATP)	adp[c] + (3) h[e] + pi[c] <==> atp[c] + (2) h[c] + h2o[c]	RLRE00753, RLRE00756, RLRE00757, RLRE00758, RLRE00759, RLRE00760, RLRE00755, RLRE00754	EC-3.6.3.14,
BTDD-RR	(R,R)-butanediol dehydrogenase	[c] : btd-RR + nad <==> actn-R + h + nadh	RLRE01049	EC-1.1.1.4
BTDt1-RR	(R,R)-butanediol transport in/out via diffusion reversible	btd-RR[c] <==> btd-RR[e]		
BTMAT1	Butyryl-[acyl-carrier protein]:malonyl-CoA C-acyltransferase	[c] : 2beacp + h + nadh --> butacp + nad	RLRE01399	EC-1.3.1.10
BTNt2i	Biotin uptake	btn[e] + h[e] --> btn[c] + h[c]		
CBIA	Cobyrinic acid a,c-diamide synthase (EC 6.3.1.-)	[c] : (2) atp + cobrnt + (2) gln-L + (2) h2o --> (2) adp + co2dam + (2) glu-L + (2) pi	RLRE00259	

Abbrev.	Name	Equation	Gene[a.]	EC number
CBIAT	Cobinamide adenyltransferase	[c] : atp + cbi + h2o <==> adcoba + pi + ppi	RLRE00244, RLRE00256	
CBMK2	Carbamate kinase	[c] : atp + co2 + nh4 --> adp + cbp + (2) h	RLRE01361	EC-2.7.2.2
CBPS	carbamoyl-phosphate synthase (glutamine-hydrolysing)	[c] : (2) atp + gln-L + h2o + hco3 --> (2) adp + cbp + glu-L + (2) h + pi	RLRE01122, RLRE00357, RLRE01123, RLRE00358	EC-6.3.5.5
CDPMEK	4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase	[c] : 4c2me + atp --> 2p4c2me + adp + h	RLRE01081	
CELBpts	cellobiose transport via PEP:Pyr PTS	cellb[e] + pep[c] --> 6pgg[c] + pyr[c]	RLRE00437, RLRE00126, RLRE00764, RLRE00785, RLRE00440	
CFAS180	cyclopropane fatty acid synthase (n18:0)	[c] : 2cocdacp + amet --> ahcys + cpocdacp + h	RLRE01213	EC-2.1.1.79
CGPT	CDP-glycerol glycerophosphotransferase	[c] : cdpglyc + unagama --> cmp + h + unagamagp	RLRE01604	EC-2.7.8.12
CHLabc	choline transport via ABC system	atp[c] + chol[e] + h2o[c] --> adp[c] + chol[c] + h[c] + pi[c]	RLRE00973, RLRE00974, RLRE00975	
CHLt6	choline transport in/out via proton symport	chol[e] + h[e] <==> chol[c] + h[c]	RLRE00976	TC-2.A.15
CLPNS_LRE	Cardiolipin Synthase (LRE specific)	[c] : (0.02) pg_LRE <==> (0.01) clpn_LRE + glyc	RLRE00851, RLRE00009	
CMLDC	4-carboxymuconolactone decarboxylase	[c] : 2c25dho --> 5odhf2a + co2 + h	RLRE00441	EC-4.1.1.44
CO2t	CO2 transport out via diffusion	co2[e] <==> co2[c]		
Coabc	Cobalt transport via ABC system	atp[c] + cobalt2[e] + h2o[c] --> adp[c] + cobalt2[c] + h[c] + pi[c]	RLRE00273, RLRE00275, RLRE01651, RLRE01650, RLRE00274, RLRE01649	
COBALTt5	cobalt transport in/out via permease (no H+)	cobalt2[c] <==> cobalt2[e]	RLRE00042	TC-1.A.35
COBAT	cob(I)alamin adenosyltransferase	[c] : cobamcoa + pi + ppi <==> atp + cbl1 + h + (0.5) h2o2	RLRE00244, RLRE00256	EC-2.5.1.17
COPREC2MT	Precorrin-2 C20-methyltransferase	[c] : amet + coprec2 --> ahcys + coprec3	RLRE00271	
COPREC3MT	Precorrin-3B C17-methyltransferase (EC 2.1.1.131)	[c] : amet + coprec3 --> ahcys + coprec4	RLRE00267	
COPREC4MT	Precorrin-4 C11-methyltransferase (EC 2.1.1.133)	[c] : amet + coprec4 --> ahcys + coprec5	RLRE00265	
COPREC6MT	Precorrin-6Y C5,15-methyltransferase [decarboxylating] E.C. 2.1.1.132	[c] : (2) amet + codhprec6 --> (2) ahcys + co2 + coprec8	RLRE00263, RLRE00264	
COPREC6R	Precorrin-6X reductase (EC 1.3.1.54)	[c] : coprec6 + h + nadh --> codhprec6 + nad	RLRE00268	
COPREC6R2	Precorrin-6X reductase (EC 1.3.1.54)	[c] : coprec6 + h + nadph --> codhprec6 + nadp	RLRE00268	
COPRECI	Precorrin-8X methylmutase (EC 5.4.1.2)	[c] : coprec8 --> cobrnt	RLRE00261	
COPRECT	Precorrin-6A synthase (deacetylating)	[c] : amet + coprec5 + h2o --> acald + ahcys + coprec6	RLRE00266, RLRE00262	
CPSS_LRE	CPS synthase complex, LRE specific	[c] : dtdp6dm + (5) h2o + (2) udpg + (2) udpgal <==> CPS_LRE + dtdp + (6) h + (3) udp + ump	RLRE00212, RLRE00222, RLRE00207, RLRE01200, RLRE00205, RLRE00201	
CPSS_LRE2	CPS synthase complex, LRE specific	[c] : (2) dtdp6dm + (4) h2o + udpg + udpgal <==> CPS_LRE2 + (2) dtdp + (5) h + udp + ump	RLRE00212, RLRE00222, RLRE00207, RLRE01200, RLRE00205, RLRE00201	
CRCT	CTP:D-ribitol-5-phosphate cytidylyltransferase	[c] : ctp + h + rbt5p --> cdprbtl + ppi		EC-2.7.7.40
CRPT_25	CDP-ribitol phosphoribitoltransferase (25 units with ribitol-P)	[c] : (25) cdprbtl + unagamagp --> (25) cmp + (25) h + unRTA	RLRE01603, RLRE01622, RLRE01623	
CSND	Cytosine deaminase	[c] : csn + h + h2o --> nh4 + ura	RLRE00559	EC-3.5.4.1
CTPS1_#1	CTP synthase (NH3)	[c] : atp + nh4 + utp --> adp + ctp + (2) h + pi	RLRE00467	EC-6.3.4.2
CTPS2	CTP synthase (glutamine)	[c] : atp + gln-L + h2o + utp --> adp + ctp + glu-L + (2) h + pi	RLRE00467	EC-6.3.4.2
Cuabc	Copper transport via ABC system	atp[c] + cu2[e] + h2o[c] --> adp[c] + cu2[c] + h[c] + pi[c]	RLRE01365+ RLRE01364	TC-3.A.3
Cut1	Copper export via ATPase	atp[c] + cu2[c] + h2o[c] --> adp[c] + cu2[e] + h[c] + pi[c]	RLRE01366	TC-3.A.3
CYSabc	L-cysteine transport via ABC system	atp[c] + cys-L[e] + h2o[c] --> adp[c] + cys-L[c] + h[c] + pi[c]	RLRE01165+RLRE01164+RLRE01166	
CYSDabc	D-cysteine transport via ABC system	atp[c] + cys-D[e] + h2o[c] --> adp[c] + cys-D[c] + h[c] + pi[c]	RLRE01165+RLRE01164+RLRE01166	
CYSS	cysteine synthase	[c] : acser + h2s --> ac + cys-L + h	RLRE00702, RLRE00008	EC-4.2.99.8
CYSt6	L-cysteine transport in via proton symport	cys-L[e] + h[e] <==> cys-L[c] + h[c]		
CYSTabc	L-cystine transport via ABC system	atp[c] + cyst-L[e] + h2o[c] --> adp[c] + cyst-L[c] + h[c] + pi[c]	RLRE01165+ RLRE01164+ RLRE01166	TC-3.A.1.3
CYSTGL	cystathionine g-lyase	[c] : cysth-L + h2o --> 2obut + cys-L + nh4	RLRE01167	EC-4.4.1.1

Abbrev.	Name	Equation	Gene[a.]	EC number
CYSTL	cystathionine b-lyase	[c] : cysth-L + h2o --> hcys-L + nh4 + pyr	RLRE00443, RLRE01167	EC-4.4.1.8
CYSTRS	Cysteinyl-tRNA synthetase	[c] : atp + cys-L + trnacys --> amp + cystrna + h + ppi	RLRE01157	EC-6.1.1.16
CYTB_B2	menaquinol oxidase (7:1 protons)	(2) h[c] + mql7[c] + (0.5) o2[c] --> (2) h[e] + h2o[c] + mqn7[c]	RLRE00740, RLRE00741, RLRE00742, RLRE00739	,
CYTD	cytidine deaminase	[c] : cytd + h + h2o --> nh4 + uri	RLRE01063	EC-3.5.4.5
CYTDK1	cytidine kinase (ATP)	[c] : atp + cytd --> adp + cmp + h		
CYTDK2	cytidine kinase (GTP)	[c] : cytd + gtp --> cmp + gdp + h		
CYTDK3	cytidine kinase (ITP)	[c] : cytd + itp --> cmp + h + idp		
CYTK1	cytidylate kinase (CMP)	[c] : atp + cmp <==> adp + cdp	RLRE01190	EC-2.7.4.14
CYTK2	cytidylate kinase (dCMP)	[c] : atp + dcmp <==> adp + dcdp	RLRE01190	EC-2.7.4.14
D-LACt2	D-lactate transport via proton symport	h[e] + lac-D[e] <==> h[c] + lac-D[c]		
DADA	Deoxyadenosine deaminase	[c] : dad-2 + h + h2o --> din + nh4	RLRE00559	
DADNK	deoxyadenosine kinase	[c] : atp + dad-2 --> adp + damp + h	RLRE00514, RLRE00131	EC-2.7.1.76
DAGGT_LRE	1,2-diacylglycerol 3-glucosyltransferase (LRE specific)	[c] : (0.01) dag_LRE + (2) udpg --> (0.01) dgdag_LRE + (2) h + (2) udp	RLRE00864, RLRE00865	EC-2.4.1.157
DAGK_LRE	Diacylglycerol kinase (LRE specific)	[c] : atp + (0.01) dag_LRE --> adp + h + (0.01) pa_LRE	RLRE00833, RLRE01059	EC-2.7.1.107
DALTAL	D-Alanine lipoteichoic acid ligase	[c] : (0.01) LTA_LRE + (25) ala-D + (25) atp --> (0.01) LTAala_LRE + (25) adp + (25) pi	RLRE00610, RLRE01220, RLRE00611, RLRE00608	EC-6.3.2.16
DAPDC	diaminopimelate decarboxylase	[c] : 26dap-M + h --> co2 + lys-L	RLRE00629	EC-4.1.1.20
DAPE	diaminopimelate epimerase	[c] : 26dap-LL <==> 26dap-M	RLRE00631	EC-5.1.1.7
DARTAL	D-Alanine ribitol teichoic acid ligase	[c] : RTA + (25) ala-D + (25) atp --> RTAala + (25) adp + (25) pi	RLRE00610, RLRE01220, RLRE00611, RLRE00608	
DASYN_LRE	CDP-Diacylglycerol synthetase (LRE specific)	[c] : ctp + h + (0.01) pa_LRE <==> (0.01) cdpdag_LRE + ppi	RLRE00672	EC-2.7.7.41
DB4PS	3,4-Dihydroxy-2-butanone-4-phosphate	[c] : ru5p-D --> db4p + for + h	RLRE01154	
DCYTD	deoxycytidine deaminase	[c] : dcyt + h + h2o --> duri + nh4	RLRE01063	EC-3.5.4.14
DDMAT5	Dodecanoyl-[acyl-carrier protein]: malonyl-CoA C-acyltransferase	[c] : 2tddacp + h + nadh --> ddeacp + nad	RLRE01399	EC-1.3.1.10
DEMAT4	Decanoyl-[acyl-carrier protein]:malonyl-CoA C-acyltransferase	[c] : 2tdeacp + h + nadh --> decacp + nad	RLRE01399	EC-1.3.1.10
DGNSK	deoxyguanosine kinase	[c] : atp + dgsn --> adp + dgmp + h	RLRE00514, RLRE00131	EC-2.7.1.113
DHAPT	Dihydroxyacetone phosphotransferase	[c] : dha + pep --> dhap + pyr		
DHAt	Dihydroxyacetone transport via facilitated diffusion	dha[e] <==> dha[c]	RLRE00229	
DHDPRy	dihydrodipicolinate reductase (NADPH)	[c] : 23dhdp + h + nadph --> nadp + thdp	RLRE00625	EC-1.3.1.26
DHDPS	dihydrodipicolinate synthase	[c] : aspsa + pyr --> 23dhdp + h + (2) h2o	RLRE00626	EC-4.2.1.52
DHFOR2	dihydrofolate reductase	[c] : dhf + nadp <==> fol + nadph	RLRE01182	EC-1.5.1.3
DHFR	dihydrofolate reductase	[c] : dhf + h + nadph <==> nadp + thf	RLRE01182	EC-1.5.1.3
DHFS	dihydrofolate synthase	[c] : atp + dhpt + glu-L --> adp + dhf + h + pi	RLRE01598, RLRE00737	EC-6.3.2.12
DHNPA	dihydroneopterin aldolase	[c] : dhnpt --> 2ahhmp + gcald	RLRE01601	EC-4.1.2.25
DHORD6	dihydoorotic acid dehydrogenase (NAD)	[c] : dhor-S + nad <==> h + nadh + orot	RLRE01217	EC-1.3.1.14
DHORTS	dihydroorotase	[c] : dhor-S + h2o <==> cbasp + h	RLRE01218	EC-3.5.2.3
DHPPDA_#1	diaminohydroxyphosphoribosylaminopyrimidine deaminase	[c] : 25dhpp + h + h2o --> 5apru + nh4	RLRE01152	EC-3.5.4.26
DHPS3	dihydropteroate synthase	[c] : 2ahhmd + 4abz --> dhpt + ppi	RLRE01596	EC-2.5.1.15
DIACTt	diacetyl diffusion	diact[c] <==> diact[e]		
DINSK	deoxyinosine kinase	[c] : atp + din --> adp + dimp	RLRE00514, RLRE00131	
DKGLCNR1	2,5-diketo-D-gluconate reductase	[c] : 25dkglcn + h + nadph --> 2dhguln + nadp	RLRE00429, RLRE01511, RLRE00002, RLRE01486	
DKGLCNR2x	2,5-diketo-D-gluconate reductase (NADH)	[c] : 25dkglcn + h + nadh --> 5dglcn + nad	RLRE00429, RLRE01511, RLRE00002, RLRE01486	
DKGLCNR2y	2,5-diketo-D-gluconate reductase (NADPH)	[c] : 25dkglcn + h + nadph --> 5dglcn + nadp	RLRE00429, RLRE01511, RLRE00002, RLRE01486	
DMATT	dimethylallyltranstransferase	[c] : dmpp + ipdp --> grdp + ppi	RLRE01562, RLRE01385	EC-2.5.1.1

Abbrev.	Name	Equation	Gene[a.]	EC number
DNAS_LRE	DNA synthesis, LRE specific	[c] : (1.37) atp + (0.28) datp + (0.22) dctp + (0.22) dgtp + (0.28) dttp + (1.37) h2o --> (0.01) DNA_LRE + (1.37) adp + (1.37) h + (1.37) pi + ppi		
DNMPPA	Dihydroneopterin monophosphate dephosphorylase	[c] : dhpmp + h2o --> dhnpt + h + pi		
DNTPPA	Dihydroneopterin triphosphate pyrophosphatase	[c] : ahdt + h2o --> dhpmp + ppi	RLRE01597	
DPCOAK	dephospho-CoA kinase	[c] : atp + dpcoa --> adp + coa + h	RLRE01539	EC-2.7.1.24
DPMVD	diphosphomevalonate decarboxylase	[c] : 5dpmev + atp --> adp + co2 + ipdp + pi	RLRE00340	EC-4.1.1.33
DPR	2-dehydropantoate 2-reductase	[c] : 2dhp + h + nadph --> nadp + pant-R	RLRE00584, RLRE00084	EC-1.1.1.169
DRBK	Deoxyribokinase	[c] : atp + drib --> 2dr5p + adp + h	RLRE01090, RLRE01556	EC-2.7.1.15
DRIBt2	deoxyribose transport in via proton symporter	drib[e] + h[e] --> drib[c] + h[c]	RLRE01554, RLRE01553	
DRPA	deoxyribose-phosphate aldolase	[c] : 2dr5p --> acald + g3p	RLRE01426	EC-4.1.2.4
DTMPK	dTMP kinase	[c] : atp + dtmp <==> adp + dtdp	RLRE00564	EC-2.7.4.9
DURADy	dihydrouracil dehydrogenase (NADP)	[c] : 56dura + nadp <==> h + nadph + ura	RLRE00535, RLRE00534	EC-1.3.1.2
DURIK1	deoxyuridine kinase (ATP:Deoxyuridine)	[c] : atp + duri --> adp + dump + h	RLRE00770	
DURIPP	purine-nucleoside phosphatase (deoxyuridine)	[c] : duri + pi <==> 2dr1p + ura	RLRE01423	EC-2.4.2.1
DUTPDP	dUTP diphosphatase	[c] : dutp + h2o --> dump + h + ppi	RLRE01160	EC-3.6.1.23
DXPS	1-deoxy-D-xylulose 5-phosphate synthase	[c] : g3p + h + pyr --> co2 + dxyl5p	RLRE01018, RLRE01369	
ENO	enolase	[c] : 2pg <==> h2o + pep		EC-4.2.1.11
EPPP	exopolyphosphatase	[c] : h2o + polypi --> (2) h + pi	RLRE01071, RLRE00572, RLRE00570	EC-3.6.1.11
ETOHt1	ethanol transport in/out via diffusion	etoh[e] <==> etoh[c]		
FA120ACPH	fatty-acyl-ACP hydrolase	[c] : ddcaACP + h2o <==> ACP + ddca + h	RLRE00569	EC-3.1.2.14
FA140ACPH	fatty-acyl-ACP hydrolase	[c] : h2o + myrsACP <==> ACP + h + ttdca	RLRE00569	EC-3.1.2.14
FA141ACPH	fatty-acyl-ACP hydrolase	[c] : h2o + tdeACP <==> ACP + h + ttdcea	RLRE00569	EC-3.1.2.14
FA160ACPH	fatty-acyl-ACP hydrolase	[c] : h2o + palmACP <==> ACP + h + hdca	RLRE00569	EC-3.1.2.14
FA161ACPH	fatty-acyl-ACP hydrolase	[c] : h2o + hdeACP <==> ACP + h + hdcea	RLRE00569	EC-3.1.2.14
FA180ACPH	fatty-acyl-ACP hydrolase	[c] : h2o + ocdcaACP <==> ACP + h + ocdca	RLRE00569	EC-3.1.2.14
FA181ACPH	fatty-acyl-ACP hydrolase	[c] : h2o + octeACP <==> ACP + h + ocdcea	RLRE00569	EC-3.1.2.14
FA182ACPH	fatty-acyl-ACP hydrolase	[c] : h2o + ocdcyaACP <==> ACP + h + ocdcya	RLRE00569	EC-3.1.2.14
FABM1	Fatty acid enoyl isomerase (FabM reaction)	[c] : 2thdeacp <==> 2chdeacp		
FABM2	Fatty acid enoyl isomerase (FabM reaction, 18:1)	[c] : 2tocdacp <==> 2cocdacp		
FBA	fructose-bisphosphate aldolase	[c] : fdp <==> dhap + g3p	RLRE00789	EC-4.1.2.13
FCLT	Heme B synthesis reaction	[c] : fe2 + ppp9 --> h + pheme	RLRE00701	EC-4.99.1.1
FLVR	flavin reductase	[c] : h + nadph + ribflv --> nadp + ribflvRD	RLRE01276	EC-1.5.1.30
FMETDF	formylmethionine deformylase	[c] : fmet + h2o --> for + met-L	RLRE01469, RLRE01638	EC-3.5.1.31
FMETTRS	Methionyl-tRNA formyltransferase	[c] : 10fthf + mettrna --> fmettrna + thf	RLRE01440	EC-2.1.2.9
FMNAT	FMN adenylyltransferase	[c] : atp + fmn + h --> fad + ppi	RLRE00661	EC-2.7.7.2
FORt	formate transport via diffusion	for[e] <==> for[c]		
FRTT	farnesyltranstransferase	[c] : frdp + ipdp --> ggdp + ppi	RLRE01562, RLRE01385	EC-2.5.1.29
FRUK	fructokinase	[c] : atp + fru --> adp + f6p + h	RLRE00333, RLRE00334	EC-2.7.1.4
FRUpts	D-fructose transport via PEP:Pyr PTS	fru[e] + pep[c] --> f1p[c] + pyr[c]	RLRE00126, RLRE00764, RLRE01568	
FTHFCL	5-formyltetrahydrofolate cyclo-ligase	[c] : 5fothf + atp --> adp + methf + pi		EC-6.3.3.2
FTHFL	formate-tetrahydrofolate ligase	[c] : atp + for + thf --> 10fthf + adp + pi	RLRE01211	EC-6.3.4.3
FUCt	L-fucose transport via proton symport	fuc-L[e] + h[e] <==> fuc-L[c] + h[c]	RLRE01554, RLRE01553	
FUM	fumarase	[c] : fum + h2o <==> mal-L	RLRE00724	EC-4.2.1.2
G1PACT	glucosamine-1-phosphate N-acetyltransferase	[c] : accoa + gam1p --> acgam1p + coa + h	RLRE01343	EC-2.3.1.157
G1PPDM	glucose-1-phosphate phosphodismutase	[c] : (2) g1p --> g16bp + glc-D	RLRE01239	EC-2.7.1.41
G1PTMT	glucose-1-phosphate thymidylyltransferase	[c] : dttp + g1p + h --> dtdpglc + ppi	RLRE00206	EC-2.7.7.24
G35DP	guanosine-3',5'-bis(diphosphate) 3'-diphosphatase	[c] : gdpdp + h2o --> gdp + ppi	RLRE00586	EC-3.1.7.2

Abbrev.	Name	Equation	Gene[a.]	EC number
G3PCT	glycerol-3-phosphate cytidylyltransferase	[c] : ctp + glyc3p + h --> cdpglyc + ppi	RLRE00672	EC-2.7.7.39
G3PD1	glycerol-3-phosphate dehydrogenase (NAD)	[c] : glyc3p + nad <==> dhap + h + nadh	RLRE00958	EC-1.1.1.94
G3PD2	glycerol-3-phosphate dehydrogenase (NADP)	[c] : glyc3p + nadp <==> dhap + h + nadph	RLRE00958	EC-1.1.1.94
G5SADs	L-glutamate 5-semialdehyde dehydratase (spontaneous)	[c] : glu5sa <==> 1pyr5c + h + h2o		
G5SD	glutamate-5-semialdehyde dehydrogenase	[c] : glu5p + h + nadph --> glu5sa + nadp + pi	RLRE00580	EC-1.2.1.41
G6PDA	glucosamine-6-phosphate deaminase	[c] : gam6p + h2o --> f6p + nh4	RLRE01192	EC-3.5.99.6
G6PDHy	glucose 6-phosphate dehydrogenase	[c] : g6p + nadp <==> 6pgl + h + nadph	RLRE00216	EC-1.1.1.49
GALK2	galactokinase	[c] : a-gal-D + atp --> adp + gal1p + h	RLRE01263	EC-2.7.1.6
GALM	aldose 1-epimerase	[c] : gal <==> a-gal-D	RLRE00717	EC-5.1.3.3
GALS3	a-galactosidase (melibiose)	[c] : h2o + melib --> gal + glc-D	RLRE00336	EC-3.2.1.22
GALSZ	beta-galactosidase	[c] : h2o + lcts --> gal + glc-D	RLRE01171, RLRE01386	EC-3.2.1.23
GALT	galactose-1-phosphate uridylyltransferase	[c] : gal1p + h + utp <==> ppi + udpgal	RLRE01262	EC-2.7.7.10
GALTpts	Galactitol transport via PEP:Pyr PTS	galt[e] + pep[c] --> galt1p[c] + pyr[c]	RLRE00126, RLRE00764	
GALU	UTP-glucose-1-phosphate uridylyltransferase	[c] : g1p + h + utp <==> ppi + udpg	RLRE00959	EC-2.7.7.9
GAPD	glyceraldehyde-3-phosphate dehydrogenase (NAD)	[c] : g3p + nad + pi <==> 13dpg + h + nadh		EC-1.2.1.12
GARFT	phosphoribosylglycinamide formyltransferase	[c] : 10fthf + gar <==> fgam + h + thf	RLRE01419	EC-2.1.2.2
GAT1_LRE	glycerol 3-phosphate acyltransferase (Lb plantarum specific)	[c] : (0.12) 2chdeacp + (0.32) 2cocdacp + (0.25) cpocdacp + glyc3p + (0.26) hdeacp + (0.02) ocdacp + (0.03) tdeacp --> acp + (0.01) agly3p_LRE		EC-2.3.1.15
GCALDt	Glycoaldehydye reversible transport	gcald[e] <==> gcald[c]		
GDHYD	glycerol dehydratase	[c] : glyc <==> 3hppnl + h2o	RLRE00236, RLRE00240, RLRE00232, RLRE00237, RLRE00233, RLRE00235, RLRE00234	EC-4.2.1.28
GF6PTA	glutamine-fructose-6-phosphate transaminase	[c] : f6p + gln-L --> gam6p + glu-L		EC-2.6.1.16
GGLUGABH	gammaglutamyl-gamma-aminobutyrate hydrolase	[c] : ggluabt + h2o --> 4abut + glu-L	RLRE01230	
GHMT	glycine hydroxymethyltransferase	[c] : ser-L + thf <==> gly + h2o + mlthf	RLRE00563, RLRE00766	EC-2.1.2.1
GK1	guanylate kinase (GMP:ATP)	[c] : atp + gmp <==> adp + gdp	RLRE01436	EC-2.7.4.8
GK2	guanylate kinase (GMP:dATP)	[c] : datp + gmp <==> dadp + gdp	RLRE01436	EC-2.7.4.8
GLCNt2	D-gluconate transport via proton symport, reversible	glcn-D[e] + h[e] <==> glcn-D[c] + h[c]	RLRE00371, RLRE00620	
GLCpts	D-glucose transport via PEP:Pyr PTS	glc-D[e] + pep[c] --> g6p[c] + pyr[c]	RLRE00126, RLRE01426, RLRE00764	
GLNabc	L-glutamine transport via ABC system	atp[c] + gln-L[e] + h2o[c] --> adp[c] + gln-L[c] + h[c] + pi[c]	RLRE00811, RLRE01672, RLRE01673, RLRE00808, RLRE00809, RLRE00810	TC-3.A.1.3
GLNS	glutamine synthetase	[c] : atp + glu-L + nh4 --> adp + gln-L + h + pi	RLRE01322	EC-6.3.1.2
GLNTAL	glutamyl-tRNA(Gln):L-glutamine amido-ligase (ADP-forming)	[c] : atp + gln-L + glutrna + h2o <==> adp + glntrna + glu-L + h + pi	RLRE01058, RLRE01057, RLRE01056	
GLTAL	Glucose lipoteichoic acid ligase	[c] : (0.01) LTA_LRE + (25) udpg --> (0.01) LTAglc_LRE + (25) h + (25) udp	RLRE01128, RLRE01626, RLRE01127	
GLU5K	glutamate 5-kinase	[c] : atp + glu-L --> adp + glu5p	RLRE00581	EC-2.7.2.11
GLUCYSL	glutamate-cysteine ligase	[c] : atp + cys-L + glu-L --> adp + glucys + h + pi	RLRE01232	EC-6.3.2.2
GLUN	glutaminase	[c] : gln-L + h2o --> glu-L + nh4	RLRE01624, RLRE00797	EC-3.5.1.2
GLUPRT	glutamine phosphoribosyldiphosphate amidotransferase	[c] : gln-L + h2o + prpp --> glu-L + ppi + pram	RLRE01417	EC-2.4.2.14
GLUR	glutamate racemase	[c] : glu-D <==> glu-L	RLRE00487	EC-5.1.1.3
GLUt4	L-glutamate:Na+ symporter	glu-L[e] + na1[e] <==> glu-L[c] + na1[c]	RLRE01257	TC-2.A.27
GLUt6	L-glutamate transport in/out via proton symporter	glu-L[e] + h[e] <==> glu-L[c] + h[c]	RLRE01257	TC-2.A.23
GLUTRR	glutamyl-tRNA reductase	[c] : glutrna + (2) h + nadph --> glu1sa + nadp + trnaglu	RLRE00278	
GLUTRS	Glutamyl-tRNA synthetase	[c] : atp + glu-L + trnaglu --> amp + glutrna + h + ppi	RLRE01323	EC-6.1.1.17

Abbrev.	Name	Equation	Gene[a.]	EC number
GLYBabc	glycine betaine transport via ABC system	atp[c] + glyb[e] + h2o[c] --> adp[c] + glyb[c] + h[c] + pi[c]	RLRE00973, RLRE00974, RLRE00975	TC-3.A.1.12
GLYBt6	betaine (glycine betaine) transport in/out via proton symport	glyb[e] + h[e] <==> glyb[c] + h[c]	RLRE00976	TC-2.A.1.6
GLYCDx	Glycerol dehydrogenase	[c] : glyc + nad <==> dha + h + nadh	RLRE00056	EC-1.1.1.6
GLYCK	glycerate kinase	[c] : atp + glyc-R --> 3pg + adp + h	RLRE00619	EC-2.7.1.31
GLYCt1	glycerol transport via uniport (facilitated diffusion)	glyc[e] --> glyc[c]	RLRE00870	
GLYCt5	glycerol transport in/out via diffusion reversible	glyc[c] <==> glyc[e]	RLRE00229	
GLYK	glycerol kinase	[c] : atp + glyc --> adp + glyc3p + h		EC-2.7.1.30
GLYt6	glycine transport in/out via proton symport	gly[e] + h[e] <==> gly[c] + h[c]		TC-2.A.3.1
GLYTRS	Glycyl-tRNA synthetase	[c] : atp + gly + trnagly --> amp + glytrna + h + ppi	RLRE00836, RLRE00837	EC-6.1.1.14
GMPR	GMP reductase	[c] : gmp + (2) h + nadph --> imp + nadp + nh4	RLRE01225	EC-1.7.1.7
GMPS2	GMP synthase (glutamine-hydrolysing)	[c] : atp + gln-L + h2o + xmp --> amp + glu-L + gmp + (2) h + ppi	RLRE01045, RLRE00424	EC-6.3.5.2
GNK	gluconokinase	[c] : atp + glcn-D --> 6pgc + adp + h	RLRE00369	EC-2.7.1.12
GPDDA1	Glycerophosphodiester phosphodiesterase (sn-Glycero-3-phosphocholine)	[c] : g3pc + h2o --> chol + glyc3p + h	RLRE01228	EC-3.1.4.46
GPDDA2	Glycerophosphodiester phosphodiesterase (Glycerophosphoethanolamine)	[c] : g3pe + h2o --> etha + glyc3p + h	RLRE01228	EC-3.1.4.46
GPDDA3	Glycerophosphodiester phosphodiesterase (Glycerophosphoserine)	[c] : g3ps + h2o --> glyc3p + h + ser-L	RLRE01228	EC-3.1.4.46
GPDDA4	Glycerophosphodiester phosphodiesterase (Glycerophosphoglycerol)	[c] : g3pg + h2o --> glyc + glyc3p + h	RLRE01228	EC-3.1.4.46
GPDDA5	Glycerophosphodiester phosphodiesterase (Glycerophosphoinositol)	[c] : g3pi + h2o --> glyc3p + h + inost	RLRE01228	EC-3.1.4.46
GRTAL	Glucose ribitol teichoic acid ligase	[c] : RTA + (25) udpg --> RTAglc + (25) h + (25) udp	RLRE00191	
GRTT	geranyltranstransferase	[c] : grdp + ipdp --> frdp + ppi	RLRE01562, RLRE01385	EC-2.5.1.10
GTHRD	glutathione-disulfide reductase	[c] : (2) gthrd + nadp <==> gthox + h + nadph	RLRE00166	EC-1.8.1.7
GTHS	glutathione synthase	[c] : atp + glucys + gly --> adp + gthrd + h + pi	RLRE01232	EC-6.3.2.3
GTPCI	GTP cyclohydrolase I	[c] : gtp + h2o --> ahdt + for + h	RLRE01599	EC-3.5.4.16
GTPCII	GTP cyclohydrolase II	[c] : gtp + (3) h2o --> 25dhpp + for + (2) h + ppi	RLRE01154	EC-3.5.4.25
GTPDPK	GTP diphosphokinase	[c] : atp + gtp --> amp + gdptp + h	RLRE01517, RLRE01237, RLRE01236, RLRE00586	EC-2.7.6.5
GUAPRT	guanine phosphoribosyltransferase	[c] : gua + prpp --> gmp + ppi	RLRE01305, RLRE00452	
GUAt2	guanine transport in via proton symport	gua[e] + h[e] --> gua[c] + h[c]		
H2Ot5	H2O transport via diffusion	h2o[e] <==> h2o[c]		
HACD1	3-hydroxyacyl-CoA dehydrogenase (acetoacetyl-CoA)	[c] : aacoa + h + nadh <==> 3hbycoa + nad	RLRE01434	EC-1.1.1.35
HBUHL1	(3R)-3-Hydroxybutanoyl-[acyl-carrier-protein] hydro-lyase	[c] : 3hbacp --> 2beacp + h2o	RLRE00538, RLRE01411, RLRE01403	EC-4.2.1.58
HBUR1	(3R)-3-Hydroxybutanoyl-[acyl-carrier protein]:NADP+ oxidoreductase	[c] : aaacp + h + nadph --> 3hbacp + nadp	RLRE01093, RLRE01406	EC-1.1.1.100
HCO3E	carbonate dehydratase (HCO3 equilibration reaction)	[c] : co2 + h2o <==> h + hco3		EC-4.2.1.1
HCYSMT	homocysteine S-methyltransferase	[c] : amet + hcys-L --> ahcys + h + met-L	RLRE01011	EC-2.1.1.10
HDDHL5	(3R)-3-Hydroxybutanoyl-[acyl-carrier-protein] hydro-lyase	[c] : 3hddacp --> 2tddacp + h2o	RLRE00538, RLRE01411, RLRE01403	EC-4.2.1.58
HDDR5	3R)-3-Hydroxydodecanoyl-[acyl-carrier-protein]:NADP+ oxidoreductase	[c] : 3oxddacp + h + nadph --> 3hddacp + nadp	RLRE01093, RLRE01406	EC-1.1.1.100
HDEHL4	(3R)-3-Hydroxybutanoyl-[acyl-carrier-protein] hydro-lyase	[c] : 3hdeacp --> 2tdeacp + h2o	RLRE00538, RLRE01411, RLRE01403	EC-4.2.1.58
HDER4	(3R)-3-Hydroxydecanoyl-[acyl-carrier-protein]:NADP+ oxidoreductase	[c] : 3oxdeacp + h + nadph --> 3hdeacp + nadp	RLRE01093, RLRE01406	EC-1.1.1.100
HDMAT7	Hexadecanoyl-[acyl-carrier protein]:malonyl-CoA C-acyltransferase	[c] : 2thdeacp + h + nadh --> hdeacp + nad	RLRE01399	EC-1.3.1.10
HEMAT2	Hexanoyl-[acyl-carrier protein]:malonyl-CoA C-acyltransferase	[c] : 2theacp + h + nadh --> hexacp + nad	RLRE01399	EC-1.3.1.10
HETZK	hydroxyethylthiazole kinase	[c] : 4mhetz + atp --> 4mpetz + adp + h	RLRE00933	EC-2.7.1.50

Abbrev.	Name	Equation	Gene[a.]	EC number
HEXTT	trans-hexaprenyltranstransferase	[c] : hexdp + ipdp --> hepdp + ppi	RLRE01385	EC-2.5.1.30
HHDHL7	(3R)-3-Hydroxypalmitoyl-[acyl-carrier-protein] hydro-lyase	[c] : 3hpaacp --> 2thdeacp + h2o	RLRE00538, RLRE01411, RLRE01403	EC-4.2.1.58
HHDR7	(3R)-3-Hydroxypalmitoyl-[acyl-carrier-protein]:NADP+ oxidoreductase	[c] : 3oxhdacp + h + nadph --> 3hpaacp + nadp	RLRE01093, RLRE01406	EC-1.1.1.100
HHYHL2	(3R)-3-Hydroxybutanoyl-[acyl-carrier-protein] hydro-lyase	[c] : 3hhacp --> 2theacp + h2o	RLRE00538, RLRE01411, RLRE01403	EC-4.2.1.58
HHYR2	(3R)-3-Hydroxyhexanoyl-[acyl-carrier-protein]:NADP+ oxidoreductase	[c] : 3oxhacp + h + nadph --> 3hhacp + nadp	RLRE01093, RLRE01406	EC-1.1.1.100
HISabc	L-histidine transport via ABC system	atp[c] + h2o[c] + his-L[e] --> adp[c] + h[c] + his-L[c] + pi[c]	RLRE00732, RLRE00731, RLRE00730	
HISDC	histidine decarboxylase	[c] : h + his-L --> co2 + hista	RLRE00048	EC-4.1.1.22
HISTAap	histide/histamine antiporter	his-L[c] + hista[e] <==> his-L[e] + hista[c]		
HISTRS	Histidyl-tRNA synthetase	[c] : atp + his-L + trnahis --> amp + h + histrna + ppi	RLRE00046, RLRE00065	EC-6.1.1.21
HMGCOAR	Hydroxymethylglutaryl CoA reductase	[c] : coa + mev-R + (2) nadp <==> (2) h + hmgcoa + (2) nadph	RLRE01072, RLRE00931	EC-1.1.1.34
HMGCOAS	Hydroxymethylglutaryl CoA synthase	[c] : coa + h + hmgcoa <==> aacoa + accoa + h2o	RLRE00912	EC-4.1.3.5
HMPabc	Hydroxymethylpyrimidine ABC transporter	4ahmmp[e] + atp[c] + h2o[c] --> 4ahmmp[c] + adp[c] + h[c] + pi[c]	RLRE00694 +RLRE00695	
HMPK1	hydroxymethylpyrimidine kinase (ATP)	[c] : 4ahmmp + atp --> 4ampm + adp + h	RLRE00934	EC-2.7.1.49
HOCHL3	((3R)-3-Hydroxybutanoyl-[acyl-carrier-protein] hydro-lyase	[c] : 3hocacp --> 2toceacp + h2o	RLRE00538, RLRE01411, RLRE01403	EC-4.2.1.58
HOCR3	(3R)-3-Hydroxyoctanoyl-[acyl-carrier-protein]:NADP+ oxidoreductase	[c] : 3oxocacp + h + nadph --> 3hocacp + nadp	RLRE01093, RLRE01406	EC-1.1.1.100
HODHL8	(3R)-3-Hydroxyoctadecanoyl-[acyl-carrier-protein] hydro-lyase	[c] : 3hocdacp --> 2tocdacp + h2o	RLRE00538, RLRE01411, RLRE01403	EC-4.2.1.58
HODR8	(3R)-3-Hydroxyoctadecanoyl-[acyl-carrier-protein]:NADP+ oxidoreductase	[c] : 3oxocdacp + h + nadph --> 3hocdacp + nadp	RLRE01093, RLRE01406	EC-1.1.1.100
HPPK	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase	[c] : 2ahhmp + atp --> 2ahhmd + amp + h	RLRE01600	EC-2.7.6.3
HSAT	Acetyl-CoA:L-homoserine O-acetyltransferase	[c] : accoa + hom-L <==> achms + coa	RLRE00007	EC-2.3.1.31
HSDy	homoserine dehydrogenase (NADPH)	[c] : hom-L + nadp <==> aspsa + h + nadph		EC-1.1.1.3
HTDHL6	(3R)-3-Hydroxypalmitoyl-[acyl-carrier-protein] hydro-lyase	[c] : 3htdacp --> 2ttdeacp + h2o	RLRE00538, RLRE01411, RLRE01403	EC-4.2.1.58
HTDR6	3R)-3-Hydroxytetradecanoyl-[acyl-carrier-protein]:NADP+ oxidoreductase	[c] : 3oxtdacp + h + nadph --> 3htdacp + nadp	RLRE01093, RLRE01406	EC-1.1.1.100
HXANt2	hypoxanthine transport in via proton symport	h[e] + hxan[e] --> h[c] + hxan[c]		
HXPRT	hypoxanthine phosphoribosyltransferase (Hypoxanthine)	[c] : hxan + prpp --> imp + ppi	RLRE01305, RLRE00452	EC-2.4.2.8
HYPOE	hypothetical enzyme	[c] : h2o + pyam5p --> pi + pydam		
ICDHy	isocitrate dehydrogenase (NADP)	[c] : icit + nadp <==> akg + co2 + nadph	RLRE00637	EC-1.1.1.42
ILEt2	L-isoleucine transport in via proton symport	h[e] + ile-L[e] --> h[c] + ile-L[c]	RLRE00300	
ILEt6	L-isoeucine transport in/out via proton symport	h[e] + ile-L[e] <==> h[c] + ile-L[c]	RLRE00027, RLRE01221, RLRE01222	TC-2.A.26
ILETRS	Isoleucyl-tRNA synthetase	[c] : atp + ile-L + trnaile --> amp + h + iletrna + ppi	RLRE00643	EC-6.1.1.5
IMPC	IMP cyclohydrolase	[c] : h2o + imp <==> fprica	RLRE01420	EC-3.5.4.10
INSt2r	inosine transport in via proton symport, reversible	h[e] + ins[e] <==> h[c] + ins[c]		
IPDDI	isopentenyl-diphosphate D-isomerase	[c] : ipdp <==> dmpp	RLRE00338	EC-5.3.3.2
kaasIII	beta-ketoacyl-ACP synthase III	[c] : accoa + h + malacp --> aaacp + co2 + coa	RLRE01409	
Kabc	potassium transport via ABC system	atp[c] + h2o[c] + k[e] --> adp[c] + h[c] + k[c] + pi[c]	RLRE01274, RLRE01273	EC-3.6.3.12
L-LACt2	L-lactate reversible transport via proton symport	h[e] + lac-L[e] <==> h[c] + lac-L[c]		
LAR	lactate racemase	[c] : lac-L <==> lac-D		EC-5.1.2.1
LCTSt6	lactose transport in/out via proton symport	h[e] + lcts[e] <==> h[c] + lcts[c]	RLRE01387	TC-2.A.1.5
LDH_D	D-lactate dehydrogenase	[c] : lac-D + nad <==> h + nadh + pyr	RLRE00289	EC-1.1.1.28
LDH_L	L-lactate dehydrogenase	[c] : lac-L + nad <==> h + nadh + pyr	RLRE00332	EC-1.1.1.27

Abbrev.	Name	Equation	Gene[a.]	EC number
LEUt2	L-leucine transport in via proton symport	h[e] + leu-L[e] --> h[c] + leu-L[c]	RLRE00300	
LEUt6	L-leucine transport in/out via proton symport	h[e] + leu-L[e] <==> h[c] + leu-L[c]	RLRE00027, RLRE01221, RLRE01222	
LEUTRS	Leucyl-tRNA synthetase	[c] : atp + leu-L + trnaleu --> amp + h + leutrna + ppi	RLRE00521	EC-6.1.1.4
LPGS_LRE	lysylphosphatidyl-glycerol synthetase	[c] : lystrna + (0.01) pg_LRE --> (0.01) lyspg_LRE + trnalys	RLRE00819	
LTAS_LRE	Lipoteichoic acid synthase (LRE specific)	[c] : (0.01) dgdag_LRE + (0.25) pg_LRE --> (0.01) LTA_LRE + (0.25) dag_LRE		EC-2.7.8.20
LYSDC	lysine decarboxylase	[c] : h + lys-L --> 15dap + co2	RLRE00184	EC-4.1.1.18
LYSt6	L-lysine transport in/out via proton symport	h[e] + lys-L[e] <==> h[c] + lys-L[c]	RLRE00213	TC-2.A.3
LYSTRS	Lysyl-tRNA synthetase	[c] : atp + lys-L + trnalys --> amp + h + lystrna + ppi	RLRE01575	EC-6.1.1.6
M1PD	mannitol-1-phosphate 5-dehydrogenase	[c] : mnl1p + nad <==> f6p + h + nadh	RLRE00025	EC-1.1.1.17
MACPMT	Malonyl-CoA:[acyl-carrier-protein] S-malonyltransferase	[c] : acp + malcoa --> coa + malacp	RLRE01407	EC-2.3.1.39
MALLAC	malolactic enzyme	[c] : h + mal-L --> co2 + lac-L	RLRE00723	
MALP	maltose phosphorylase	[c] : malt + pi <==> g1p-B + glc-D	RLRE01240, RLRE00455	EC-2.4.1.8
MALT	alpha-glucosidase	[c] : h2o + malt --> (2) glc-D	RLRE00937	EC-3.2.1.20
MALt6	malate symporter	h[e] + mal-L[e] <==> h[c] + mal-L[c]	RLRE00444	
MAN6PI	mannose-6-phosphate isomerase	[c] : man6p <==> f6p	RLRE01659	EC-5.3.1.8
MANpts	D-mannose transport via PEP:Pyr PTS	man[e] + pep[c] --> man6p[c] + pyr[c]	RLRE00126, RLRE00764, RLRE00727, RLRE00729	
MDH	malate dehydrogenase	[c] : mal-L + nad <==> h + nadh + oaa	RLRE00649	EC-1.1.1.37
METabc	L-methionine transport via ABC system	atp[c] + h2o[c] + met-L[e] --> adp[c] + h[c] + met-L[c] + pi[c]	RLRE00967, RLRE00968, RLRE00969	
METACH	O-Acetyl-L-homoserine acetate-lyase (adding methanethiol)	[c] : achms + h2s <==> ac + h + hcys-L	RLRE00091	EC-4.2.99.10
METAT	methionine adenosyltransferase	[c] : atp + h2o + met-L --> amet + pi + ppi	RLRE00522	EC-2.5.1.6
METDabc	D-methionine transport via ABC system	atp[c] + h2o[c] + met-D[e] --> adp[c] + h[c] + met-D[c] + pi[c]	RLRE00967, RLRE00968, RLRE00969	
METt6	L-methionine transport in/out via proton symport	h[e] + met-L[e] <==> h[c] + met-L[c]		TC-2.A.3.8
METTRS	Methionyl-tRNA synthetase	[c] : atp + met-L + trnamet --> amp + h + mettrna + ppi	RLRE01076	EC-6.1.1.10
MEVK	mevalonate kinase	[c] : atp + mev-R --> 5pmev + adp + h	RLRE00341	EC-2.7.1.36
MEVK2	mevalonate kinase (ctp)	[c] : ctp + mev-R --> 5pmev + cdp + h	RLRE00341	EC-2.7.1.36
MEVK3	mevalonate kinase (gtp)	[c] : gtp + mev-R --> 5pmev + gdp + h	RLRE00341	EC-2.7.1.36
MEVK4	mevalonate kinase (utp)	[c] : mev-R + utp --> 5pmev + h + udp	RLRE00341	EC-2.7.1.36
MGt5	magnesium transport in/out via permease (no H+)	mg2[c] <==> mg2[e]	RLRE00042	TC-1.A.35
MI1PP	myo-inositol 1-phosphatase	[c] : h2o + mi1p-D --> inost + pi	RLRE00516	EC-3.1.3.25
MNabc	manganese transport via ABC system	atp[c] + h2o[c] + mn2[e] --> adp[c] + h[c] + mn2[c] + pi[c]	RLRE01341 +RLRE01339+RLRE01340	TC-3.A.1.15
MNLpts	mannitol transport via PEP:Pyr PTS	mnl[e] + pep[c] --> mnl1p[c] + pyr[c]	RLRE00022, RLRE00126, RLRE00024, RLRE00764	
MNt2	manganese transport in via proton symport	h[e] + mn2[e] --> h[c] + mn2[c]	RLRE01271, RLRE00077	TC-2.A.55
MNt6	manganese transport in/out via proton symport	h[e] + mn2[e] <==> h[c] + mn2[c]	RLRE01271, RLRE00077	TC-2.A.55
MOADCST	MoaD:cysteine sulfur transferase	[c] : MCOOH + atp + cys-L + h2o --> MCOSH + amp + ppi + ser-L	RLRE00745, RLRE00638, RLRE00318, RLRE00744	
MTAN	methylthioadenosine nucleosidase	[c] : 5mta + h2o --> 5mtr + ade	RLRE00639	EC-3.2.2.16
MTHFC	methenyltetrahydrofolate cyclohydrolase	[c] : h2o + methf <==> 10fthf + h	RLRE01565, RLRE01566	EC-3.5.4.9
MTHFD	methylenetetrahydrofolate dehydrogenase (NADP)	[c] : mlthf + nadp <==> methf + nadph	RLRE01565, RLRE01566	EC-1.5.1.5
MTHPTGHM	5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase	[c] : 5mthglu + hcys-L --> met-L + thglu	RLRE00087, RLRE01382	EC-2.1.1.14
NACUP	Nicotinic acid uptake	nac[e] --> nac[c]		
NADH4	NADH dehydrogenase (Menaquinone 7 & no proton)	[c] : h + mqn7 + nadh --> mql7 + nad	RLRE00738	EC-1.6.5.3
NADK	NAD kinase	[c] : atp + nad --> adp + h + nadp	RLRE00383	EC-2.7.1.23
NADN	NAD nucleosidase	[c] : h2o + nad --> adprib + h + ncam		EC-3.2.2.5
NADPH12	Quinone oxidoreductase	[c] : h + nadph + ubq8 --> nadp + ubq8h2	RLRE01687	EC-1.6.5.5

Abbrev.	Name	Equation	Gene[a.]	EC number
NADS1	NAD synthase (nh4)	[c] : atp + dnad + nh4 --> amp + h + nad + ppi	RLRE01545	EC-6.3.1.5
NAPRT	NAPRTase	[c] : h + nac + prpp --> nicrnt + ppi	RLRE01086, RLRE01546	EC-2.4.2.11
NAt7	sodium transport in/out via proton antiport (one H+)	h[e] + na1[c] <==> h[c] + na1[e]	RLRE01691, RLRE00698, RLRE01690, RLRE01066	TC-2.A.36
NDPK1	nucleoside-diphosphate kinase (ATP:GDP)	[c] : atp + gdp <==> adp + gtp	RLRE01068	EC-2.7.4.6
NDPK2	nucleoside-diphosphate kinase (ATP:UDP)	[c] : atp + udp <==> adp + utp	RLRE01068	EC-2.7.4.6
NDPK3	nucleoside-diphosphate kinase (ATP:CDP)	[c] : atp + cdp <==> adp + ctp	RLRE01068	EC-2.7.4.6
NDPK4	nucleoside-diphosphate kinase (ATP:dTDP)	[c] : atp + dtdp <==> adp + dttp	RLRE01068	EC-2.7.4.6
NDPK5	nucleoside-diphosphate kinase (ATP:dGDP)	[c] : atp + dgdp <==> adp + dgtp	RLRE01068	EC-2.7.4.6
NDPK6	nucleoside-diphosphate kinase (ATP:dUDP)	[c] : atp + dudp <==> adp + dutp	RLRE01068	EC-2.7.4.6
NDPK7	nucleoside-diphosphate kinase (ATP:dCDP)	[c] : atp + dcdp <==> adp + dctp	RLRE01068	EC-2.7.4.6
NDPK8	nucleoside-diphosphate kinase (ATP:dADP)	[c] : atp + dadp <==> adp + datp	RLRE01068	EC-2.7.4.6
NDPK9	nucleoside-diphophate kinase (ATP:IDP)	[c] : atp + idp <==> adp + itp	RLRE01068	EC-2.7.4.6
NH3t	ammonia transport via diffusion	nh3[e] <==> nh3[c]	RLRE00165	
NH4DIS	nh4 Dissociation	[c] : nh4 <==> h + nh3		
NH4DISex	nh4 Dissociation extracellular	[e] : nh4 <==> h + nh3		
NNAMr	nicotinamidase, reversible	[c] : h2o + ncam <==> nac + nh4	RLRE01087	EC-3.5.1.19
NNAT	nicotinate-nucleotide adenylyltransferase	[c] : atp + h + nicrnt --> dnad + ppi		EC-2.7.7.18
NNDMBRT	nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase	[c] : dmbzid + nicrnt --> 5prdmbz + h + nac	RLRE00286	EC-2.4.2.21
NOX1	NADH oxidase (H2O2 forming)	[c] : h + nadh + o2 --> h2o2 + nad	RLRE01226	
NOX2	NADH oxidase (H2O forming)	[c] : (2) h + (2) nadh + o2 --> (2) h2o + (2) nad	RLRE01226	
NPHS	naphthoate synthase	[c] : sbzcoa --> coa + dhna	RLRE00312	EC-4.1.3.36
NTD1	5'-nucleotidase (dUMP)	[c] : dump + h2o --> duri + pi	RLRE01195	EC-3.1.3.5
NTD10	5'-nucleotidase (XMP)	[c] : h2o + xmp --> pi + xtsn	RLRE01195	EC-3.1.3.5
NTD11	5'-nucleotidase (IMP)	[c] : h2o + imp --> ins + pi	RLRE01195	EC-3.1.3.5
NTD2	5'-nucleotidase (UMP)	[c] : h2o + ump --> pi + uri	RLRE01195	EC-3.1.3.5
NTD3	5'-nucleotidase (dCMP)	[c] : dcmp + h2o --> dcyt + pi	RLRE01195	EC-3.1.3.5
NTD4	5'-nucleotidase (CMP)	[c] : cmp + h2o --> cytd + pi	RLRE01195	EC-3.1.3.5
NTD5	5'-nucleotidase (dTMP)	[c] : dtmp + h2o --> pi + thymd	RLRE01195	EC-3.1.3.5
NTD6	5'-nucleotidase (dAMP)	[c] : damp + h2o --> dad-2 + pi	RLRE01195	EC-3.1.3.5
NTD7	5'-nucleotidase (AMP)	[c] : amp + h2o --> adn + pi	RLRE01195	EC-3.1.3.5
NTD8	5'-nucleotidase (dGMP)	[c] : dgmp + h2o --> dgsn + pi	RLRE01195	EC-3.1.3.5
NTD9	5'-nucleotidase (GMP)	[c] : gmp + h2o --> gsn + pi	RLRE01195	EC-3.1.3.5
NTR3B	nitrate reductase	(2) h[c] + mql7[c] + no3[c] --> (2) h[e] + h2o[c] + mqn7[c] + no2[c]	RLRE01249	EC-1.7.99.4,
OCBT	ornithine carbamoyltransferase	[c] : cbp + orn-L <==> citr-L + h + pi	RLRE01360	EC-2.1.3.3
OCDMAT8	Octadecanoyl-[acyl-carrier protein]:malonyl-CoA C-acyltransferase	[c] : 2tocdacp + h + nadh --> nad + ocdacp	RLRE01399	EC-1.3.1.10
OCMAT3	Octanoyl-[acyl-carrier protein]:malonyl-CoA C-acyltransferase	[c] : 2toceacp + h + nadh --> nad + octacp	RLRE01399	EC-1.3.1.10
OMPDC	orotidine-5'-phosphate decarboxylase	[c] : h + orot5p --> co2 + ump	RLRE01216	EC-4.1.1.23
ORNt2	ornithine transport in via proton symport	h[e] + orn-L[e] --> h[c] + orn-L[c]	RLRE00773	
OROt6	Orotic acid transport in/out via proton symporter	h[e] + orot[e] <==> h[c] + orot[c]		
ORPT	orotate phosphoribosyltransferase	[c] : orot5p + ppi <==> orot + prpp	RLRE01215	EC-2.4.2.10
P5CRr	pyrroline-5-carboxylate reductase	[c] : 1pyr5c + (2) h + nadph <==> nadp + pro-L	RLRE00579	EC-1.5.1.2
PABAt	para aminobenzoic acid transport	4abz[e] <==> 4abz[c]		
PAPPT5	phospho-N-acetylmuramoyl-pentapeptide-transferase (meso-2,6-diaminopimelate)	[c] : udcpp + ugmdalac --> uagmdalac + ump	RLRE01108	EC-2.7.8.13
PDH	pyruvate dehydrogenase	[c] : coa + nad + pyr --> accoa + co2 + nadh	RLRE01634, RLRE01637, RLRE01636, RLRE01635	EC-1.2.1.51
PFK	phosphofructokinase	[c] : atp + f6p --> adp + fdp + h	RLRE00632	EC-2.7.1.11
PGAMT	phosphoglucosamine mutase	[c] : gam1p <==> gam6p	RLRE01550	EC-5.4.2.10

Abbrev.	Name	Equation	Gene[a.]	EC number
PGDH	phosphogluconate dehydrogenase	[c] : 6pgc + nadp --> co2 + nadph + ru5p-D		EC-1.1.1.44
PGGH	6-phospho-beta-glucosidase	[c] : 6pgg + h2o --> g6p + glc-D	RLRE00441	EC-3.2.1.86
PGGT4	peptidoglycan glycosyltransferase	[c] : uaagmdalac --> PGlac2 + udcpdp	RLRE01695, RLRE00346, RLRE01109	
PGI	glucose-6-phosphate isomerase	[c] : g6p <==> f6p	RLRE01355	EC-5.3.1.9
PGK	phosphoglycerate kinase	[c] : 13dpg + adp <==> 3pg + atp		EC-2.7.2.3
PGL	6-phosphogluconolactonase	[c] : 6pgl + h2o --> 6pgc + h	RLRE01117	EC-3.1.1.31
PGLYCP	phosphoglycolate phosphatase	[c] : 2pglyc + h2o --> glyclt + pi	RLRE00686	EC-3.1.3.18
PGM	phosphoglycerate mutase	[c] : 3pg <==> 2pg	RLRE00790, RLRE01035, RLRE00635, RLRE01422, RLRE01169, RLRE00251, RLRE00053	EC-5.4.2.1
PGMT	phosphoglucomutase	[c] : g1p <==> g6p	RLRE01496	EC-5.4.2.2
PGMT_B	b-phosphoglucomutase	[c] : g6p-B <==> g1p-B	RLRE01239	EC-5.4.2.6
PGPP_LRE	Phosphatidylglycerol phosphate phosphatase (LRE specific)	[c] : h2o + (0.01) pgp_LRE --> (0.01) pg_LRE + pi	RLRE00987	EC-3.1.3.27
PGSA_LRE	Phosphatidylglycerol synthase (LRE specific)	[c] : (0.01) cdpdag_LRE + glyc3p <==> cmp + h + (0.01) pgp_LRE	RLRE00726	EC-2.7.8.5
PHEt6	L-phenylalanine transport in/out via proton symport	h[e] + phe-L[e] <==> h[c] + phe-L[c]		TC-2.A.3.1
PHETRS	Phenylalanyl-tRNA synthetase	[c] : atp + phe-L + trnaphe --> amp + h + phetrna + ppi		EC-6.1.1.20
PIabc	phosphate transport via ABC system	atp[c] + h2o[c] + pi[e] --> adp[c] + h[c] + (2) pi[c]	RLRE01283, RLRE01281, RLRE01280, RLRE01282, RLRE01284	TC-3.A.1.7
PIt6	phosphate transport in/out via proton symporter	h[e] + pi[e] <==> h[c] + pi[c]		TC-2.A.20
PKL	phosphoketolase	[c] : pi + xu5p-D <==> actp + g3p + h2o	RLRE00295	EC-4.1.2.9
PMANM	phosphomannomutase	[c] : man1p <==> man6p	RLRE01496	EC-5.4.2.8
PMEVK	phosphomevalonate kinase	[c] : 5pmev + atp --> 5dpmev + adp	RLRE00339	EC-2.7.4.2
PMPK	phosphomethylpyrimidine kinase	[c] : 4ampm + atp --> 2mahmp + adp	RLRE00934	EC-2.7.4.7
PNS1	purine nucleosidase (adenosine)	[c] : adn + h2o --> ade + rib-D	RLRE00691	EC-3.2.2.1
PNS2	purine nucleosidase (guanosine)	[c] : gsn + h2o --> gua + rib-D	RLRE00691	EC-3.2.2.1
PNS3	purine nucleosidase (inosine)	[c] : h2o + ins --> hxan + rib-D	RLRE00691	EC-3.2.2.1
PNS4	purine nucleosidase (xanthosine)	[c] : h2o + xtsn --> rib-D + xan	RLRE00691	EC-3.2.2.1
PNTK	pantothenate kinase	[c] : atp + pnto-R --> 4ppan + adp + h	RLRE01043	EC-2.7.1.33
PNTOt2	Pantothenate reversible transport via proton symport	h[e] + pnto-R[e] <==> h[c] + pnto-R[c]		
PPA	inorganic diphosphatase	[c] : h2o + ppi --> h + (2) pi	RLRE00363	EC-3.6.1.1
PPCDC	phosphopantothenoylcysteine decarboxylase	[c] : 4ppcys + h --> co2 + pan4p	RLRE01438	EC-4.1.1.36
PPDt1	1,3-propanediol transport in/out via diffusion reversible	13ppd[c] <==> 13ppd[e]		
PPIK	polyphosphate kinase	[c] : atp + ppi --> adp + pppi	RLRE00571	EC-2.7.4.1
PPM	phosphopentomutase	[c] : r1p <==> r5p	RLRE01425, RLRE00979	EC-5.4.2.7
PPM2	phosphopentomutase (deoxyribose)	[c] : 2dr1p <==> 2dr5p	RLRE01425, RLRE00979	EC-5.4.2.7
PPN13D	1,3-propanediol dehydrogenase	[c] : 13ppd + nad <==> 3hppnl + h + nadh	RLRE00989	EC-1.1.1.202
PPNCL	phosphopantothenate-cysteine ligase	[c] : 4ppan + ctp + cys-L --> 4ppcys + cdp + h + pi	RLRE01438	EC-6.3.2.5
PRAGS	phosphoribosylglycinamide synthetase	[c] : atp + gly + pram --> adp + gar + h + pi	RLRE01421	EC-6.3.4.13
PRAIS	phosphoribosylaminoimidazole synthetase	[c] : atp + fpram --> adp + air + h + pi	RLRE01418	EC-6.3.3.1
PRASCS	phosphoribosylaminoimidazolesuccinocarboxamide synthase	[c] : 5aizc + asp-L + atp <==> 25aics + adp + h + pi	RLRE01413	EC-6.3.2.6
PRFGS	phosphoribosylformylglycinamidine synthase	[c] : atp + fgam + gln-L + h2o --> adp + fpram + glu-L + (2) h + pi	RLRE01416, RLRE01414, RLRE01415	EC-6.3.5.3
PROt6	L-proline transport in/out via proton symport	h[e] + pro-L[e] <==> h[c] + pro-L[c]		TC-2.A.3.1
PROTRS	Prolyl-tRNA synthetase	[c] : atp + pro-L + trnapro --> amp + h + ppi + protrna	RLRE00670	EC-6.1.1.15

Abbrev.	Name	Equation	Gene[a.]	EC number
PROTS_LRE_v6.0	protein synthesis_LRE belonging to biomass version 6.0	[c] : (0.125) alatrna + (0.04) argtrna + (0.06) asntrna + (0.06) asptrna + (0.306) atp + (0.011) cystrna + (0.083) glntrna + (0.023) glutrna + (0.084) glytrna + (2) gtp + (2.306) h2o + (0.017) histrna + (0.043) iletrna + (0.078) leutrna + (0.066) lystrna + (0.022) mettrna + (0.034) phetrna + (0.04) protrna + (0.056) sertrna + (0.064) thrtrna + (0.006) trptrna + (0.028) tyrtrna + (0.06) valtrna --> (0.001) PROT_LRE_v6.0 + (0.306) adp + (2) gdp + (2.306) h + (2.306) pi + (0.125) trnaala + (0.04) trnaarg + (0.06) trnaasn + (0.06) trnaasp + (0.011) trnacys + (0.106) trnaglu + (0.084) trnagly + (0.017) trnahis + (0.043) trnaile + (0.078) trnaleu + (0.066) trnalys + (0.022) trnamet + (0.034) trnaphe + (0.04) trnapro + (0.056) trnaser + (0.064) trnathr + (0.006) trnatrp + (0.028) trnatyr + (0.06) trnaval		
PRPPS	phosphoribosylpyrophosphate synthetase	[c] : atp + r5p <==> amp + h + prpp	RLRE01344	EC-2.7.6.1
PSUDS	pseudouridylate synthase	[c] : r5p + ura --> h2o + psd5p	RLRE01648, RLRE00818, RLRE00662, RLRE00356, RLRE00041	EC-4.2.1.70
PTAr	phosphotransacetylase	[c] : accoa + pi <==> actp + coa	RLRE01504	EC-2.3.1.8
PTPAT	pantetheine-phosphate adenylyltransferase	[c] : atp + h + pan4p <==> dpcoa + ppi	RLRE01586	EC-2.7.7.3
PTPATi	pantetheine-phosphate adenylyltransferase	[c] : atp + h + pan4p --> dpcoa + ppi	RLRE01586	EC-2.7.7.3
PTRCabc	putrescine transport via ABC system	atp[c] + h2o[c] + ptrc[e] --> adp[c] + h[c] + pi[c] + ptrc[c]	RLRE00679, RLRE00677, RLRE00678, RLRE00680	TC-3.A.1.11
PUNP1	purine-nucleoside phosphorylase (Adenosine)	[c] : adn + pi <==> ade + r1p	RLRE01423	EC-2.4.2.1
PUNP2	purine-nucleoside phosphorylase (Deoxyadenosine)	[c] : dad-2 + pi <==> 2dr1p + ade	RLRE01423	EC-2.4.2.1
PUNP3	purine-nucleoside phosphorylase (Guanosine)	[c] : gsn + pi <==> gua + r1p	RLRE01423	EC-2.4.2.1
PUNP4	purine-nucleoside phosphorylase (Deoxyguanosine)	[c] : dgsn + pi <==> 2dr1p + gua	RLRE01423	EC-2.4.2.1
PUNP5	purine-nucleoside phosphorylase (Inosine)	[c] : ins + pi <==> hxan + r1p	RLRE01423	EC-2.4.2.1
PUNP6	purine-nucleoside phosphorylase (Deoxyinosine)	[c] : din + pi <==> 2dr1p + hxan	RLRE01423	EC-2.4.2.1
PUNP7	purine-nucleoside phosphorylase (Xanthosine)	[c] : pi + xtsn <==> r1p + xan	RLRE01423	EC-2.4.2.1
PYDAMK	pyridoxal kinase (Pyridoxamine)	[c] : atp + pydam --> adp + h + pyam5p		EC-2.7.1.35
PYDAMt	pyridoxamine transport	h[e] + pydam[e] <==> h[c] + pydam[c]		
PYDXK	pyridoxal kinase (Pyridoxal)	[c] : atp + pydx --> adp + (2) h + pydx5p		EC-2.7.1.35
PYDXPP	Pyridoxal 5-phosphate phosphatase	[c] : h + h2o + pydx5p --> pi + pydx		
PYK	pyruvate kinase	[c] : adp + h + pep --> atp + pyr	RLRE00847	EC-2.7.1.40
PYNP1	pyrimidine-nucleoside phosphorylase (cytosine)	[c] : csn + r1p --> cytd + pi	RLRE01424	EC-2.4.2.2
PYNP1r	pyrimidine-nucleoside phosphorylase (cytosine)	[c] : csn + r1p <==> cytd + pi	RLRE01424	EC-2.4.2.2
PYNP2	pyrimidine-nucleoside phosphorylase (uracil)	[c] : pi + uri <==> r1p + ura	RLRE01424	EC-2.4.2.2
PYNP3	pyrimidine-nucleoside phosphorylase (thymine)	[c] : 2dr1p + thym --> pi + thymd	RLRE01424	EC-2.4.2.2
PYNP4	pyrimidine-nucleoside phosphorylase (pyrimidine nucleoside)	[c] : pi + pyrin <==> pyrimid + r1p	RLRE01424	EC-2.4.2.2
PYRNS1	pyrimidine nucleosidase (uridine)	[c] : h2o + uri --> rib-D + ura	RLRE01151	EC-3.2.2.8
PYRt2	pyruvate reversible transport via proton symport	h[e] + pyr[e] <==> h[c] + pyr[c]		
PYRZAM	Pyrazinamidase	[c] : h2o + malm --> male + nh4	RLRE01087	
QUIND	quinate dehydrogenase (pyrroloquinoline-quinone)	[c] : pqqox + quin --> 3dhq + h + pqqrd	RLRE01095	EC-1.1.99.25
R05220	Cob(I)yrinate a,c diamide <=> Adenosyl cobyrinate a,c diamide	[c] : atp + co1dam + h2o + (0.5) nadh --> adcobdam + (0.5) h + (0.5) nad + pi + ppi	RLRE00244, RLRE00256	
R05223	Adenosine-GDP-cobinamide + alpha-Ribazole <=> Cobamide coenzyme	[c] : adgcoba + (0.5) h + (0.5) nad + rdmbzi --> cobamcoa + gmp + (0.5) nadh	RLRE00283	
R05225	Adenosyl cobyrinate a,c diamide <=> Adenosyl cobyrinate hexaamide	[c] : adcobdam + (4) atp + (4) gln-L + (4) h2o --> adcobhex + (4) adp + (4) glu-L + (4) h + (4) pi	RLRE00276	
R05226	Adenosyl cobyrinate hexaamide l <=> Adenosyl cobinamide	[c] : 1ap2ol + adcobhex --> adcoba + h2o	RLRE00260	
RAFGH	Raffinose galactohydrolase	[c] : h2o + raffin --> gal + sucr	RLRE00336	EC-3.2.1.22
RBFK	riboflavin kinase	[c] : atp + ribflv --> adp + fmn + h	RLRE00661	EC-2.7.1.26
RBFSa	riboflavin synthase	[c] : 4r5au + db4p --> dmlz + (2) h2o + pi	RLRE01153	EC-2.5.1.9
RBK	ribokinase	[c] : atp + rib-D --> adp + h + r5p	RLRE01090, RLRE01556	EC-2.7.1.15
RBLK1	L-ribulokinase (L-ribulose)	[c] : atp + rbl-L --> adp + h + ru5p-L	RLRE01515	EC-2.7.1.16

Abbrev.	Name	Equation	Gene[a.]	EC number
RBLK2	L-ribulokinase (ribitol)	[c] : atp + rbt --> adp + h + rbt5p	RLRE01515	EC-2.7.1.16
RBP4E	L-ribulose-phosphate 4-epimerase	[c] : ru5p-L <==> xu5p-D	RLRE01514	EC-5.1.3.4
RBT5PDHy	ribitol-5-phosphate 2-dehydrogenase (NADP)	[c] : nadp + rbt5p <==> h + nadph + ru5p-D		EC-1.1.1.137
RBZP	alpha-ribazole-5-phosphatase	[c] : 5prdmbz + h2o --> pi + rdmbzi	RLRE00284	
RHC	ribosylhomocysteinase	[c] : h2o + rhcys --> hcys-L + rib-D	RLRE01300	EC-3.2.1.148
RIBFLVt2	riboflavin transport in via proton symport	h[e] + ribflv[e] --> h[c] + ribflv[c]	RLRE01699	
RNAS_LRE	RNA synthesis, L.plantarum specific	[c] : (0.66) atp + (0.2) ctp + (0.32) gtp + (0.4) h2o + (0.22) utp --> (0.01) RNA_LRE + (0.4) adp + (0.4) h + (0.4) pi + ppi		
RNDR1	ribonucleoside-diphosphate reductase (ADP)	[c] : adp + trdrd --> dadp + h2o + trdox	RLRE00556, RLRE00557	EC-1.17.4.1
RNDR2	ribonucleoside-diphosphate reductase (GDP)	[c] : gdp + trdrd --> dgdp + h2o + trdox	RLRE00556, RLRE00557	EC-1.17.4.1
RNDR3	ribonucleoside-diphosphate reductase (CDP)	[c] : cdp + trdrd --> dcdp + h2o + trdox	RLRE00556, RLRE00557	EC-1.17.4.1
RNDR4	ribonucleoside-diphosphate reductase (UDP)	[c] : trdrd + udp --> dudp + h2o + trdox	RLRE00556, RLRE00557	EC-1.17.4.1
RNTR1	ribonucleoside-triphosphate reductase (ATP)	[c] : atp + trdrd --> datp + h2o + trdox	RLRE00542, RLRE00350, RLRE00219	EC-1.17.4.2
RNTR2	ribonucleoside-triphosphate reductase (GTP)	[c] : gtp + trdrd --> dgtp + h2o + trdox	RLRE00542, RLRE00350, RLRE00219	EC-1.17.4.2
RNTR3	ribonucleoside-triphosphate reductase (CTP)	[c] : ctp + trdrd --> dctp + h2o + trdox	RLRE00542, RLRE00350, RLRE00219	EC-1.17.4.2
RNTR4	ribonucleoside-triphosphate reductase (UTP)	[c] : trdrd + utp --> dutp + h2o + trdox	RLRE00542, RLRE00350, RLRE00219	EC-1.17.4.2
RPE	ribulose 5-phosphate 3-epimerase	[c] : ru5p-D <==> xu5p-D	RLRE01445	EC-5.1.3.1
RPI	ribose-5-phosphate isomerase	[c] : r5p <==> ru5p-D	RLRE01162	EC-5.3.1.6
SBTpts	D-sorbitol transport via PEP:Pyr PTS	pep[c] + sbt-D[e] --> pyr[c] + sbt6p[c]	RLRE00126, RLRE00764	
SDPDS	succinyl-diaminopimelate desuccinylase	[c] : h2o + sl26da --> 26dap-LL + succ	RLRE00970, RLRE00964	EC-3.5.1.18
SECYSL	selenocysteine lyase	[c] : dtt_rd + scys-L --> H2Se + ala-L + dtt_ox	RLRE00745, RLRE00638, RLRE00318	EC-4.4.1.16
SERAT	serine O-acetyltransferase	[c] : accoa + ser-L <==> acser + coa		EC-2.3.1.30
SERD_L	L-serine deaminase	[c] : ser-L --> nh4 + pyr		EC-4.3.1.17
SERt3	L-serine transport out via proton antiport	h[e] + ser-L[c] --> h[c] + ser-L[e]	RLRE00801, RLRE00824, RLRE00800	
SERt6	L-serine transport in/out via proton symport	h[e] + ser-L[e] <==> h[c] + ser-L[c]		TC-2.A.42
SERTRS	Seryl-tRNA synthetase	[c] : atp + ser-L + trnaser --> amp + h + ppi + sertrna	RLRE01242	EC-6.1.1.11
SHCHD2	sirohydrochlorin dehydrogenase (NAD)	[c] : nad + shcl --> (2) h + nadh + srch	RLRE00277	
SPMDabc	spermidine transport via ABC system	atp[c] + h2o[c] + spmd[e] --> adp[c] + h[c] + pi[c] + spmd[c]	RLRE00679, RLRE00677, RLRE00678, RLRE00680	TC-3.A.1.11
SSALx	succinate-semialdehyde dehydrogenase (NAD)	[c] : h2o + nad + sucsal --> (2) h + nadh + succ		EC-1.2.1.16
SSALy	succinate-semialdehyde dehydrogenase (NADP)	[c] : h2o + nadp + sucsal --> (2) h + nadph + succ		EC-1.2.1.16
SUCBZL	o-succinylbenzoate-CoA ligase	[c] : atp + coa + sucbz --> amp + ppi + sbzcoa	RLRE00313	EC-6.2.1.26
SUCpts	sucrose transport via PEP:Pyr PTS	pep[c] + sucr[e] --> pyr[c] + suc6p[c]	RLRE00126, RLRE00764, RLRE01426	
TAPGL4	teichoic acid peptidoglycan ligase	[c] : unRTA --> RTA + udcpp		
TDMAT6	Tetradecanoyl-[acyl-carrier protein]:malonyl-CoA C-acyltransferase	[c] : 2ttdeacp + h + nadh --> nad + tdeacp	RLRE01399	EC-1.3.1.10
TDPDRE	dTDP-4-dehydrorhamnose 3,5-epimerase	[c] : dtdpddg <==> dtdpddm		EC-5.1.3.13
TDPDRR	dTDP-4-dehydrorhamnose reductase	[c] : dtdp6dm + nadp <==> dtdpddm + h + nadph	RLRE01021	EC-1.1.1.133
TDPGDH	dTDPglucose 4,6-dehydratase	[c] : dtdpglc --> dtdpddg + h2o	RLRE01022	EC-4.2.1.46
THDPS	tetrahydropicolinate succinylase	[c] : h2o + succoa + thdp --> coa + sl2a6o	RLRE00628	EC-2.3.1.117
THFGLUS	Tetrahydrofolate:L-glutamate gamma-ligase (ADP-forming)	[c] : atp + glu-L + thf <==> adp + h + pi + thfglu	RLRE01598, RLRE00737	EC-6.3.2.17
THMabc	thiamine transport via ABC system	atp[c] + h2o[c] + thm[e] --> adp[c] + h[c] + pi[c] + thm[c]		
THMDt2r	thymidine transport in via proton symport, reversible	h[e] + thymd[e] <==> h[c] + thymd[c]		

Abbrev.	Name	Equation	Gene[a.]	EC number
THRPDC	Threonine-phosphate decarboxylase (EC 4.1.1.81)	[c] : thrp --> 1ap2olp + co2	RLRE00258	
THRt2	L-threonine transport in via proton symport	h[e] + thr-L[e] --> h[c] + thr-L[c]		
THRt3	L-threonine transport out via proton antiport	h[e] + thr-L[c] --> h[c] + thr-L[e]	RLRE00801, RLRE00824, RLRE00800	
THRTRS	Threonyl-tRNA synthetase	[c] : atp + thr-L + trnathr --> amp + h + ppi + thrtrna	RLRE00079	EC-6.1.1.3
THZPSN	thiazole phosphate synthesis	[c] : atp + cys-L + dxyl5p + tyr-L --> 4hba + 4mpetz + ala-L + amp + co2 + h + h2o + ppi	RLRE00820	
THZPSN2	thiazole phosphate synthesis	[c] : MCOSH + dxyl5p + gly + nadp --> 4mpetz + MCOOH + co2 + (2) h + (2) h2o + nadph	RLRE00820	
TMDK1	thymidine kinase (ATP:thymidine)	[c] : atp + thymd --> adp + dtmp + h	RLRE00770	EC-2.7.1.21
TMDK2	thymidine kinase (GTP:Thymidine)	[c] : gtp + thymd --> dtmp + gdp + h	RLRE00770	EC-2.7.1.21
TMDPK	thiamine diphosphokinase	[c] : atp + thm --> amp + h + thmpp		EC-2.7.6.2
TMDS	thymidylate synthase	[c] : dump + mlthf --> dhf + dtmp	RLRE01183	EC-2.1.1.45
TMPPP	thiamine-phosphate diphosphorylase	[c] : 2mahmp + 4mpetz + h --> ppi + thmmp	RLRE00935	EC-2.5.1.3
TPI	triose-phosphate isomerase	[c] : dhap <==> g3p	RLRE00788, RLRE01102, RLRE01103	EC-5.3.1.1
TRDR	thioredoxin reductase (NADPH)	[c] : h + nadph + trdox --> nadp + trdrd	RLRE01590, RLRE00776, RLRE00963	EC-1.8.1.9
TREpts	trehalose transport via PEP:Pyr PTS	pep[c] + tre[e] --> pyr[c] + tre6p[c]	RLRE00126, RLRE00764, RLRE00243, RLRE00454	
TRPt6	L-tryptophan transport in/out via proton symport	h[e] + trp-L[e] <==> h[c] + trp-L[c]		TC-2.A.3.1
TRPTRS	Tryptophanyl-tRNA synthetase	[c] : atp + trnatrp + trp-L --> amp + h + ppi + trptrna	RLRE01070	EC-6.1.1.2
TYRt6	L-tyrosine transport in/out via proton symport	h[e] + tyr-L[e] <==> h[c] + tyr-L[c]	RLRE01097, RLRE00045	TC-2.A.3.1
TYRTRS	Tyrosyl-tRNA synthetase	[c] : atp + trnatyr + tyr-L --> amp + h + ppi + tyrtrna		EC-6.1.1.1
UAAGDS	UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-meso-2,6-diaminopimelate synthetase	[c] : 26dap-M + atp + uamag --> adp + h + pi + ugmd	RLRE00771	EC-6.3.2.13
UAAGLS1	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate-lysine synthetase (alpha-glutamate)	[c] : atp + lys-L + uamag --> adp + (2) h + pi + uAgl	RLRE00464	EC-6.3.2.7
UAG2E	UDP-N-acetylglucosamine 2-epimerase	[c] : uacgam <==> uacmam	RLRE00546, RLRE00188	EC-5.1.3.14
UAG2EMA	UDP-N-acetyl-D-glucosamine 2-epimerase (Hydrolysis)	[c] : h2o + uacgam <==> acmana + h + udp	RLRE00546, RLRE00188	EC-5.1.3.14
UAGCVT	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	[c] : pep + uacgam --> pi + uaccg	RLRE00783, RLRE00752	EC-2.5.1.7
UAGDP	UDP-N-acetylglucosamine diphosphorylase	[c] : acgam1p + h + utp --> ppi + uacgam	RLRE01343	EC-2.7.7.23
UAGPT5	UDP-N-acetylglucosamine-N-acetylmuramyl-(pentapeptide)pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	[c] : uacgam + uagmdalac --> h + uaagmdalac + udp	RLRE01106	
UAMAGS	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase	[c] : atp + glu-D + uama --> adp + h + pi + uamag	RLRE01107	EC-6.3.2.9
UAMAS	UDP-N-acetylmuramoyl-L-alanine synthetase	[c] : ala-L + atp + uamr --> adp + h + pi + uama	RLRE01620	EC-6.3.2.8
UAPGR	UDP-N-acetylenolpyruvoylglucosamine reductase	[c] : h + nadph + uaccg --> nadp + uamr	RLRE01557	EC-1.1.1.158
UDCPDP	undecaprenyl-diphosphatase	[c] : h2o + udcpdp --> h + pi + udcpp		EC-3.6.1.27
UDCPDPS	Undecaprenyl diphosphate synthase	[c] : frdp + (8) ipdp --> (8) ppi + udcpdp	RLRE01385, RLRE00673, RLRE01562	
UDCPK	undecaprenol kinase	[c] : atp + udcp --> adp + h + udcpp		EC-2.7.1.66
UDPDPS	undecaprenyl-diphosphate synthase	[c] : decdp + ipdp --> ppi + udcpdp	RLRE00673	EC-2.5.1.31
UDPG4E	UDPglucose 4-epimerase	[c] : udpg <==> udpgal	RLRE00573	EC-5.1.3.2
UDPGALM	UDPgalactopyranose mutase	[c] : udpgal --> udpgalfur	RLRE00204	EC-5.4.99.9
UGLDDS1	UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine synthetase (alpha-glutamate)	[c] : alaala + atp + uAgl --> adp + pi + uAgla	RLRE00794	EC-6.3.2.10
UGLDDS2	UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine synthetase (gamma-glutamate)	[c] : alaala + atp + uGgl --> adp + h + pi + uGgla	RLRE00794	EC-6.3.2.10
UGLDDS3	UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-lactate synthetase (alpha-glutamate)	[c] : alalac + atp + uAgl --> adp + pi + uAglac	RLRE00794	EC-6.3.2.10
UGMDDS2	UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-meso-2,6-diaminopimeloyl-D-alanyl-D-lactate synthetase	[c] : alalac + atp + ugmd --> adp + h + pi + ugmdalac	RLRE00794	

Abbrev.	Name	Equation	Gene[a.]	EC number
UNAGAMAMT	N-acetylglucosaminyldiphospho-undecaprenol-N-acetyl-beta-D-mannosaminyltransferase	[c] : uacmam + unaga --> h + udp + unagama		EC-2.4.1.187
UPPRT	uracil phosphoribosyltransferase	[c] : prpp + ura --> ppi + ump	RLRE00765	EC-2.4.2.9
URAt2	uracil transport in via proton symport	h[e] + ura[e] --> h[c] + ura[c]	RLRE00761	TC-2.A.40
URIDK1	uridylate kinase (UMP)	[c] : atp + ump --> adp + udp	RLRE01190	
URIDK2	uridylate kinase (dUMP)	[c] : atp + dump --> adp + dudp	RLRE01190	
URIK1	uridine kinase (ATP:Uridine)	[c] : atp + uri --> adp + h + ump		EC-2.7.1.48
URIK2	uridine kinase (GTP:Uridine)	[c] : gtp + uri --> gdp + h + ump		EC-2.7.1.48
URIK3	uridine kinase (ITP:Uridine)	[c] : itp + uri --> h + idp + ump		EC-2.7.1.48
VALt2	L-valine transport in via proton symport	h[e] + val-L[e] --> h[c] + val-L[c]	RLRE00300	
VALt6	L-valine transport in/out via proton symport	h[e] + val-L[e] <==> h[c] + val-L[c]	RLRE00027, RLRE01221, RLRE01222	TC-2.A.26
VALTRS	Valyl-tRNA synthetase	[c] : atp + trnaval + val-L --> amp + h + ppi + valtrna	RLRE00743	EC-6.1.1.9
XANt2	xanthine transport in via proton symport	h[e] + xan[e] --> h[c] + xan[c]	RLRE00044	TC-2.A.40
XPPT	xanthine phosphoribosyltransferase	[c] : prpp + xan --> ppi + xmp	RLRE01051	EC-2.4.2.22
XYLK	xylulokinase	[c] : atp + xylu-D --> adp + h + xu5p-D	RLRE01357	EC-2.7.1.17
YUMPS	yUMP synthetase	[c] : r5p + ura <==> h2o + psd5p	RLRE01648, RLRE00818, RLRE00662, RLRE00356, RLRE00041	EC-4.2.1.70

[a.] Association between RLRE and Lreu numbering system is available upon request.

Table 4.S1.2. Complete list of model metabolites and their abbreviation.

Abbreviation	Official name	Formula
10fthf	10-Formyltetrahydrofolate	C20H21N7O7
13dpg	3-Phospho-D-glyceroyl phosphate	C3H4O10P2
13ppd	Propane-1,3-diol	C3H8O2
15dap	1,5-Diaminopentane	C5H16N2
1ap2ol	1-Aminopropan-2-ol	C3H10NO
1ap2olp	D-1-Aminopropan-2-ol O-phosphate	C3H10NO4P
1pyr5c	1-Pyrroline-5-carboxylate	C5H6NO2
23dhdp	2,3-Dihydrodipicolinate	C7H5NO4
25aics	(S)-2-[5-Amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamido]succinate	C13H15N4O12P
25dhpp	2,5-Diamino-6-hydroxy-4-(5'-phosphoribosylamino)-pyrimidine	C9H14N5O8P
25dkglcn	2,5-diketo-D-gluconate	C6H7O7
26dap-LL	LL-2,6-Diaminoheptanedioate	C7H14N2O4
26dap-M	meso-2,6-Diaminoheptanedioate	C7H14N2O4
2ahbut	(S)-2-Aceto-2-hydroxybutanoate	C6H9O4
2ahhmd	2-Amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine diphosphate	C7H8N5O8P2
2ahhmp	2-Amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine	C7H9N5O2
2beacp	But-2-enoyl-[acyl-carrier protein]	C4H4OX
2c25dho	2-Carboxy-2,5-dihydro-5-oxofuran-2-acetate	C7H6O6
2chdeacp	cis-Hexadec-2-enoyl-[acyl-carrier protein]	C16H28OX
2cocdacp	cis-Octadec-2-enoyl-[acyl-carrier-protein]	C18H32OX
2dhguln	2-Dehydro-L-gulonate	C6H9O7
2dhp	2-Dehydropantoate	C6H9O4
2dmmq7	2-Demethylmenaquinone 7	C45H62O2
2dmmq8	2-Demethylmenaquinone 8	C50H70O2
2dr1p	2-Deoxy-D-ribose 1-phosphate	C5H9O7P
2dr5p	2-Deoxy-D-ribose 5-phosphate	C5H9O7P
2hxic-L	L-2-hydroxyisocaproate	C6H11O3
2mahmp	2-Methyl-4-amino-5-hydroxymethylpyrimidine diphosphate	C6H8N3O7P2
2mpal	2-methylpropanal	C4H8O
2mpol	2-methylpropanol	C4H10O
2obut	2-Oxobutanoate	C4H5O3
2p4c2me	2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol	C14H22N3O17P3
2pg	D-Glycerate 2-phosphate	C3H4O7P
2pglyc	2-Phosphoglycolate	C2H2O6P
2tddacp	trans-Dodec-2-enoyl-[acyl-carrier protein]	C12H20OX
2tdeacp	trans-Dec-2-enoyl-[acyl-carrier protein]	C10H16OX
2thdeacp	trans-Hexadec-2-enoyl-[acyl-carrier protein]	C16H28OX
2theacp	trans-Hex-2-enoyl-[acp]	C6H8OX
2tocdacp	trans-Octadec-2-enoyl-[acyl-carrier-protein]	C18H32OX
2toceacp	trans-Oct-2-enoyl-[acp]	C8H12OX
2ttdeacp	trans-Tetradec-2-enoyl-[acyl-carrier protein]	C14H24OX
34hpp	3-(4-Hydroxyphenyl)pyruvate	C9H7O4
3dhq	3-Dehydroquinate	C7H9O6
3hbacp	(3R)-3-Hydroxybutanoyl-[acyl-carrier protein]	C4H6O2X
3hbycoa	(S)-3-Hydroxybutyryl-CoA	C25H38N7O18P3S
3hddacp	(R)-3-Hydroxydodecanoyl-[acyl-carrier protein]	C12H22O2X
3hdeacp	(3R)-3-Hydroxydecanoyl-[acyl-carrier protein]	C10H18O2X
3hhacp	(R)-3-Hydroxyhexanoyl-[acp]	C6H10O2X
3hocacp	(R)-3-Hydroxyoctanoyl-[acyl-carrier protein]	C8H14O2X
3hocdacp	(3R)-3-Hydroxyoctadecanoyl-[acyl-carrier protein	C18H34O2X
3hpaacp	(3R)-3-Hydroxypalmitoyl-[acyl-carrier protein]	C16H30O2X

Abbreviation	Official name	Formula
3hppnl	3-Hydroxypropanal	C3H6O2
3htdacp	(3R)-3-Hydroxytetradecanoyl-[acyl-carrier protein]	C14H26O2X
3mop	(S)-3-Methyl-2-oxopentanoate	C6H9O3
3oxddacp	3-Oxododecanoyl-[acyl-carrier protein]	C12H20O2X
3oxdeacp	3-Oxodecanoyl-[acyl-carrier protein]	C10H16O2X
3oxhacp	3-Oxohexanoyl-[acyl-carrier protein]	C6H8O2X
3oxhdacp	3-Oxohexadecanoyl-[acp]	C16H28O2X
3oxocacp	3-Oxooctanoyl-[acyl-carrier protein]	C8H12O2X
3oxocdacp	3-Oxooctadecanoyl-[acp]	C18H32O2X
3oxtdacp	3-Oxotetradecanoyl-[acyl-carrier protein]	C14H24O2X
3pg	3-Phospho-D-glycerate	C3H4O7P
4abut	4-Aminobutanoate	C4H9NO2
4abz	4-Aminobenzoate	C7H6NO2
4adcho	4-amino-4-deoxychorismate	C10H10NO5
4ahmmp	4-Amino-5-hydroxymethyl-2-methylpyrimidine	C6H9N3O
4ampm	4-Amino-2-methyl-5-phosphomethylpyrimidine	C6H8N3O4P
4c2me	4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol	C14H23N3O14P2
4h2oxg	D-4-Hydroxy-2-oxoglutarate	C5H4O6
4hba	4-Hydroxy-benzyl alcohol	C7H8O2
4hglu	4-Hydroxy-L-glutamate	C5H8NO5
4mhetz	4-Methyl-5-(2-hydroxyethyl)-thiazole	C6H9NOS
4mop	4-Methyl-2-oxopentanoate	C6H9O3
4mpetz	4-Methyl-5-(2-phosphoethyl)-thiazole	C6H8NO4PS
4pasp	4-Phospho-L-aspartate	C4H6NO7P
4ppan	D-4'-Phosphopantothenate	C9H15NO8P
4ppcys	N-((R)-4-Phosphopantothenoyl)-L-cysteine	C12H20N2O9PS
4r5au	4-(1-D-Ribitylamino)-5-aminouracil	C9H16N4O6
56dura	5,6-dihydrouracil	C4H6N2O2
5aizc	5-amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxylate	C9H11N3O9P
5aop	5-Amino-4-oxopentanoate	C5H9NO3
5aprbu	5-Amino-6-(5'-phosphoribitylamino)uracil	C9H15N4O9P
5apru	5-Amino-6-(5'-phosphoribosylamino)uracil	C9H13N4O9P
5dglcn	5-Dehydro-D-gluconate	C6H9O7
5dpmev	(R)-5-Diphosphomevalonate	C6H10O10P2
5fothf	5-Formyltetrahydrofolate	C20H21N7O7
5mta	5-Methylthioadenosine	C11H15N5O3S
5mthglu	5-Methyltetrahydropteroyltri-L-glutamate	C25H34N8O12
5mtr	5-Methylthio-D-ribose	C6H12O4S
5odhf2a	5-Oxo-4,5-dihydrofuran-2-acetate	C6H5O4
5pmev	(R)-5-Phosphomevalonate	C6H10O7P
5prdmbz	N1-(5-Phospho-alpha-D-ribosyl)-5,6-dimethylbenzimidazole	C14H17N2O7P
6pgc	6-Phospho-D-gluconate	C6H10O10P
6pgg	6-Phospho-beta-D-glucosyl-(1,4)-D-glucose	C12H21O14P
6pgl	6-phospho-D-glucono-1,5-lactone	C6H9O9P
ACP	acyl carrier protein	C11H21N2O7PRS
CPS_LRE	capsular polysaccharide linkage unit, LRE specific	C30H59O32P1
CPS_LRE2	capsular polysaccharide linkage unit, LRE specific	C24H47O25P1
DNA_LRE	DNA, LRE specific	C978H1128N372O600P100
H2Se	Selenide	H2Se
LTA_LRE	lipoteichoic acid (n=25, LRE specific)	C12474H24072O14000P2500
LTAala_LRE	Lipoteichoic acid (n=25) with 100% D-Ala substitutions (LRE specific)	C19974H39072N2500O16500P2500
LTAglc_LRE	Lipoteichoic acid (n=25 with glucose residues, LRE specific)	C27474H49072O26500P2500

Abbreviation	Official name	Formula
Lglyald	L-Glyceraldehyde	C3H6O3
MCOOH	MPT synthase small subunit MoaD	MCO2
MCOSH	MPT synthase sulfurylated small subunit (MoaD-SH)	MCHOS
PGlac2	peptidoglycan with D-lac as C-terminal residue to form pentadepsipeptide	C40H62N7O22
PROT_LRE_v 6.0	L. reuteri-specific protein composition for biomass version 6.0	C4708H7526N1369O1457S33
RNA_LRE	RNA, LRE specific	C958H1078N394O696P100
RTA	teichoic acid containing ribitol-P	C144H283N2O193P27
RTAala	ribitol teichoic acid (n=25) with D-Ala substitutions	C219H433N27O218P27
RTAglc	ribitol teichoic acid (n=25) with glucose substitutions	C294H533N2O318P27
a-gal-D	alpha-D galactose	C6H12O6
aaacp	Acetoacetyl-[acyl-carrier protein]	C4H4O2X
aacoa	Acetoacetyl-CoA	C25H36N7O18P3S
abt-L	L-Arabinitol	C5H12O5
ac	Acetate	C2H3O2
acald	Acetaldehyde	C2H4O
acamoxm	N-Acetyl-L-2-amino-6-oxopimelate	C9H11NO6
accoa	Acetyl-CoA	C23H34N7O17P3S
acgal6p	N-acetylgalactosamine 6-phosphate	C8H14NO9P
acgala	N-Acetyl-D-galactosamine	C8H15NO6
acgam	N-Acetyl-D-glucosamine	C8H15NO6
acgam1p	N-Acetyl-D-glucosamine 1-phosphate	C8H14NO9P
acgam6p	N-Acetyl-D-glucosamine 6-phosphate	C8H14NO9P
achms	O-Acetyl-L-homoserine	C6H11NO4
acmam	N-Acetyl-D-muramoate	C11H18NO8
acmama	N-Acetyl-D-muramoyl-L-alanine	C14H23N2O9
acmana	N-Acetyl-D-mannosamine	C8H15NO6
acmanap	N-Acetyl-D-mannosamine 6-phosphate	C8H14NO9P
acp	Acyl-carrier Protein	X
acser	O-Acetyl-L-serine	C5H9NO4
actn-R	(R)-Acetoin	C4H8O2
actp	Acetyl phosphate	C2H3O5P
adcoba	Adenosyl cobinamide	C58H87CoN16O11
adcobap	Adenosyl cobinamide phosphate	C58H88CoN16O14P
adcobdam	Adenosyl cobyrinate diamide	C55H71CoN11O15
adcobhex	adenosyl-cobyric acid	C55H79CoN15O11
ade	Adenine	C5H5N5
adgcoba	Adenosine-GDP-cobinamide	C68H98CoN21O21P2
adn	Adenosine	C10H13N5O4
adp	ADP	C10H12N5O10P2
adprib	ADPribose	C15H21N5O14P2
agly3p_LRE	1-Acyl-sn-glycerol 3-phosphate (Lb reuteri specific)	C2037H3836O700P100
ahcys	S-Adenosyl-L-homocysteine	C14H20N6O5S
ahdt	2-Amino-4-hydroxy-6-(erythro-1,2,3-trihydroxypropyl)dihydropteridine triphosphate	C9H12N5O13P3
aicar	5-Amino-1-(5-Phospho-D-ribosyl)imidazole-4-carboxamide	C9H13N4O8P
air	5-amino-1-(5-phospho-D-ribosyl)imidazole	C8H12N3O7P
akg	2-Oxoglutarate	C5H4O5
al26da	N6-Acetyl-LL-2,6-diaminoheptanedioate	C9H14N2O5
ala-D	D-Alanine	C3H7NO2
ala-L	L-Alanine	C3H7NO2
alaala	D-Alanyl-D-alanine	C6H12N2O3
alac-S	(S)-2-Acetolactate	C5H7O4

Abbreviation	Official name	Formula
alalac	D-Alanyl-D-lactate	C6H11NO4
alatrna	L-Alanyl-tRNA(Ala)	C18H28NO17P2R3
amet	S-Adenosyl-L-methionine	C15H23N6O5S
amp	AMP	C10H12N5O7P
antim	Antimonite	H4OSb
ap4a	P1,P4-Bis(5'-adenosyl) tetraphosphate	C20H24N10O19P4
apoACP	apoprotein [acyl carrier protein]	RHO
arab-L	L-Arabinose	C5H10O5
arg-L	L-Arginine	C6H15N4O2
argsuc	N(omega)-(L-Arginino)succinate	C10H17N4O6
argtrna	L-Arginyl-tRNA(Arg)	C21H36N4O17P2R3
arsna	Arsenate	H1AsO4
arsni2	arsenite	AsH3O3
asn-L	L-Asparagine	C4H8N2O3
asntrna	L-Asparaginyl-tRNA(Asn)	C19H29N2O18P2R3
asp-D	D-Aspartate	C4H6NO4
asp-L	L-Aspartate	C4H6NO4
aspsa	L-Aspartate 4-semialdehyde	C4H7NO3
asptrna	L-Aspartyl-tRNA(Asp)	C19H27NO19P2R3
atp	ATP	C10H12N5O13P3
btd-RR	(R,R)-2,3-Butanediol	C4H10O2
btn	Biotin	C10H15N2O3S
butacp	Butyryl-[acyl-carrier protein]	C4H6OX
cbasp	N-Carbamoyl-L-aspartate	C5H6N2O5
cbi	Cobinamide	C48H75CoN11O8
cbl1	Cob(I)alamin	C62H92CoN13O14P
cbp	Carbamoyl phosphate	CH2NO5P
cdp	CDP	C9H12N3O11P2
cdpdag_LRE	CDPdiacylglycerol (LRE specific)	C4674H8172N300O1500P200
cdpglyc	CDPglycerol	C12H19N3O13P2
cdprbtl	CDPribitol	C14H23N3O15P2
cellb	cellobiose	C12H22O11
chol	Choline	C5H14NO
citr-L	L-Citrulline	C6H13N3O3
clpn_LRE	Cardiolipin (LRE specific)	C7848H14544O1700P200
cmp	CMP	C9H12N3O8P
co1dam	Cob(I)yrinate a,c diamide	C45H59CoN6O12
co2	CO2	CO2
co2dam	Cob(II)yrinate a,c diamide	C45H60CoN6O12
coa	Coenzyme A	C21H32N7O16P3S
cobalt2	Co2+	Co
cobamcoa	Cobamide coenzyme	C72H104CoN18O17P
cobrnt	Cobyrinate	C45H54CoN4O14
codhprec6	Cobalt-dihydro-precorrin 6	C44H48CoN4O16
coprec2	cobalt-precorrin2	C42H36CoN4O16
coprec3	Cobalt-precorrin 3	C43H39CoN4O16
coprec4	Cobalt-precorrin 4	C44H42CoN4O16
coprec5	Cobalt-precorrin 5	C45H45CoN4O16
coprec6	Cobalt-precorrin 6	C44H46CoN4O16
coprec8	Cobalt-precorrin 8	C45H54CoN4O14
cpocdacp	cyclopropanoyl octadecanoyl-[acyl-carrier protein]	C19H34OX
csn	Cytosine	C4H5N3O
ctp	CTP	C9H12N3O14P3

Abbreviation	Official name	Formula
cu2	Cu2+	Cu
cys-D	D-Cysteine	C3H7NO2S
cys-L	L-Cysteine	C3H7NO2S
cyst-L	L-Cystine	C6H12N2O4S2
cysth-L	L-Cystathionine	C7H14N2O4S
cystrna	L-Cysteinyl-tRNA(Cys)	C18H28NO17P2SR3
cytd	Cytidine	C9H13N3O5
dad-2	Deoxyadenosine	C10H13N5O3
dadp	dADP	C10H12N5O9P2
dag_LRE	1,2-Diacylglycerol, LRE specific	C3774H7072O500
damp	dAMP	C10H12N5O6P
datp	dATP	C10H12N5O12P3
db4p	3,4-dihydroxy-2-butanone 4-phosphate	C4H7O6P
dcamp	N6-(1,2-Dicarboxyethyl)-AMP	C14H14N5O11P
dcdp	dCDP	C9H12N3O10P2
dcmp	dCMP	C9H12N3O7P
dctp	dCTP	C9H12N3O13P3
dcyt	Deoxycytidine	C9H13N3O4
ddca	Dodecanoate	C12H23O2
ddcaACP	Dodecanoyl-ACP (n-C12:0ACP)	C23H43N2O8PRS
ddeacp	Dodecanoyl-[acyl-carrier protein]	C12H22OX
decacp	Decanoyl-[acyl-carrier protein]	C10H18OX
decdp	all-trans-Decaprenyl diphosphate	C50H81O7P2
dgdag_LRE	Diglucosyl-diacylglycerol	C4974H9072O1500
dgdp	dGDP	C10H12N5O10P2
dgmp	dGMP	C10H12N5O7P
dgsn	Deoxyguanosine	C10H13N5O4
dgtp	dGTP	C10H12N5O13P3
dha	Dihydroxyacetone	C3H6O3
dhap	Dihydroxyacetone phosphate	C3H5O6P
dhf	7,8-Dihydrofolate	C19H19N7O6
dhna	1,4-Dihydroxy-2-naphthoate	C11H7O4
dhnpt	2-Amino-4-hydroxy-6-(D-erythro-1,2,3-trihydroxypropyl)-7,8-dihydropteridine	C9H13N5O4
dhor-S	(S)-Dihydroorotate	C5H5N2O4
dhpmp	Dihydroneopterin monophosphate	C9H13N5O7P
dhpt	Dihydropteroate	C14H13N6O3
diact	Diacetyl	C4H6O2
dimp	dIMP	C10H12N4O7P
din	Deoxyinosine	C10H12N4O4
dmbzid	5,6-Dimethylbenzimidazole	C9H10N2
dmlz	6,7-Dimethyl-8-(1-D-ribityl)lumazine	C13H18N4O6
dmpp	Dimethylallyl diphosphate	C5H9O7P2
dnad	Deamino-NAD+	C21H24N6O15P2
dpcoa	Dephospho-CoA	C21H33N7O13P2S
drib	Deoxyribose	C5H10O4
dtdp	dTDP	C10H13N2O11P2
dtdp6dm	dTDP-6-deoxy-L-mannose	C16H24N2O15P2
dtdpddg	dTDP-4-dehydro-6-deoxy-D-glucose	C16H22N2O15P2
dtdpddm	dTDP-4-dehydro-6-deoxy-L-mannose	C16H22N2O15P2
dtdpglc	dTDPglucose	C16H24N2O16P2
dtmp	dTMP	C10H13N2O8P
dtt_ox	Oxidized dithiothreitol	C4H8O2S2

Abbreviation	Official name	Formula
dtt_rd	Reduced dithiothreitol	C4H10O2S2
dttp	dTTP	C10H13N2O14P3
dudp	dUDP	C9H11N2O11P2
dump	dUMP	C9H11N2O8P
duri	Deoxyuridine	C9H12N2O5
dutp	dUTP	C9H11N2O14P3
dxyl5p	1-deoxy-D-xylulose 5-phosphate	C5H9O7P
etha	Ethanolamine	C2H8NO
etoh	Ethanol	C2H6O
f1p	D-Fructose 1-phosphate	C6H11O9P
f6p	D-Fructose 6-phosphate	C6H11O9P
fad	FAD	C27H31N9O15P2
fdp	D-Fructose 1,6-bisphosphate	C6H10O12P2
fe2	Fe2+	Fe
fgam	N2-Formyl-N1-(5-phospho-D-ribosyl)glycinamide	C8H13N2O9P
fmet	N-Formyl-L-methionine	C6H10NO3S
fmettrna	N-Formylmethionyl-tRNA	C21H32NO18P2SR3
fmn	flavin mononucleotide	C17H19N4O9P
fol	Folate	C19H18N7O6
for	Formate	CH1O2
fpram	2-(Formamido)-N1-(5-phospho-D-ribosyl)acetamidine	C8H14N3O8P
fprica	5-Formamido-1-(5-phospho-D-ribosyl)imidazole-4-carboxamide	C10H13N4O9P
frdp	Farnesyl diphosphate	C15H25O7P2
fru	D-Fructose	C6H12O6
fuc-L	L-Fucose	C6H12O5
fum	Fumarate	C4H2O4
g16bp	D-Glucose 1,6-bisphosphate	C6H10O12P2
g1p	D-Glucose 1-phosphate	C6H11O9P
g1p-B	beta-D-Glucose 1-phosphate	C6H11O9P
g3p	Glyceraldehyde 3-phosphate	C3H5O6P
g3pc	sn-Glycero-3-phosphocholine	C8H20NO6P
g3pe	sn-Glycero-3-phosphoethanolamine	C5H14NO6P
g3pg	Glycerophosphoglycerol	C6H14O8P
g3pi	sn-Glycero-3-phospho-1-inositol	C9H18O11P
g3ps	Glycerophosphoserine	C6H13NO8P
g6p	D-Glucose 6-phosphate	C6H11O9P
g6p-B	beta-D-glucose 6-phosphate	C6H11O9P
gal	D-Galactose	C6H12O6
gal1p	alpha-D-Galactose 1-phosphate	C6H11O9P
galt	Galactitol	C6H14O6
galt1p	Galactitol 1-phosphate	C6H13O9P
gam1p	D-Glucosamine 1-phosphate	C6H13NO8P
gam6p	D-Glucosamine 6-phosphate	C6H13NO8P
gar	N1-(5-Phospho-D-ribosyl)glycinamide	C7H14N2O8P
gcald	Glycolaldehyde	C2H4O2
gdp	GDP	C10H12N5O11P2
gdpdp	Guanosine 3',5'-bis(diphosphate)	C10H11N5O17P4
gdptp	Guanosine 3'-diphosphate 5'-triphosphate	C10H11N5O20P5
ggdp	Geranylgeranyl diphosphate	C20H33O7P2
ggluabt	gammaglutamyl-gamma-aminobutyrate	C9H15N2O5
glc-D	D-Glucose	C6H12O6
glcn-D	D-Gluconate	C6H11O7
gln-L	L-Glutamine	C5H10N2O3

Abbreviation	Official name	Formula
glntrna	L-Glutaminyl-tRNA(Gln)	C20H31N2O18P2R3
glu-D	D-Glutamate	C5H8NO4
glu-L	L-Glutamate	C5H8NO4
glu1sa	L-Glutamate 1-semialdehyde	C5H9NO3
glu5p	L-Glutamate 5-phosphate	C5H8NO7P
glu5sa	L-Glutamate 5-semialdehyde	C5H9NO3
glucys	gamma-L-Glutamyl-L-cysteine	C8H13N2O5S
glutrna	L-Glutamyl-tRNA(Glu)	C20H29NO19P2R3
gly	Glycine	C2H5NO2
glyald	D-Glyceraldehyde	C3H6O3
glyb	Glycine betaine	C5H11NO2
glyc	Glycerol	C3H8O3
glyc-R	(R)-Glycerate	C3H5O4
glyc3p	sn-Glycerol 3-phosphate	C3H7O6P
glyclt	Glycolate	C2H3O3
glytrna	Glycyl-tRNA(Gly)	C17H26NO17P2R3
gmp	GMP	C10H12N5O8P
grdp	Geranyl diphosphate	C10H17O7P2
gsn	Guanosine	C10H13N5O5
gthox	Oxidized glutathione	C20H30N6O12S2
gthrd	Reduced glutathione	C10H16N3O6S
gtp	GTP	C10H12N5O14P3
gua	Guanine	C5H5N5O
h	H+	H
h2o	H2O	H2O
h2o2	Hydrogen peroxide	H2O2
h2s	Hydrogen sulfide	H2S
hco3	Bicarbonate	CHO3
hcys-L	L-Homocysteine	C4H9NO2S
hdca	Hexadecanoate (n-C16:0)	C16H31O2
hdcea	hexadecenoate (n-C16:1)	C16H29O2
hdeACP	Hexadecenoyl-ACP (n-C16:1ACP)	C27H49N2O8PRS
hdeacp	Hexadecanoyl-[acyl-carrier protein]	C16H30OX
hepdp	all-trans-Heptaprenyl diphosphate	C35H57O7P2
hexacp	Hexanoyl-[acyl-carrier protein]	C6H10OX
hexdp	all-trans-Hexaprenyl diphosphate	C30H49O7P2
his-L	L-Histidine	C6H9N3O2
hista	Histamine	C5H10N3
histrna	L-Histidyl-tRNA(His)	C21H30N3O17P2R3
hmbil	Hydroxymethylbilane	C40H38N4O17
hmgcoa	Hydroxymethylglutaryl-CoA	C27H39N7O20P3S
hom-L	L-Homoserine	C4H9NO3
hxan	Hypoxanthine	C5H4N4O
icit	Isocitrate	C6H5O7
idp	IDP	C10H11N4O11P2
ile-L	L-Isoleucine	C6H13NO2
iletrna	L-Isoleucyl-tRNA(Ile)	C21H34NO17P2R3
imp	IMP	C10H11N4O8P
indpyr	Indolepyruvate	C11H8NO3
inost	myo-Inositol	C6H12O6
ins	Inosine	C10H12N4O5
ipdp	Isopentenyl diphosphate	C5H9O7P2
itp	ITP	C10H11N4O14P3

Abbreviation	Official name	Formula
k	K+	K
lac-D	D-Lactate	C3H5O3
lac-L	L-Lactate	C3H5O3
lcts	Lactose	C12H22O11
leu-L	L-Leucine	C6H13NO2
leutrna	L-Leucyl-tRNA(Leu)	C21H34O17P2R3N
lys-L	L-Lysine	C6H15N2O2
lyspg_LRE	1-lysyl-phosphatidyl glycerol (LRE specific)	C4674H8972N200O1100P100
lystrna	L-Lysine-tRNA (Lys)	C21H36N2O17P2R3
mal-L	L-Malate	C4H4O5
malacp	Malonyl-[acyl-carrier protein]	C3HO3X
malcoa	Malonyl-CoA	C24H33N7O19P3S
male	Maleate	C4H2O4
malm	Maleamate	C4H4NO3
malt	Maltose	C12H22O11
man	D-Mannose	C6H12O6
man1p	D-Mannose 1-phosphate	C6H11O9P
man6p	D-Mannose 6-phosphate	C6H11O9P
melib	Melibiose	C12H22O11
mercppyr	Mercaptopyruvate	C3H4O3S
met-D	D-Methionine	C5H10NO2S
met-L	L-Methionine	C5H11NO2S
methf	5,10-Methenyltetrahydrofolate	C20H20N7O6
mettrna	L-Methionyl-tRNA (Met)	C20H32NO17P2SR3
mev-R	(R)-Mevalonate	C6H11O4
mg2	Mg	Mg
mi1p-D	1D-myo-Inositol 1-phosphate	C6H11O9P
mlthf	5,10-Methylenetetrahydrofolate	C20H21N7O6
mn2	Mn2+	Mn
mnl	D-Mannitol	C6H14O6
mnl1p	D-Mannitol 1-phosphate	C6H13O9P
mql7	Menaquinol 7	C46H66O2
mqn7	Menaquinone 7	C46H64O2
mqn8	Menaquinone 8	C51H72O2
myrsACP	Myristoyl-ACP (n-C14:0ACP)	C25H47N2O8PRS
na1	Sodium	Na
nac	Nicotinate	C6H4NO2
nad	Nicotinamide adenine dinucleotide	C21H26N7O14P2
nadh	Nicotinamide adenine dinucleotide - reduced	C21H27N7O14P2
nadp	Nicotinamide adenine dinucleotide phosphate	C21H25N7O17P3
nadph	Nicotinamide adenine dinucleotide phosphate - reduced	C21H26N7O17P3
ncam	Nicotinamide	C6H6N2O
nh3	Ammonia	H3N
nh4	Ammonium	H4N
nicrnt	Nicotinate D-ribonucleotide	C11H12NO9P
no2	Nitrite	NO2
no3	Nitrate	NO3
o2	O2	O2
oaa	Oxaloacetate	C4H2O5
ocdacp	Octadecanoyl-[acyl-carrier protein]	C18H34OX
ocdca	octadecanoate (n-C18:0)	C18H35O2
ocdcaACP	Octadecanoyl-ACP (n-C18:0ACP)	C29H55N2O8PRS
ocdcea	octadecenoate (n-C18:1)	C18H33O2

Abbreviation	Official name	Formula
ocdcya	octadecynoate (n-C18:2)	C18H31O2
ocdcyaACP	Octadecynoyl-ACP (n-C18:2ACP)	C29H51N2O8PRS
octacp	Octanoyl-[acyl-carrier protein]	C8H14OX
octeACP	Octadecenoyl-ACP (n-C18:1ACP)	C29H53N2O8PRS
orn-L	L-Ornithine	C5H13N2O2
orot	Orotate	C5H3N2O4
orot5p	Orotidine 5'-phosphate	C10H10N2O11P
pa_LRE	phosphatidic acid (Lb reuteri specific)	C3774H6972O800P100
pacald	Phenylacetaldehyde	C8H8O
palmACP	Palmitoyl-ACP (n-C16:0ACP)	C27H51N2O8PRS
pan4p	Pantetheine 4'-phosphate	C11H21N2O7PS
pant-R	(R)-Pantoate	C6H11O4
pap	Adenosine 3',5'-bisphosphate	C10H11N5O10P2
pea	Phenylethyl alcohol	C8H10O
pep	Phosphoenolpyruvate	C3H2O6P
pg_LRE	Phospatidylglycerol (LRE specific)	C4074H7672O1000P100
pgp_LRE	Phosphatidylglycerophosphate (LRE specific)	C4074H7572O1300P200
phe-L	L-Phenylalanine	C9H11NO2
pheme	Protoheme	C34H31FeN4O4
phetrna	L-Phenylalanyl-tRNA(Phe)	C24H32NO17P2R3
phpyr	Phenylpyruvate	C9H7O3
pi	Phosphate	HO4P
pnto-R	(R)-Pantothenate	C9H16NO5
polypi	Polyphosphate	HO3P
ppbng	Porphobilinogen	C10H13N2O4
ppi	Diphosphate	HO7P2
ppp9	Protoporphyrin	C34H32N4O4
pppi	Inorganic triphosphate	HO10P3
pqqox	PQQ (Pyrroloquinoline-quinone) oxidized form	C14H3N2O8
pqqrd	PQQ (pyrroloquinoline-quinone) reduced form	C14H5N2O8
pram	5-Phospho-beta-D-ribosylamine	C5H11NO7P
pro-L	L-Proline	C5H9NO2
protrna	L-Prolyl-tRNA(Pro)	C20H30NO17P2R3
prpp	5-Phospho-alpha-D-ribose 1-diphosphate	C5H8O14P3
psd5p	Pseudouridine 5'-phosphate	C9H11N2O9P
ptrc	Putrescine	C4H14N2
pyam5p	Pyridoxamine 5'-phosphate	C8H11N2O5P
pydam	Pyridoxamine	C8H12N2O2
pydx	Pyridoxal	C8H9NO3
pydx5p	Pyridoxal 5'-phosphate	C8H7NO6P
pyr	Pyruvate	C3H3O3
pyrimid	Pyrimidine	C4H4N2
pyrin	Pyrimidine nucleoside	C9H14N2O4
quin	Quinate	C7H12O6
r1p	alpha-D-Ribose 1-phosphate	C5H9O8P
r5p	alpha-D-Ribose 5-phosphate	C5H9O8P
raffin	Raffinose	C18H32O16
rbl-L	L-Ribulose	C5H10O5
rbt	Ribitol	C5H12O5
rbt5p	D-Ribitol 5-phosphate	C5H11O8P
rdmbzi	N1-(alpha-D-ribosyl)-5,6-dimethylbenzimidazole	C14H18N2O4
rhcys	S-Ribosyl-L-homocysteine	C9H17NO6S
rib-D	D-Ribose	C5H10O5

Abbreviation	Official name	Formula
ribflv	Riboflavin	C17H20N4O6
ribflvRD	Reduced riboflavin	C17H22N4O6
ru5p-D	D-Ribulose 5-phosphate	C5H9O8P
ru5p-L	L-Ribulose 5-phosphate	C5H9O8P
sbt-D	D-Sorbitol	C6H14O6
sbt6p	D-Sorbitol 6-phosphate	C6H13O9P
sbzcoa	O-Succinylbenzoyl-CoA	C32H39N7O20P3S
scys-L	L-Selenocysteine	C3H7NO2Se
ser-L	L-Serine	C3H7NO3
sertrna	L-Seryl-tRNA(Ser)	C18H28NO18P2R3
shcl	Sirohydrochlorin	C42H41N4O16
sl26da	N-Succinyl-LL-2,6-diaminoheptanedioate	C11H16N2O7
sl2a6o	N-Succinyl-2-L-amino-6-oxoheptanedioate	C11H12NO8
spmd	Spermidine	C7H22N3
srch	Sirochlorin	C42H38N4O16
suc6p	Sucrose 6-phosphate	C12H21O14P
sucbz	o-Succinylbenzoate	C11H8O5
succ	Succinate	C4H4O4
succoa	Succinyl-CoA	C25H35N7O19P3S
sucr	Sucrose	C12H22O11
sucsal	Succinic semialdehyde	C4H5O3
tdeACP	Tetradecenoyl-ACP (n-C14:1ACP)	C25H45N2O8PRS
tdeacp	Tetradecanoyl-[acyl-carrier protein]	C14H26OX
thdp	2,3,4,5-Tetrahydrodipicolinate	C7H7NO4
thf	5,6,7,8-Tetrahydrofolate	C19H21N7O6
thfglu	Tetrahydrofolyl-[Glu](2)	C24H27N8O9
thglu	Tetrahydropteroyltri-L-glutamate	C24H32N8O12
thm	Thiamin	C12H17N4OS
thmmp	Thiamin monophosphate	C12H16N4O4PS
thmpp	Thiamine diphosphate	C12H16N4O7P2S
thr-L	L-Threonine	C4H9NO3
thrp	L-Threonine phosphate	C4H10NO6P
thrtrna	L-Threonyl-tRNA(Thr)	C19H30NO18P2R3
thym	Thymine	C5H6N2O2
thymd	Thymidine	C10H14N2O5
trdox	Oxidized thioredoxin	X
trdrd	Reduced thioredoxin	XH2
tre	Trehalose	C12H22O11
tre6p	alpha,alpha'-Trehalose 6-phosphate	C12H21O14P
trnaala	tRNA(Ala)	C15H23O16P2R3
trnaarg	tRNA(Arg)	C15H23O16P2R3
trnaasn	tRNA(Asn)	C15H23O16P2R3
trnaasp	tRNA(Asp)	C15H23O16P2R3
trnacys	tRNA(Cys)	C15H23O16P2R3
trnaglu	tRNA (Glu)	C15H23O16P2R3
trnagly	tRNA(Gly)	C15H23O16P2R3
trnahis	tRNA(His)	C15H23O16P2R3
trnaile	tRNA(Ile)	C15H23O16P2R3
trnaleu	tRNA(Leu)	C15H23O16P2R3
trnalys	tRNA(Lys)	C15H23O16P2R3
trnamet	tRNA(Met)	C15H23O16P2R3
trnaphe	tRNA(Phe)	C15H23O16P2R3
trnapro	tRNA(Pro)	C15H23O16P2R3

Abbreviation	Official name	Formula
trnaser	tRNA(Ser)	C15H23O16P2R3
trnathr	tRNA(Thr)	C15H23O16P2R3
trnatrp	tRNA(Trp)	C15H23O16P2R3
trnatyr	tRNA(Tyr)	C15H23O16P2R3
trnaval	tRNA(Val)	C15H23O16P2R3
trp-L	L-Tryptophan	C11H12N2O2
trptrna	L-Tryptophanyl-tRNA(Trp)	C26H33N2O17P2R3
ttdca	tetradecanoate	C14H27O2
ttdcea	tetradecenoate (n-C14:1)	C14H25O2
tyr-L	L-Tyrosine	C9H11NO3
tyrtrna	L-Tyrosyl-tRNA(Tyr)	C24H32NO18P2R3
uAgl	UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-L-lysine	C34H51N7O24P2
uAgla	UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine	C40H62N9O26P2
uAglac	UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-lactate	C40H61N8O27P2
uGgl	UDP-N-acetylmuramoyl-L-alanyl-gamma-D-glutamyl-L-lysine	C34H52N7O24P2
uGgla	UDP-N-acetylmuramoyl-L-alanyl-gamma-D-glutamyl-L-lysyl-D-alanyl-D-alanine	C40H62N9O26P2
uaagmdalac	Undecaprenyl-diphospho-N-acetylmuramoyl-(N-acetylglucosamine)-L-alanyl-D-glutamyl-meso-2,6-diaminopimeloyl-D-alanyl-D-lactate	C95H151N7O29P2
uaccg	UDP-N-acetyl-3-O-(1-carboxyvinyl)-D-glucosamine	C20H26N3O19P2
uacgam	UDP-N-acetyl-D-glucosamine	C17H25N3O17P2
uacmam	UDP-N-acetyl-D-mannosamine	C17H25N3O17P2
uagmdalac	Undecaprenyl-diphospho-N-acetylmuramoyl-L-alanyl-D-glutamyl-meso-2,6-diaminopimeloyl-D-alanyl-D-lactate	C87H138N6O24P2
uama	UDP-N-acetylmuramoyl-L-alanine	C23H33N4O20P2
uamag	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate	C28H39N5O23P2
uamr	UDP-N-acetylmuramate	C20H28N3O19P2
ubq8	Ubiquinone-8	C49H74O4
ubq8h2	Ubiquinol-8	C49H76O4
udcp	Undecaprenol	C55H90O
udcpdp	Undecaprenyl diphosphate	C55H89O7P2
udcpp	Undecaprenyl phosphate	C55H89O4P
udp	UDP	C9H11N2O12P2
udpg	UDPglucose	C15H22N2O17P2
udpgal	UDPgalactose	C15H22N2O17P2
udpgalfur	UDP-D-galacto-1,4-furanose	C15H22N2O17P2
ugmd	UDP-N-acetylmuramoyl-L-alanyl-D-gamma-glutamyl-meso-2,6-diaminopimelate	C35H51N7O26P2
ugmdalac	UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-meso-2,6-diaminopimeloyl-D-alanyl-D-lactate	C41H60N8O29P2
ump	UMP	C9H11N2O9P
unRTA	undecaprenyl-teichoic acid with ribitol	C199H372N2O197P28
unaga	Undecaprenyl diphospho N-acetyl-glucosamine	C63H103NO12P2
unagama	Undecaprenyl-diphospho-N-acetylglucosamine-N-acetylmannosamine	C71H116N2O17P2
unagamagp	Undecaprenyl-diphospho-N-acetylglucosamine-N-acetylmannosamine-glycerolphosphate	C74H122N2O22P3
uppg3	Uroporphyrinogen III	C40H36N4O16
ura	Uracil	C4H4N2O2
uri	Uridine	C9H12N2O6
utp	UTP	C9H11N2O15P3
val-L	L-Valine	C5H11NO2
valtrna	L-Valyl-tRNA(Val)	C20H32NO17P2R3
xan	Xanthine	C5H4N4O2
xmp	Xanthosine 5'-phosphate	C10H11N4O9P
xtsn	Xanthosine	C10H12N4O6
xu5p-D	D-Xylulose 5-phosphate	C5H9O8P

Abbreviation	Official name	Formula
xylu-D	D-Xylulose	C5H10O5

Content of supplementary material S2 to Chapter 4:
Model simulations

- Table 4.S2.1. Full details of the several *in silico* simulations carried out in this study. In simulation A the measured uptake fluxes of CDM components were used as constraints. In simulation B the measured uptake fluxes of components of CDM supplemented with glycerol were entered as constraints. Simulation C is similar to simulation B, except that now the fluxes GLYCDx and GDHYD were made irreversible in resemblance to what is suggested by the expression data. For list of reaction abbreviations, please see supplementary material 1. Page 142.

Table 4.S2.1. Full details of the several *in silico* simulations carried out in this study. In simulation A the measured uptake fluxes of CDM components were used as constraints. In simulation B the measured uptake fluxes of components of CDM supplemented with glycerol were entered as constraints. Simulation C is similar to simulation B, except that now the fluxes GLYCDx, GDHYD, G3PD and G3PD2 were made irreversible in resemblance to what is suggested by the expression data. For list of reaction abbreviations, please see supplementary material 1.

Reaction	Simulation A				Simulation B				Simulation C			
	LB	UB	Min	Max	LB	UB	Min	Max	LB	UB	Min	Max
EX_13ppd(e)	0.0E+00	∞	0.0E+00	8.2E-04	0.0E+00	∞	1.1E+01	2.2E+01	0.0E+00	∞	2.2E+01	2.2E+01
EX_4abz(e)	-1.0E-01	1.0E+00	-4.5E-06	-4.5E-06	-1.0E-01	1.0E+00	-5.1E-06	-5.1E-06	-1.0E-01	1.0E+00	-5.1E-06	-5.1E-06
EX_ac(e)	0.0E+00	∞	2.8E-01	2.8E-01	0.0E+00	∞	0.0E+00	1.1E+01	0.0E+00	∞	1.1E+01	1.1E+01
EX_acald(e)	0.0E+00	∞	0.0E+00	1.4E-13	0.0E+00	∞	0.0E+00	-1.6E-12	0.0E+00	∞	0.0E+00	5.2E-13
EX_actn-R(e)	0.0E+00	∞	0.0E+00	8.5E-14	0.0E+00	∞	0.0E+00	-8.1E-13	0.0E+00	∞	0.0E+00	1.8E-13
EX_ade(e)	-1.0E-01	1.0E+00	2.2E-02	2.2E-02	-1.0E-01	1.0E+00	2.5E-02	2.5E-02	-1.0E-01	1.0E+00	2.5E-02	2.5E-02
EX_ala-L(e)	-3.6E-01	1.0E+00	-2.4E-01	-2.4E-01	-4.0E-01	1.0E+00	-1.4E-01	-1.4E-01	-4.0E-01	1.0E+00	-1.4E-01	-1.4E-01
EX_arg-L(e)	-6.3E-01	1.0E+00	-4.4E-02	-4.4E-02	-4.8E-01	1.0E+00	-5.0E-02	-5.0E-02	-4.8E-01	1.0E+00	-5.0E-02	-5.0E-02
EX_asp-L(e)	-4.2E-01	1.0E+00	-4.2E-01	-4.2E-01	-6.1E-01	1.0E+00	-6.1E-01	-6.1E-01	-6.1E-01	1.0E+00	-6.1E-01	-6.1E-01
EX_btd-RR(e)	0.0E+00	∞	0.0E+00	6.8E-13	0.0E+00	∞	0.0E+00	-1.2E-12	0.0E+00	∞	0.0E+00	8.9E-13
EX_btn(e)	-1.0E-01	1.0E+00	0.0E+00	0.0E+00	-1.0E-01	1.0E+00	0.0E+00	0.0E+00	-1.0E-01	1.0E+00	0.0E+00	0.0E+00
EX_co2(e)	-∞	∞	1.8E+01	4.8E+01	-∞	∞	1.4E+01	4.9E+01	-∞	∞	1.4E+01	3.7E+01
EX_cobalt2(e)	-1.0E-01	1.0E+00	0.0E+00	0.0E+00	-1.0E-01	1.0E+00	0.0E+00	0.0E+00	-1.0E-01	1.0E+00	0.0E+00	0.0E+00
EX_cys-L(e)	-4.5E-01	1.0E+00	9.4E-03	9.4E-03	-7.5E-01	1.0E+00	1.1E-02	1.1E-02	-7.5E-01	1.0E+00	1.1E-02	1.1E-02
EX_dha(e)	0.0E+00	∞	0.0E+00	6.6E-14	0.0E+00	∞	0.0E+00	-7.8E-13	0.0E+00	∞	0.0E+00	6.8E-14
EX_diact(e)	0.0E+00	∞	0.0E+00	1.8E-13	0.0E+00	∞	0.0E+00	-4.6E-13	0.0E+00	∞	0.0E+00	2.7E-13
EX_etoh(e)	0.0E+00	∞	1.6E+01	4.7E+01	0.0E+00	∞	6.9E-01	4.7E+01	0.0E+00	∞	6.9E-01	2.5E+01
EX_for(e)	0.0E+00	∞	4.5E-06	4.5E-06	0.0E+00	∞	5.1E-06	5.1E-06	0.0E+00	∞	5.1E-06	5.1E-06
EX_gcald(e)	0.0E+00	∞	4.5E-06	4.5E-06	0.0E+00	∞	5.1E-06	5.1E-06	0.0E+00	∞	5.1E-06	5.1E-06
EX_glc(e)	-2.5E+01	0.0E+00	-2.5E+01	-2.5E+01	-1.9E+01	0.0E+00	-1.9E+01	-1.9E+01	-1.9E+01	0.0E+00	-1.9E+01	-1.9E+01
EX_gln-L(e)	-4.3E-01	1.0E+00	-4.3E-01	-4.3E-01	-6.7E-01	1.0E+00	-4.9E-01	-4.9E-01	-6.7E-01	1.0E+00	-4.9E-01	-4.9E-01
EX_glu-L(e)	-2.4E-01	1.0E+00	-2.4E-01	-2.4E-01	-3.6E-01	1.0E+00	-3.6E-01	-3.6E-01	-3.6E-01	1.0E+00	-3.6E-01	-3.6E-01
EX_glyc(e)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	-2.2E+01	0.0E+00	-2.2E+01	-2.2E+01	-2.2E+01	0.0E+00	-2.2E+01	-2.2E+01
EX_gua(e)	-1.0E-01	1.0E+00	-2.5E-02	-2.5E-02	-1.0E-01	1.0E+00	-2.9E-02	-2.9E-02	-1.0E-01	1.0E+00	-2.9E-02	-2.9E-02
EX_h(e)	-∞	∞	-7.4E-01	3.0E+01	-∞	∞	-1.4E+00	3.3E+01	-∞	∞	9.9E+00	3.3E+01
EX_h2o(e)	-∞	∞	4.6E+00	4.6E+00	-∞	∞	1.6E+01	1.6E+01	-∞	∞	1.6E+01	1.6E+01
EX_his-L(e)	-1.0E-01	1.0E+00	-1.9E-02	-1.9E-02	-1.0E-01	1.0E+00	-2.1E-02	-2.1E-02	-1.0E-01	1.0E+00	-2.1E-02	-2.1E-02
EX_hxan(e)	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
EX_ile-L(e)	-2.5E-01	1.0E+00	-4.7E-02	-4.7E-02	-3.3E-01	1.0E+00	-5.4E-02	-5.4E-02	-3.3E-01	1.0E+00	-5.4E-02	-5.4E-02
EX_ins(e)	-1.0E-01	1.0E+00	-1.9E-12	4.4E-16	-1.0E-01	1.0E+00	4.9E-12	-3.9E-13	-1.0E-01	1.0E+00	-1.9E-12	4.9E-14
EX_lac-D(e)	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00	0.0E+00
EX_lac-L(e)	0.0E+00	∞	0.0E+00	3.0E+01	0.0E+00	∞	0.0E+00	3.5E+01	0.0E+00	∞	0.0E+00	2.4E+01
EX_leu-L(e)	-3.3E-01	1.0E+00	-8.6E-02	-8.6E-02	-4.0E-01	1.0E+00	-9.8E-02	-9.8E-02	-4.0E-01	1.0E+00	-9.8E-02	-9.8E-02
EX_lys-L(e)	-2.6E-01	1.0E+00	-8.0E-02	-8.0E-02	-2.2E-01	1.0E+00	-9.1E-02	-9.1E-02	-2.2E-01	1.0E+00	-9.1E-02	-9.1E-02
EX_mal-L(e)	0.0E+00	∞	0.0E+00	8.0E-13	0.0E+00	∞	0.0E+00	-3.7E-12	0.0E+00	∞	0.0E+00	5.8E-13
EX_met-L(e)	-7.0E-02	1.0E+00	-4.6E-02	-4.6E-02	-1.7E-01	1.0E+00	-5.2E-02	-5.2E-02	-1.7E-01	1.0E+00	-5.2E-02	-5.2E-02
EX_nac(e)	-1.0E-01	1.0E+00	-9.1E-04	-9.1E-04	-1.0E-01	1.0E+00	-1.0E-03	-1.0E-03	-1.0E-01	1.0E+00	-1.0E-03	-1.0E-03
EX_nh3(e)	-∞	∞	2.1E-01	2.1E-01	-∞	∞	2.2E-01	2.2E-01	-∞	∞	2.2E-01	2.2E-01
EX_orn-L(e)	0.0E+00	∞	0.0E+00	-7.2E-15	0.0E+00	∞	0.0E+00	-4.9E-13	0.0E+00	∞	0.0E+00	2.9E-13
EX_phe-L(e)	-2.2E-01	1.0E+00	-3.7E-02	-3.7E-02	-2.0E-01	1.0E+00	-4.3E-02	-4.3E-02	-2.0E-01	1.0E+00	-4.3E-02	-4.3E-02
EX_pi(e)	-∞	∞	-5.7E-01	-5.7E-01	-∞	∞	-6.5E-01	-6.5E-01	-∞	∞	-6.5E-01	-6.5E-01
EX_pnto-R(e)	-1.0E-01	1.0E+00	-9.1E-05	-9.1E-05	-1.0E-01	1.0E+00	-1.0E-04	-1.0E-04	-1.0E-01	1.0E+00	-1.0E-04	-1.0E-04
EX_pro-L(e)	-2.4E-01	1.0E+00	4.4E-01	4.4E-01	-4.1E-01	1.0E+00	5.9E-01	5.9E-01	-4.1E-01	1.0E+00	5.9E-01	5.9E-01
EX_pydam(e)	-1.0E-01	1.0E+00	-4.5E-07	-4.5E-07	-1.0E-01	1.0E+00	-5.1E-07	-5.1E-07	-1.0E-01	1.0E+00	-5.1E-07	-5.1E-07
EX_pyr(e)	0.0E+00	∞	0.0E+00	1.7E-13	0.0E+00	∞	0.0E+00	-1.6E-12	0.0E+00	∞	0.0E+00	3.6E-13
EX_ribflv(e)	-1.0E-01	1.0E+00	0.0E+00	0.0E+00	-1.0E-01	1.0E+00	0.0E+00	0.0E+00	-1.0E-01	1.0E+00	0.0E+00	0.0E+00

Reaction	Simulation A				Simulation B				Simulation C			
	LB	UB	Min	Max	LB	UB	Min	Max	LB	UB	Min	Max
EX_ser-L(e)	-5.7E-01	1.0E+00	-5.7E-01	-5.7E-01	-6.3E-01	1.0E+00	-6.3E-01	-6.3E-01	-6.3E-01	1.0E+00	-6.3E-01	-6.3E-01
EX_thm(e)	-1.0E-01	1.0E+00	-4.5E-06	-4.5E-06	-1.0E-01	1.0E+00	-5.1E-06	-5.1E-06	-1.0E-01	1.0E+00	-5.1E-06	-5.1E-06
EX_thr-L(e)	-3.0E-01	1.0E+00	-7.0E-02	-7.0E-02	-2.7E-01	1.0E+00	-8.0E-02	-8.0E-02	-2.7E-01	1.0E+00	-8.0E-02	-8.0E-02
EX_trp-L(e)	-3.0E-02	1.0E+00	-6.6E-03	-6.6E-03	-7.0E-02	1.0E+00	-7.5E-03	-7.5E-03	-7.0E-02	1.0E+00	-7.5E-03	-7.5E-03
EX_tyr-L(e)	-3.2E-01	1.0E+00	-3.1E-02	-3.1E-02	-1.4E-01	1.0E+00	-3.5E-02	-3.5E-02	-1.4E-01	1.0E+00	-3.5E-02	-3.5E-02
EX_ura(e)	-1.0E-01	1.0E+00	-6.7E-02	-6.7E-02	-1.0E-01	1.0E+00	-7.6E-02	-7.6E-02	-1.0E-01	1.0E+00	-7.6E-02	-7.6E-02
EX_val-L(e)	-2.8E-01	1.0E+00	-6.6E-02	-6.6E-02	-2.4E-01	1.0E+00	-7.5E-02	-7.5E-02	-2.4E-01	1.0E+00	-7.5E-02	-7.5E-02
EX_xan(e)	-1.0E-01	1.0E+00	1.7E-14	0.0E+00	-1.0E-01	1.0E+00	4.1E-13	0.0E+00	-1.0E-01	1.0E+00	-9.5E-14	0.0E+00
2HXICDH	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
2MPDH	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
AALDH	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
ABTA	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
ACACT1	0.0E+00	∞	1.0E-03	1.0E-03	0.0E+00	∞	1.1E-03	1.1E-03	0.0E+00	∞	1.1E-03	1.1E-03
ACALD	-∞	∞	-4.7E+01	-1.6E+01	-∞	∞	-4.7E+01	-6.9E-01	-∞	∞	-2.5E+01	-6.9E-01
ACALDt	-∞	∞	-1.4E-13	0.0E+00	-∞	∞	1.6E-12	0.0E+00	-∞	∞	-5.2E-13	0.0E+00
ACBIPGT	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
ACCOAC	0.0E+00	∞	6.5E-01	6.5E-01	0.0E+00	∞	7.4E-01	7.4E-01	0.0E+00	∞	7.4E-01	7.4E-01
ACGALpts	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
ACGAMT	0.0E+00	∞	6.2E-03	6.2E-03	0.0E+00	∞	7.1E-03	7.1E-03	0.0E+00	∞	7.1E-03	7.1E-03
ACGApts	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
ACHBS	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
ACKr	-∞	∞	-2.2E-01	-2.2E-01	-∞	∞	-1.1E+01	7.5E-02	-∞	∞	-1.1E+01	-1.1E+01
ACLDC	0.0E+00	∞	0.0E+00	6.8E-13	0.0E+00	∞	0.0E+00	-1.2E-12	0.0E+00	∞	0.0E+00	4.1E-13
ACLS	0.0E+00	∞	0.0E+00	6.8E-13	0.0E+00	∞	0.0E+00	-1.2E-12	0.0E+00	∞	0.0E+00	4.1E-13
ACPS2	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
ACt6	-∞	∞	-2.8E-01	-2.8E-01	-∞	∞	-1.1E+01	0.0E+00	-∞	∞	-1.1E+01	-1.1E+01
ACTD	-∞	∞	0.0E+00	1.8E-13	-∞	∞	0.0E+00	-4.6E-13	-∞	∞	0.0E+00	2.7E-13
ACTNdiff	-∞	∞	-8.5E-14	0.0E+00	-∞	∞	8.1E-13	0.0E+00	-∞	∞	-1.8E-13	0.0E+00
ACTPASE	0.0E+00	∞	0.0E+00	-2.0E-14	0.0E+00	∞	0.0E+00	-8.1E-13	0.0E+00	∞	0.0E+00	1.6E-13
ADA	0.0E+00	∞	0.0E+00	1.1E-14	0.0E+00	∞	0.0E+00	-2.1E-13	0.0E+00	∞	0.0E+00	4.6E-14
ADCL	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
ADCOBAK	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
ADCOBAPS	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
ADEt2	-∞	∞	-2.2E-02	-2.2E-02	-∞	∞	-2.5E-02	-2.5E-02	-∞	∞	-2.5E-02	-2.5E-02
ADK1	-∞	∞	-∞	1.4E+00	-∞	∞	-∞	1.6E+00	-∞	∞	-∞	1.6E+00
ADK2	-∞	∞	0.0E+00	∞	-∞	∞	0.0E+00	∞	-∞	∞	0.0E+00	∞
ADPDS	0.0E+00	∞	6.6E-02	6.6E-02	0.0E+00	∞	7.5E-02	7.5E-02	0.0E+00	∞	7.5E-02	7.5E-02
ADPRDP	0.0E+00	∞	0.0E+00	-1.0E-14	0.0E+00	∞	0.0E+00	-1.3E-13	0.0E+00	∞	0.0E+00	2.3E-14
ADPT	0.0E+00	∞	0.0E+00	-1.0E-13	0.0E+00	∞	0.0E+00	-4.8E-13	0.0E+00	∞	0.0E+00	1.4E-14
ADPTA	-∞	∞	-6.6E-02	-6.6E-02	-∞	∞	-7.5E-02	-7.5E-02	-∞	∞	-7.5E-02	-7.5E-02
ADSL1	0.0E+00	∞	6.3E-02	6.3E-02	0.0E+00	∞	7.2E-02	7.2E-02	0.0E+00	∞	7.2E-02	7.2E-02
ADSL2	0.0E+00	∞	8.5E-02	8.5E-02	0.0E+00	∞	9.6E-02	9.6E-02	0.0E+00	∞	9.6E-02	9.6E-02
ADSS	0.0E+00	∞	6.3E-02	6.3E-02	0.0E+00	∞	7.2E-02	7.2E-02	0.0E+00	∞	7.2E-02	7.2E-02
AGAT_LRE	0.0E+00	∞	4.3E-02	4.3E-02	0.0E+00	∞	4.9E-02	4.9E-02	0.0E+00	∞	4.9E-02	4.9E-02
AGDC	0.0E+00	∞	0.0E+00	1.2E-15	0.0E+00	∞	0.0E+00	-2.3E-13	0.0E+00	∞	0.0E+00	1.4E-13
AHCYSNS	0.0E+00	∞	2.2E-02	2.2E-02	0.0E+00	∞	2.5E-02	2.5E-02	0.0E+00	∞	2.5E-02	2.5E-02
AHMMPS	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
AICART	-∞	∞	8.5E-02	8.5E-02	-∞	∞	9.6E-02	9.6E-02	-∞	∞	9.6E-02	9.6E-02
AIRC	0.0E+00	∞	8.5E-02	8.5E-02	0.0E+00	∞	9.6E-02	9.6E-02	0.0E+00	∞	9.6E-02	9.6E-02
ALA_Lt6	-∞	∞	2.4E-01	2.4E-01	-∞	∞	1.4E-01	1.4E-01	-∞	∞	1.4E-01	1.4E-01
ALALAC	-∞	∞	6.6E-02	6.6E-02	-∞	∞	7.5E-02	7.5E-02	-∞	∞	7.5E-02	7.5E-02
ALAR	-∞	∞	6.6E-02	6.6E-02	-∞	∞	7.5E-02	7.5E-02	-∞	∞	7.5E-02	7.5E-02
ALATA_L	-∞	∞	-2.9E-02	-2.9E-02	-∞	∞	-1.7E-01	-1.7E-01	-∞	∞	-1.7E-01	-1.7E-01
ALATA_Lr	-∞	∞	-4.5E-07	-4.5E-07	-∞	∞	-5.1E-07	-5.1E-07	-∞	∞	-5.1E-07	-5.1E-07
ALATRS	0.0E+00	∞	1.4E-01	1.4E-01	0.0E+00	∞	1.6E-01	1.6E-01	0.0E+00	∞	1.6E-01	1.6E-01
ALCD19	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
ALCD19_D	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
ALCD19_L	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00

Reaction	Simulation A				Simulation B				Simulation C			
	LB	UB	Min	Max	LB	UB	Min	Max	LB	UB	Min	Max
ALCD2x	-∞	∞	-4.7E+01	-1.6E+01	-∞	∞	-4.7E+01	-6.9E-01	-∞	∞	-2.5E+01	-6.9E-01
ALDD2x	0.0E+00	∞	0.0E+00	2.8E-14	0.0E+00	∞	0.0E+00	-8.0E-13	0.0E+00	∞	0.0E+00	1.4E-13
ALDD8x	0.0E+00	∞	0.0E+00	-2.5E-14	0.0E+00	∞	0.0E+00	-1.1E-12	0.0E+00	∞	0.0E+00	-4.1E-14
ALKP	0.0E+00	∞	0.0E+00	-9.3E-14	0.0E+00	∞	0.0E+00	-7.0E-13	0.0E+00	∞	0.0E+00	1.5E-13
ALOX	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
AMAA	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
AMANAPE	0.0E+00	∞	0.0E+00	1.2E-15	0.0E+00	∞	0.0E+00	-2.3E-13	0.0E+00	∞	0.0E+00	1.4E-13
AMANK	0.0E+00	∞	0.0E+00	1.2E-15	0.0E+00	∞	0.0E+00	-2.3E-13	0.0E+00	∞	0.0E+00	1.4E-13
AMMQT7	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
AMMQT8_2	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
ANTIMt1	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
AP4AH	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
APAT	-∞	∞	6.6E-02	6.6E-02	-∞	∞	7.5E-02	7.5E-02	-∞	∞	7.5E-02	7.5E-02
APRAUR	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
ARABRr	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
ARAI	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
araphe2	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
aratry2	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
aratyr2	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
aratyr4	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
ARBt2	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
ARGabc	0.0E+00	∞	0.0E+00	3.6E-14	0.0E+00	∞	0.0E+00	-1.4E-12	0.0E+00	∞	0.0E+00	4.5E-13
ARGDI	0.0E+00	∞	0.0E+00	8.2E-14	0.0E+00	∞	0.0E+00	-4.8E-13	0.0E+00	∞	0.0E+00	1.8E-13
ARGORNt3	-∞	∞	0.0E+00	4.4E-02	-∞	∞	0.0E+00	5.0E-02	-∞	∞	0.0E+00	5.0E-02
ARGSL	-∞	∞	0.0E+00	-1.7E-14	-∞	∞	0.0E+00	-2.8E-13	-∞	∞	0.0E+00	1.0E-13
ARGSS	0.0E+00	∞	0.0E+00	-1.7E-14	0.0E+00	∞	0.0E+00	-2.8E-13	0.0E+00	∞	0.0E+00	1.0E-13
ARGt2	0.0E+00	∞	0.0E+00	4.4E-02	0.0E+00	∞	0.0E+00	5.0E-02	0.0E+00	∞	0.0E+00	5.0E-02
ARGTRS	0.0E+00	∞	4.4E-02	4.4E-02	0.0E+00	∞	5.0E-02	5.0E-02	0.0E+00	∞	5.0E-02	5.0E-02
ARSNAt1	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
ARSt1	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
ASAD	-∞	∞	-4.5E-02	-4.5E-02	-∞	∞	-5.1E-02	-5.1E-02	-∞	∞	-5.1E-02	-5.1E-02
ASNN	0.0E+00	∞	0.0E+00	-4.5E-14	0.0E+00	∞	0.0E+00	-3.9E-13	0.0E+00	∞	0.0E+00	1.1E-13
ASNS1	0.0E+00	∞	0.0E+00	-8.1E-14	0.0E+00	∞	0.0E+00	-1.3E-12	0.0E+00	∞	0.0E+00	3.1E-13
ASNS2	0.0E+00	∞	6.6E-02	6.6E-02	0.0E+00	∞	7.5E-02	7.5E-02	0.0E+00	∞	7.5E-02	7.5E-02
ASNt6	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
ASNTRS	0.0E+00	∞	6.6E-02	6.6E-02	0.0E+00	∞	7.5E-02	7.5E-02	0.0E+00	∞	7.5E-02	7.5E-02
ASPCT	0.0E+00	∞	0.0E+00	8.2E-14	0.0E+00	∞	0.0E+00	-4.8E-13	0.0E+00	∞	0.0E+00	1.8E-13
ASPK	-∞	∞	4.5E-02	4.5E-02	-∞	∞	5.1E-02	5.1E-02	-∞	∞	5.1E-02	5.1E-02
ASPR	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
ASPt6	-∞	∞	4.2E-01	4.2E-01	-∞	∞	6.1E-01	6.1E-01	-∞	∞	6.1E-01	6.1E-01
ASPTA1	-∞	∞	9.5E-02	9.5E-02	-∞	∞	2.4E-01	2.4E-01	-∞	∞	2.4E-01	2.4E-01
ASPTA2	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
ASPTA4	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
ASPTA5	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
ASPTA6	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
ASPTRS	0.0E+00	∞	6.6E-02	6.6E-02	0.0E+00	∞	7.5E-02	7.5E-02	0.0E+00	∞	7.5E-02	7.5E-02
ATPM	1.0E+00	1.0E+00	1.0E+00	1.0E+00	1.0E+00	1.0E+00	1.0E+00	1.0E+00	1.0E+00	1.0E+00	1.0E+00	1.0E+00
ATPS3r	-∞	∞	-3.8E-01	-3.8E-01	-∞	∞	-3.6E-01	-3.6E-01	-∞	∞	-3.6E-01	-3.6E-01
BTDD-RR	-∞	∞	-6.8E-13	0.0E+00	-∞	∞	1.2E-12	0.0E+00	-∞	∞	-8.9E-13	0.0E+00
BTDt1-RR	-∞	∞	0.0E+00	6.8E-13	-∞	∞	0.0E+00	-1.2E-12	-∞	∞	0.0E+00	8.9E-13
BTMAT1	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
BTNt2i	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
CBIA	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
CBIAT	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
CBMK2	0.0E+00	∞	0.0E+00	4.9E-14	0.0E+00	∞	0.0E+00	-2.9E-13	0.0E+00	∞	0.0E+00	1.1E-13
CBPS	0.0E+00	∞	0.0E+00	-4.3E-14	0.0E+00	∞	0.0E+00	-2.1E-13	0.0E+00	∞	0.0E+00	6.5E-14
CDPMEK	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
CELBpts	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00

Reaction	Simulation A				Simulation B				Simulation C			
	LB	UB	Min	Max	LB	UB	Min	Max	LB	UB	Min	Max
CFAS180	0.0E+00	∞	2.2E-02	2.2E-02	0.0E+00	∞	2.5E-02	2.5E-02	0.0E+00	∞	2.5E-02	2.5E-02
CGPT	0.0E+00	∞	6.2E-03	6.2E-03	0.0E+00	∞	7.1E-03	7.1E-03	0.0E+00	∞	7.1E-03	7.1E-03
CHLabc	0.0E+00	∞	0.0E+00	-1.0E-13	0.0E+00	∞	0.0E+00	-1.1E-12	0.0E+00	∞	0.0E+00	6.3E-13
CHLt6	-∞	∞	1.0E-13	0.0E+00	-∞	∞	1.1E-12	0.0E+00	-∞	∞	-6.3E-13	0.0E+00
CLPNS_LRE	-∞	∞	8.2E-04	8.2E-04	-∞	∞	9.3E-04	9.3E-04	-∞	∞	9.3E-04	9.3E-04
CMLDC	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
CO2t	-∞	∞	-4.8E+01	-1.8E+01	-∞	∞	-4.9E+01	-1.4E+01	-∞	∞	-3.7E+01	-1.4E+01
Coabc	0.0E+00	∞	0.0E+00	-8.7E-15	0.0E+00	∞	0.0E+00	-7.5E-13	0.0E+00	∞	0.0E+00	1.6E-13
COBALTt5	-∞	∞	0.0E+00	-8.7E-15	-∞	∞	0.0E+00	-7.5E-13	-∞	∞	0.0E+00	1.6E-13
COBAT	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
COPREC2MT	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
COPREC3MT	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
COPREC4MT	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
COPREC6MT	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
COPREC6R	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
COPREC6R2	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
COPRECI	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
COPRECT	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
CPSS_LRE	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
CPSS_LRE2	-∞	∞	5.9E-02	5.9E-02	-∞	∞	6.7E-02	6.7E-02	-∞	∞	6.7E-02	6.7E-02
CRCT	0.0E+00	∞	1.6E-01	1.6E-01	0.0E+00	∞	1.8E-01	1.8E-01	0.0E+00	∞	1.8E-01	1.8E-01
CRPT_25	0.0E+00	∞	6.2E-03	6.2E-03	0.0E+00	∞	7.1E-03	7.1E-03	0.0E+00	∞	7.1E-03	7.1E-03
CSND	0.0E+00	∞	0.0E+00	1.9E-14	0.0E+00	∞	0.0E+00	-4.1E-13	0.0E+00	∞	0.0E+00	6.8E-14
CTPS1_#1	0.0E+00	∞	3.1E-02	3.9E-02	0.0E+00	∞	3.6E-02	4.5E-02	0.0E+00	∞	3.6E-02	4.5E-02
CTPS2	0.0E+00	∞	0.0E+00	-7.3E-14	0.0E+00	∞	0.0E+00	-1.0E-12	0.0E+00	∞	0.0E+00	4.2E-13
Cuabc	0.0E+00	∞	0.0E+00	-4.3E-15	0.0E+00	∞	0.0E+00	-3.7E-13	0.0E+00	∞	0.0E+00	7.9E-14
Cut1	0.0E+00	∞	0.0E+00	-4.3E-15	0.0E+00	∞	0.0E+00	-3.7E-13	0.0E+00	∞	0.0E+00	7.9E-14
CYSabc	0.0E+00	∞	0.0E+00	-1.0E-13	0.0E+00	∞	0.0E+00	-1.1E-12	0.0E+00	∞	0.0E+00	6.3E-13
CYSDabc	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
CYSS	0.0E+00	∞	2.2E-02	2.2E-02	0.0E+00	∞	2.5E-02	2.5E-02	0.0E+00	∞	2.5E-02	2.5E-02
CYSt6	-∞	∞	-9.4E-03	-9.4E-03	-∞	∞	-1.1E-02	-1.1E-02	-∞	∞	-1.1E-02	-1.1E-02
CYSTabc	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
CYSTGL	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
CYSTL	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
CYSTRS	0.0E+00	∞	1.2E-02	1.2E-02	0.0E+00	∞	1.4E-02	1.4E-02	0.0E+00	∞	1.4E-02	1.4E-02
CYTB_B2	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
CYTD	0.0E+00	∞	0.0E+00	1.9E-14	0.0E+00	∞	0.0E+00	-4.1E-13	0.0E+00	∞	0.0E+00	6.8E-14
CYTDK1	0.0E+00	∞	0.0E+00	-1.5E-14	0.0E+00	∞	0.0E+00	-8.1E-13	0.0E+00	∞	0.0E+00	1.7E-13
CYTDK2	0.0E+00	∞	0.0E+00	-1.8E-14	0.0E+00	∞	0.0E+00	-7.0E-13	0.0E+00	∞	0.0E+00	1.7E-13
CYTDK3	0.0E+00	∞	0.0E+00	-1.8E-14	0.0E+00	∞	0.0E+00	-7.0E-13	0.0E+00	∞	0.0E+00	1.7E-13
CYTK1	-∞	∞	3.5E-01	3.5E-01	-∞	∞	3.9E-01	3.9E-01	-∞	∞	3.9E-01	3.9E-01
CYTK2	-∞	∞	-7.8E-03	0.0E+00	-∞	∞	-8.9E-03	0.0E+00	-∞	∞	-8.9E-03	0.0E+00
DADA	0.0E+00	∞	0.0E+00	1.1E-14	0.0E+00	∞	0.0E+00	-2.1E-13	0.0E+00	∞	0.0E+00	4.6E-14
DADNK	0.0E+00	∞	0.0E+00	-1.5E-14	0.0E+00	∞	0.0E+00	-8.1E-13	0.0E+00	∞	0.0E+00	1.7E-13
DAGGT_LRE	0.0E+00	∞	5.9E-03	5.9E-03	0.0E+00	∞	6.7E-03	6.7E-03	0.0E+00	∞	6.7E-03	6.7E-03
DAGK_LRE	0.0E+00	∞	1.4E-01	1.4E-01	0.0E+00	∞	1.6E-01	1.6E-01	0.0E+00	∞	1.6E-01	1.6E-01
DALTAL	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
DAPDC	0.0E+00	∞	0.0E+00	-2.0E-13	0.0E+00	∞	0.0E+00	-8.3E-13	0.0E+00	∞	0.0E+00	2.8E-13
DAPE	-∞	∞	6.6E-02	6.6E-02	-∞	∞	7.5E-02	7.5E-02	-∞	∞	7.5E-02	7.5E-02
DARTAL	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
DASYN_LRE	-∞	∞	1.8E-01	1.8E-01	-∞	∞	2.1E-01	2.1E-01	-∞	∞	2.1E-01	2.1E-01
DB4PS	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
DCYTD	0.0E+00	∞	0.0E+00	7.8E-03	0.0E+00	∞	0.0E+00	8.9E-03	0.0E+00	∞	0.0E+00	8.9E-03
DDMAT5	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
DEMAT4	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
DGNSK	0.0E+00	∞	0.0E+00	-1.5E-14	0.0E+00	∞	0.0E+00	-8.1E-13	0.0E+00	∞	0.0E+00	1.7E-13
DHAPT	0.0E+00	∞	0.0E+00	8.2E-04	0.0E+00	∞	0.0E+00	1.1E+01	0.0E+00	∞	0.0E+00	2.2E-13
DHAt	-∞	∞	-6.6E-14	0.0E+00	-∞	∞	7.8E-13	0.0E+00	-∞	∞	-6.8E-14	0.0E+00

Reaction	Simulation A				Simulation B				Simulation C			
	LB	UB	Min	Max	LB	UB	Min	Max	LB	UB	Min	Max
DHDPRy	0.0E+00	∞	6.6E-02	6.6E-02	0.0E+00	∞	7.5E-02	7.5E-02	0.0E+00	∞	7.5E-02	7.5E-02
DHDPS	0.0E+00	∞	6.6E-02	6.6E-02	0.0E+00	∞	7.5E-02	7.5E-02	0.0E+00	∞	7.5E-02	7.5E-02
DHFOR2	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
DHFR	-∞	∞	7.8E-03	7.8E-03	-∞	∞	8.9E-03	8.9E-03	-∞	∞	8.9E-03	8.9E-03
DHFS	0.0E+00	∞	4.5E-06	4.5E-06	0.0E+00	∞	5.1E-06	5.1E-06	0.0E+00	∞	5.1E-06	5.1E-06
DHNPA	0.0E+00	∞	4.5E-06	4.5E-06	0.0E+00	∞	5.1E-06	5.1E-06	0.0E+00	∞	5.1E-06	5.1E-06
DHORD6	-∞	∞	0.0E+00	8.2E-14	-∞	∞	0.0E+00	-4.8E-13	-∞	∞	0.0E+00	1.8E-13
DHORTS	-∞	∞	-8.2E-14	0.0E+00	-∞	∞	4.8E-13	0.0E+00	-∞	∞	-1.8E-13	0.0E+00
DHPPDA_#1	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
DHPS3	0.0E+00	∞	4.5E-06	4.5E-06	0.0E+00	∞	5.1E-06	5.1E-06	0.0E+00	∞	5.1E-06	5.1E-06
DIACTt	-∞	∞	0.0E+00	1.8E-13	-∞	∞	0.0E+00	-4.6E-13	-∞	∞	0.0E+00	2.7E-13
DINSK	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
DKGLCNR1	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
DKGLCNR2x	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
DKGLCNR2y	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
D-LACt2	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
DMATT	0.0E+00	∞	9.1E-05	9.1E-05	0.0E+00	∞	1.0E-04	1.0E-04	0.0E+00	∞	1.0E-04	1.0E-04
DNAS_LRE	0.0E+00	∞	2.8E-02	2.8E-02	0.0E+00	∞	3.2E-02	3.2E-02	0.0E+00	∞	3.2E-02	3.2E-02
DNMPPA	0.0E+00	∞	4.5E-06	4.5E-06	0.0E+00	∞	5.1E-06	5.1E-06	0.0E+00	∞	5.1E-06	5.1E-06
DNTPPA	0.0E+00	∞	4.5E-06	4.5E-06	0.0E+00	∞	5.1E-06	5.1E-06	0.0E+00	∞	5.1E-06	5.1E-06
DPCOAK	0.0E+00	∞	9.1E-05	9.1E-05	0.0E+00	∞	1.0E-04	1.0E-04	0.0E+00	∞	1.0E-04	1.0E-04
DPMVD	0.0E+00	∞	1.0E-03	1.0E-03	0.0E+00	∞	1.1E-03	1.1E-03	0.0E+00	∞	1.1E-03	1.1E-03
DPR	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
DRBK	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
DRIBt2	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
DRPA	0.0E+00	∞	0.0E+00	2.1E-14	0.0E+00	∞	0.0E+00	-4.2E-13	0.0E+00	∞	0.0E+00	2.1E-13
DTMPK	-∞	∞	7.8E-03	7.8E-03	-∞	∞	8.9E-03	8.9E-03	-∞	∞	8.9E-03	8.9E-03
DURADy	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
DURIK1	0.0E+00	∞	0.0E+00	7.8E-03	0.0E+00	∞	0.0E+00	8.9E-03	0.0E+00	∞	0.0E+00	8.9E-03
DURIPP	-∞	∞	0.0E+00	2.5E-14	-∞	∞	0.0E+00	-4.2E-13	-∞	∞	0.0E+00	2.1E-13
DUTPDP	0.0E+00	∞	0.0E+00	7.8E-03	0.0E+00	∞	0.0E+00	8.9E-03	0.0E+00	∞	0.0E+00	8.9E-03
DXPS	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
ENO	-∞	∞	3.0E+01	3.0E+01	-∞	∞	2.3E+01	3.4E+01	-∞	∞	2.3E+01	2.3E+01
EPPP	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
ETOHt1	-∞	∞	-4.7E+01	-1.6E+01	-∞	∞	-4.7E+01	-6.9E-01	-∞	∞	-2.5E+01	-6.9E-01
FA120ACPH	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
FA140ACPH	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
FA141ACPH	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
FA160ACPH	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
FA161ACPH	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
FA180ACPH	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
FA181ACPH	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
FA182ACPH	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
FABM1	-∞	∞	1.0E-02	1.0E-02	-∞	∞	1.2E-02	1.2E-02	-∞	∞	1.2E-02	1.2E-02
FABM2	-∞	∞	4.9E-02	4.9E-02	-∞	∞	5.6E-02	5.6E-02	-∞	∞	5.6E-02	5.6E-02
FBA	-∞	∞	6.5E+00	6.5E+00	-∞	∞	5.0E+00	5.0E+00	-∞	∞	5.0E+00	5.0E+00
FCLT	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
FLVR	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
FMETDF	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
FMETTRS	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
FMNAT	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
FORt	-∞	∞	-4.5E-06	-4.5E-06	-∞	∞	-5.1E-06	-5.1E-06	-∞	∞	-5.1E-06	-5.1E-06
FRTT	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
FRUK	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
FRUpts	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
FTHFCL	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
FTHFL	0.0E+00	∞	0.0E+00	-1.1E-14	0.0E+00	∞	0.0E+00	-8.1E-14	0.0E+00	∞	0.0E+00	1.8E-14
FUCt	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00

Reaction	Simulation A				Simulation B				Simulation C			
	LB	UB	Min	Max	LB	UB	Min	Max	LB	UB	Min	Max
FUM	-∞	∞	1.5E-01	1.5E-01	-∞	∞	1.7E-01	1.7E-01	-∞	∞	1.7E-01	1.7E-01
G1PACT	0.0E+00	∞	1.4E-01	1.4E-01	0.0E+00	∞	1.6E-01	1.6E-01	0.0E+00	∞	1.6E-01	1.6E-01
G1PPDM	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
G1PTMT	0.0E+00	∞	1.2E-01	1.2E-01	0.0E+00	∞	1.3E-01	1.3E-01	0.0E+00	∞	1.3E-01	1.3E-01
G1SAT	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
G35DP	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
G3PCT	0.0E+00	∞	6.2E-03	6.2E-03	0.0E+00	∞	7.1E-03	7.1E-03	0.0E+00	∞	7.1E-03	7.1E-03
G3PD1	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	0.0E+00	-2.7E-01	0.0E+00
G3PD2	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	0.0E+00	-2.7E-01	0.0E+00
G5SADs	-∞	∞	4.8E-01	4.8E-01	-∞	∞	6.4E-01	6.4E-01	-∞	∞	6.4E-01	6.4E-01
G5SD	0.0E+00	∞	4.8E-01	4.8E-01	0.0E+00	∞	6.4E-01	6.4E-01	0.0E+00	∞	6.4E-01	6.4E-01
G6PDA	0.0E+00	∞	0.0E+00	-1.5E-13	0.0E+00	∞	0.0E+00	-1.2E-12	0.0E+00	∞	0.0E+00	4.6E-13
G6PDHy	0.0E+00	∞	1.8E+01	1.8E+01	0.0E+00	∞	1.4E+01	1.4E+01	0.0E+00	∞	1.4E+01	1.4E+01
G6PI	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
GALK2	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
GALM	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
GALS3	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
GALSZ	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
GALT	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
GALTpts	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
GALU	-∞	∞	2.9E-01	2.9E-01	-∞	∞	3.2E-01	3.2E-01	-∞	∞	3.2E-01	3.2E-01
GAPD	-∞	∞	3.0E+01	3.0E+01	-∞	∞	2.3E+01	3.4E+01	-∞	∞	2.3E+01	2.3E+01
GARFT	-∞	∞	8.5E-02	8.5E-02	-∞	∞	9.6E-02	9.6E-02	-∞	∞	9.6E-02	9.6E-02
GAT1_LRE	0.0E+00	∞	4.3E-02	4.3E-02	0.0E+00	∞	4.9E-02	4.9E-02	0.0E+00	∞	4.9E-02	4.9E-02
GCALDt	-∞	∞	-4.5E-06	-4.5E-06	-∞	∞	-5.1E-06	-5.1E-06	-∞	∞	-5.1E-06	-5.1E-06
GDHYD	-∞	∞	0.0E+00	8.2E-04	-∞	∞	1.1E+01	2.2E+01	-∞	∞	2.2E+01	2.2E+01
GF6PTA	0.0E+00	∞	1.4E-01	1.4E-01	0.0E+00	∞	1.6E-01	1.6E-01	0.0E+00	∞	1.6E-01	1.6E-01
GGLUGABH	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
GHMT	-∞	∞	1.8E-01	1.8E-01	-∞	∞	2.0E-01	2.0E-01	-∞	∞	2.0E-01	2.0E-01
GK1	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
GK2	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
GLCNt2	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
GLCpts	0.0E+00	∞	2.5E+01	2.5E+01	0.0E+00	∞	1.9E+01	1.9E+01	0.0E+00	∞	1.9E+01	1.9E+01
GLNabc	0.0E+00	∞	4.3E-01	4.3E-01	0.0E+00	∞	4.9E-01	4.9E-01	0.0E+00	∞	4.9E-01	4.9E-01
GLNS	0.0E+00	∞	0.0E+00	5.7E-14	0.0E+00	∞	0.0E+00	-2.4E-12	0.0E+00	∞	0.0E+00	6.8E-13
GLNTAL	-∞	∞	9.1E-02	9.1E-02	-∞	∞	1.0E-01	1.0E-01	-∞	∞	1.0E-01	1.0E-01
GLTAL	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
GLU5K	0.0E+00	∞	4.8E-01	4.8E-01	0.0E+00	∞	6.4E-01	6.4E-01	0.0E+00	∞	6.4E-01	6.4E-01
GLUCYSL	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
GLUN	0.0E+00	∞	0.0E+00	-1.5E-13	0.0E+00	∞	0.0E+00	-1.2E-12	0.0E+00	∞	0.0E+00	4.6E-13
GLUPRT	0.0E+00	∞	8.5E-02	8.5E-02	0.0E+00	∞	9.6E-02	9.6E-02	0.0E+00	∞	9.6E-02	9.6E-02
GLUR	-∞	∞	-6.6E-02	-6.6E-02	-∞	∞	-7.5E-02	-7.5E-02	-∞	∞	-7.5E-02	-7.5E-02
GLUt4	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
GLUt6	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
GLUTRR	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
GLUTRS	0.0E+00	∞	1.2E-01	1.2E-01	0.0E+00	∞	1.3E-01	1.3E-01	0.0E+00	∞	1.3E-01	1.3E-01
GLYBabc	0.0E+00	∞	0.0E+00	-1.0E-13	0.0E+00	∞	0.0E+00	-1.1E-12	0.0E+00	∞	0.0E+00	6.3E-13
GLYBt6	-∞	∞	1.0E-13	0.0E+00	-∞	∞	1.1E-12	0.0E+00	-∞	∞	-6.3E-13	0.0E+00
GLYCDx	-∞	∞	9.3E-14	8.2E-04	-∞	∞	7.0E-13	1.1E+01	-∞	0.0E+00	-2.0E-13	0.0E+00
GLYCK	0.0E+00	∞	0.0E+00	-2.5E-14	0.0E+00	∞	0.0E+00	-1.1E-12	0.0E+00	∞	0.0E+00	-4.1E-14
GLYCt1	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞
GLYCt5	-∞	∞	0.0E+00	∞	-∞	∞	-2.2E+01	∞	-∞	∞	-2.2E+01	∞
GLYK	0.0E+00	∞	0.0E+00	8.2E-04	0.0E+00	∞	0.0E+00	1.1E+01	0.0E+00	∞	0.0E+00	2.7E-01
GLYt6	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
GLYTRS	0.0E+00	∞	9.2E-02	9.2E-02	0.0E+00	∞	1.1E-01	1.1E-01	0.0E+00	∞	1.1E-01	1.1E-01
GMPR	0.0E+00	∞	0.0E+00	1.5E-14	0.0E+00	∞	0.0E+00	-2.6E-13	0.0E+00	∞	0.0E+00	6.7E-14
GMPS2	0.0E+00	∞	2.1E-02	2.1E-02	0.0E+00	∞	2.4E-02	2.4E-02	0.0E+00	∞	2.4E-02	2.4E-02
GNK	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00

Reaction	Simulation A				Simulation B				Simulation C			
	LB	UB	Min	Max	LB	UB	Min	Max	LB	UB	Min	Max
GPDDA1	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
GPDDA2	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
GPDDA3	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
GPDDA4	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
GPDDA5	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
GRTAL	0.0E+00	∞	6.2E-03	6.2E-03	0.0E+00	∞	7.1E-03	7.1E-03	0.0E+00	∞	7.1E-03	7.1E-03
GRTT	0.0E+00	∞	9.1E-05	9.1E-05	0.0E+00	∞	1.0E-04	1.0E-04	0.0E+00	∞	1.0E-04	1.0E-04
GTHRD	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
GTHS	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
GTPCI	0.0E+00	∞	4.5E-06	4.5E-06	0.0E+00	∞	5.1E-06	5.1E-06	0.0E+00	∞	5.1E-06	5.1E-06
GTPCII	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
GTPDPK	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
GUAPRT	0.0E+00	∞	2.5E-02	2.5E-02	0.0E+00	∞	2.9E-02	2.9E-02	0.0E+00	∞	2.9E-02	2.9E-02
GUAt2	0.0E+00	∞	2.5E-02	2.5E-02	0.0E+00	∞	2.9E-02	2.9E-02	0.0E+00	∞	2.9E-02	2.9E-02
H2Ot5	-∞	∞	-4.6E+00	-4.6E+00	-∞	∞	-1.6E+01	-1.6E+01	-∞	∞	-1.6E+01	-1.6E+01
HACD1	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
HBUHL1	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
HBUR1	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
HCO3E	-∞	∞	6.5E-01	6.5E-01	-∞	∞	7.4E-01	7.4E-01	-∞	∞	7.4E-01	7.4E-01
HCYSMT	0.0E+00	∞	0.0E+00	-4.6E-15	0.0E+00	∞	0.0E+00	-2.4E-13	0.0E+00	∞	0.0E+00	5.5E-14
HDDHL5	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
HDDR5	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
HDEHL4	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
HDER4	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
HDMAT7	0.0E+00	∞	7.4E-02	7.4E-02	0.0E+00	∞	8.4E-02	8.4E-02	0.0E+00	∞	8.4E-02	8.4E-02
HEMAT2	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
HETZK	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
HEXTT	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
HHDHL7	0.0E+00	∞	8.4E-02	8.4E-02	0.0E+00	∞	9.5E-02	9.5E-02	0.0E+00	∞	9.5E-02	9.5E-02
HHDR7	0.0E+00	∞	8.4E-02	8.4E-02	0.0E+00	∞	9.5E-02	9.5E-02	0.0E+00	∞	9.5E-02	9.5E-02
HHYHL2	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
HHYR2	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
HISabc	0.0E+00	∞	1.9E-02	1.9E-02	0.0E+00	∞	2.1E-02	2.1E-02	0.0E+00	∞	2.1E-02	2.1E-02
HISDC	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
HISTAap	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
HISTRS	0.0E+00	∞	1.9E-02	1.9E-02	0.0E+00	∞	2.1E-02	2.1E-02	0.0E+00	∞	2.1E-02	2.1E-02
HMBS	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
HMGCOAR	-∞	∞	-1.0E-03	-1.0E-03	-∞	∞	-1.1E-03	-1.1E-03	-∞	∞	-1.1E-03	-1.1E-03
HMGCOAS	-∞	∞	-1.0E-03	-1.0E-03	-∞	∞	-1.1E-03	-1.1E-03	-∞	∞	-1.1E-03	-1.1E-03
HMPabc	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
HMPK1	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
HOCHL3	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
HOCR3	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
HODHL8	0.0E+00	∞	5.1E-02	5.1E-02	0.0E+00	∞	5.8E-02	5.8E-02	0.0E+00	∞	5.8E-02	5.8E-02
HODR8	0.0E+00	∞	5.1E-02	5.1E-02	0.0E+00	∞	5.8E-02	5.8E-02	0.0E+00	∞	5.8E-02	5.8E-02
HPPK	0.0E+00	∞	4.5E-06	4.5E-06	0.0E+00	∞	5.1E-06	5.1E-06	0.0E+00	∞	5.1E-06	5.1E-06
HSAT	-∞	∞	-2.2E-02	-2.2E-02	-∞	∞	-2.5E-02	-2.5E-02	-∞	∞	-2.5E-02	-2.5E-02
HSDy	-∞	∞	2.2E-02	2.2E-02	-∞	∞	2.5E-02	2.5E-02	-∞	∞	2.5E-02	2.5E-02
HTDHL6	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
HTDR6	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
HXANt2	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
HXPRT	0.0E+00	∞	0.0E+00	-2.1E-13	0.0E+00	∞	0.0E+00	-4.8E-12	0.0E+00	∞	0.0E+00	1.6E-12
HYPOE	0.0E+00	∞	0.0E+00	-8.7E-15	0.0E+00	∞	0.0E+00	-7.5E-13	0.0E+00	∞	0.0E+00	1.6E-13
ICDHy	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
ILEt2	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞
ILEt6	-∞	∞	-∞	4.7E-02	-∞	∞	-∞	5.4E-02	-∞	∞	-∞	5.4E-02
ILETRS	0.0E+00	∞	4.7E-02	4.7E-02	0.0E+00	∞	5.4E-02	5.4E-02	0.0E+00	∞	5.4E-02	5.4E-02
IMPC	-∞	∞	-8.5E-02	-8.5E-02	-∞	∞	-9.6E-02	-9.6E-02	-∞	∞	-9.6E-02	-9.6E-02

Reaction	Simulation A				Simulation B				Simulation C			
	LB	UB	Min	Max	LB	UB	Min	Max	LB	UB	Min	Max
INSt2r	-∞	∞	-4.4E-16	1.9E-12	-∞	∞	3.9E-13	-4.9E-12	-∞	∞	-4.9E-14	1.9E-12
IPDDI	-∞	∞	9.1E-05	9.1E-05	-∞	∞	1.0E-04	1.0E-04	-∞	∞	1.0E-04	1.0E-04
kaasIII	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
Kabc	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
LAR	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
LCTSt6	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
LDH_D	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
LDH_L	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
LEUt2	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞
LEUt6	-∞	∞	-∞	8.6E-02	-∞	∞	-∞	9.8E-02	-∞	∞	-∞	9.8E-02
LEUTRS	0.0E+00	∞	8.6E-02	8.6E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
L-LACt2	-∞	∞	-3.0E+01	0.0E+00	-∞	∞	-3.5E+01	0.0E+00	-∞	∞	-2.4E+01	0.0E+00
LPGS_LRE	0.0E+00	∞	7.4E-03	7.4E-03	0.0E+00	∞	8.4E-03	8.4E-03	0.0E+00	∞	8.4E-03	8.4E-03
LTAS_LRE	0.0E+00	∞	5.9E-03	5.9E-03	0.0E+00	∞	6.7E-03	6.7E-03	0.0E+00	∞	6.7E-03	6.7E-03
LYSDC	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
LYSt6	-∞	∞	8.0E-02	8.0E-02	-∞	∞	9.1E-02	9.1E-02	-∞	∞	9.1E-02	9.1E-02
LYSTRS	0.0E+00	∞	8.0E-02	8.0E-02	0.0E+00	∞	9.1E-02	9.1E-02	0.0E+00	∞	9.1E-02	9.1E-02
M1PD	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
MACPMT	0.0E+00	∞	6.5E-01	6.5E-01	0.0E+00	∞	7.4E-01	7.4E-01	0.0E+00	∞	7.4E-01	7.4E-01
MALLAC	0.0E+00	∞	2.4E-01	2.4E-01	0.0E+00	∞	4.1E-01	4.1E-01	0.0E+00	∞	4.1E-01	4.1E-01
MALP	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
MALT	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
MALt6	-∞	∞	-8.0E-13	0.0E+00	-∞	∞	3.7E-12	0.0E+00	-∞	∞	-5.8E-13	0.0E+00
MAN6PI	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
MANpts	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
MCMAT2	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
MCMAT3	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
MCMAT4	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
MCMAT5	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
MCMAT6	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
MCMAT7	0.0E+00	∞	8.4E-02	8.4E-02	0.0E+00	∞	9.5E-02	9.5E-02	0.0E+00	∞	9.5E-02	9.5E-02
MCMAT8	0.0E+00	∞	5.1E-02	5.1E-02	0.0E+00	∞	5.8E-02	5.8E-02	0.0E+00	∞	5.8E-02	5.8E-02
MDH	-∞	∞	-9.5E-02	-9.5E-02	-∞	∞	-2.4E-01	-2.4E-01	-∞	∞	-2.4E-01	-2.4E-01
METabc	0.0E+00	∞	0.0E+00	-1.0E-13	0.0E+00	∞	0.0E+00	-1.1E-12	0.0E+00	∞	0.0E+00	6.3E-13
METACH	-∞	∞	-2.2E-02	-2.2E-02	-∞	∞	-2.5E-02	-2.5E-02	-∞	∞	-2.5E-02	-2.5E-02
METAT	0.0E+00	∞	2.2E-02	2.2E-02	0.0E+00	∞	2.5E-02	2.5E-02	0.0E+00	∞	2.5E-02	2.5E-02
METDabc	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
METt6	-∞	∞	4.6E-02	4.6E-02	-∞	∞	5.2E-02	5.2E-02	-∞	∞	5.2E-02	5.2E-02
METTRS	0.0E+00	∞	2.4E-02	2.4E-02	0.0E+00	∞	2.8E-02	2.8E-02	0.0E+00	∞	2.8E-02	2.8E-02
MEVK	0.0E+00	∞	0.0E+00	1.0E-03	0.0E+00	∞	0.0E+00	1.1E-03	0.0E+00	∞	0.0E+00	1.1E-03
MEVK2	0.0E+00	∞	0.0E+00	1.0E-03	0.0E+00	∞	0.0E+00	1.1E-03	0.0E+00	∞	0.0E+00	1.1E-03
MEVK3	0.0E+00	∞	0.0E+00	1.0E-03	0.0E+00	∞	0.0E+00	1.1E-03	0.0E+00	∞	0.0E+00	1.1E-03
MEVK4	0.0E+00	∞	0.0E+00	1.0E-03	0.0E+00	∞	0.0E+00	1.1E-03	0.0E+00	∞	0.0E+00	1.1E-03
MGt5	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
MI1PP	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
MNabc	0.0E+00	∞	0.0E+00	-1.0E-13	0.0E+00	∞	0.0E+00	-1.2E-12	0.0E+00	∞	0.0E+00	6.3E-13
MNLpts	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
MNt2	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞
MNt6	-∞	∞	-∞	0.0E+00	-∞	∞	-∞	0.0E+00	-∞	∞	-∞	0.0E+00
MNTD	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
MNTD2	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
MOADCST	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
MTAN	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
MTHFC	-∞	∞	1.7E-01	1.7E-01	-∞	∞	1.9E-01	1.9E-01	-∞	∞	1.9E-01	1.9E-01
MTHFD	-∞	∞	1.7E-01	1.7E-01	-∞	∞	1.9E-01	1.9E-01	-∞	∞	1.9E-01	1.9E-01
MTHPTGHM	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
NACUP	0.0E+00	∞	9.1E-04	9.1E-04	0.0E+00	∞	1.0E-03	1.0E-03	0.0E+00	∞	1.0E-03	1.0E-03
NADH4	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00

Reaction	Simulation A				Simulation B				Simulation C			
	LB	UB	Min	Max	LB	UB	Min	Max	LB	UB	Min	Max
NADK	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
NADN	0.0E+00	∞	0.0E+00	-1.0E-14	0.0E+00	∞	0.0E+00	-1.3E-13	0.0E+00	∞	0.0E+00	2.3E-14
NADPH12	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
NADS1	0.0E+00	∞	9.1E-04	9.1E-04	0.0E+00	∞	1.0E-03	1.0E-03	0.0E+00	∞	1.0E-03	1.0E-03
NAPRT	0.0E+00	∞	9.1E-04	9.1E-04	0.0E+00	∞	1.0E-03	1.0E-03	0.0E+00	∞	1.0E-03	1.0E-03
NAt7	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
NDPK1	-∞	∞	2.3E+00	2.4E+00	-∞	∞	2.6E+00	2.7E+00	-∞	∞	2.6E+00	2.7E+00
NDPK2	-∞	∞	4.9E-01	5.0E-01	-∞	∞	5.6E-01	5.7E-01	-∞	∞	5.6E-01	5.7E-01
NDPK3	-∞	∞	3.3E-01	3.5E-01	-∞	∞	3.8E-01	4.0E-01	-∞	∞	3.8E-01	4.0E-01
NDPK4	-∞	∞	1.3E-01	1.3E-01	-∞	∞	1.4E-01	1.4E-01	-∞	∞	1.4E-01	1.4E-01
NDPK5	-∞	∞	0.0E+00	6.1E-03	-∞	∞	0.0E+00	7.0E-03	-∞	∞	0.0E+00	7.0E-03
NDPK6	-∞	∞	0.0E+00	7.8E-03	-∞	∞	0.0E+00	8.9E-03	-∞	∞	0.0E+00	8.9E-03
NDPK7	-∞	∞	-7.8E-03	6.1E-03	-∞	∞	-8.9E-03	7.0E-03	-∞	∞	-8.9E-03	7.0E-03
NDPK8	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞	-∞	∞
NDPK9	-∞	∞	0.0E+00	6.7E-02	-∞	∞	0.0E+00	7.6E-02	-∞	∞	0.0E+00	7.6E-02
NH3t	-∞	∞	-2.1E-01	-2.1E-01	-∞	∞	-2.2E-01	-2.2E-01	-∞	∞	-2.2E-01	-2.2E-01
NH4DIS	-∞	∞	2.1E-01	2.1E-01	-∞	∞	2.2E-01	2.2E-01	-∞	∞	2.2E-01	2.2E-01
NH4DISex	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
NNAMr	-∞	∞	0.0E+00	-1.0E-14	-∞	∞	0.0E+00	-1.3E-13	-∞	∞	0.0E+00	2.3E-14
NNAT	0.0E+00	∞	9.1E-04	9.1E-04	0.0E+00	∞	1.0E-03	1.0E-03	0.0E+00	∞	1.0E-03	1.0E-03
NNDMBRT	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
NOX1	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
NOX2	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
NPHS	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
NTD1	0.0E+00	∞	0.0E+00	1.2E-13	0.0E+00	∞	0.0E+00	-7.9E-13	0.0E+00	∞	0.0E+00	2.9E-13
NTD10	0.0E+00	∞	0.0E+00	-3.2E-14	0.0E+00	∞	0.0E+00	-4.0E-13	0.0E+00	∞	0.0E+00	7.8E-14
NTD11	0.0E+00	∞	0.0E+00	-2.2E-14	0.0E+00	∞	0.0E+00	-4.3E-13	0.0E+00	∞	0.0E+00	1.0E-13
NTD2	0.0E+00	∞	0.0E+00	-9.2E-15	0.0E+00	∞	0.0E+00	-7.4E-13	0.0E+00	∞	0.0E+00	1.1E-13
NTD3	0.0E+00	∞	0.0E+00	7.8E-03	0.0E+00	∞	0.0E+00	8.9E-03	0.0E+00	∞	0.0E+00	8.9E-03
NTD4	0.0E+00	∞	0.0E+00	5.2E-15	0.0E+00	∞	0.0E+00	-7.5E-13	0.0E+00	∞	0.0E+00	1.7E-13
NTD5	0.0E+00	∞	0.0E+00	-1.5E-14	0.0E+00	∞	0.0E+00	-8.1E-13	0.0E+00	∞	0.0E+00	1.7E-13
NTD6	0.0E+00	∞	0.0E+00	-1.5E-14	0.0E+00	∞	0.0E+00	-8.1E-13	0.0E+00	∞	0.0E+00	1.7E-13
NTD7	0.0E+00	∞	0.0E+00	8.5E-14	0.0E+00	∞	0.0E+00	-4.6E-12	0.0E+00	∞	0.0E+00	1.2E-12
NTD8	0.0E+00	∞	0.0E+00	-1.5E-14	0.0E+00	∞	0.0E+00	-8.1E-13	0.0E+00	∞	0.0E+00	1.7E-13
NTD9	0.0E+00	∞	0.0E+00	3.2E-14	0.0E+00	∞	0.0E+00	-4.2E-13	0.0E+00	∞	0.0E+00	9.9E-14
NTR3B	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
OCBT	-∞	∞	-8.2E-14	0.0E+00	-∞	∞	4.8E-13	0.0E+00	-∞	∞	-1.8E-13	0.0E+00
OCDMAT8	0.0E+00	∞	1.7E-03	1.7E-03	0.0E+00	∞	2.0E-03	2.0E-03	0.0E+00	∞	2.0E-03	2.0E-03
OCMAT3	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
OMPDC	0.0E+00	∞	0.0E+00	8.2E-14	0.0E+00	∞	0.0E+00	-4.8E-13	0.0E+00	∞	0.0E+00	1.8E-13
ORNt2	0.0E+00	∞	0.0E+00	4.4E-02	0.0E+00	∞	0.0E+00	5.0E-02	0.0E+00	∞	0.0E+00	5.0E-02
OROt6	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
ORPT	-∞	∞	-8.2E-14	0.0E+00	-∞	∞	4.8E-13	0.0E+00	-∞	∞	-1.8E-13	0.0E+00
P5CRr	-∞	∞	4.8E-01	4.8E-01	-∞	∞	6.4E-01	6.4E-01	-∞	∞	6.4E-01	6.4E-01
PABAt	-∞	∞	4.5E-06	4.5E-06	-∞	∞	5.1E-06	5.1E-06	-∞	∞	5.1E-06	5.1E-06
PAPPT5	0.0E+00	∞	6.6E-02	6.6E-02	0.0E+00	∞	7.5E-02	7.5E-02	0.0E+00	∞	7.5E-02	7.5E-02
PDH	0.0E+00	∞	0.0E+00	3.0E+01	0.0E+00	∞	0.0E+00	3.5E+01	0.0E+00	∞	0.0E+00	2.4E+01
PFK	0.0E+00	∞	6.5E+00	6.5E+00	0.0E+00	∞	5.0E+00	5.0E+00	0.0E+00	∞	5.0E+00	5.0E+00
PGAMT	-∞	∞	-1.4E-01	-1.4E-01	-∞	∞	-1.6E-01	-1.6E-01	-∞	∞	-1.6E-01	-1.6E-01
PGDH	0.0E+00	∞	1.8E+01	1.8E+01	0.0E+00	∞	1.4E+01	1.4E+01	0.0E+00	∞	1.4E+01	1.4E+01
PGGH	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
PGGT4	0.0E+00	∞	6.6E-02	6.6E-02	0.0E+00	∞	7.5E-02	7.5E-02	0.0E+00	∞	7.5E-02	7.5E-02
PGI	-∞	∞	6.7E+00	6.7E+00	-∞	∞	5.2E+00	5.2E+00	-∞	∞	5.2E+00	5.2E+00
PGK	-∞	∞	3.0E+01	3.0E+01	-∞	∞	2.3E+01	3.4E+01	-∞	∞	2.3E+01	2.3E+01
PGL	0.0E+00	∞	1.8E+01	1.8E+01	0.0E+00	∞	1.4E+01	1.4E+01	0.0E+00	∞	1.4E+01	1.4E+01
PGLYCP	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
PGM	-∞	∞	3.0E+01	3.0E+01	-∞	∞	2.3E+01	3.4E+01	-∞	∞	2.3E+01	2.3E+01
PGMT	-∞	∞	-4.0E-01	-4.0E-01	-∞	∞	-4.6E-01	-4.6E-01	-∞	∞	-4.6E-01	-4.6E-01

Reaction	Simulation A				Simulation B				Simulation C			
	LB	UB	Min	Max	LB	UB	Min	Max	LB	UB	Min	Max
PGMT_B	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
PGPP_LRE	0.0E+00	∞	1.8E-01	1.8E-01	0.0E+00	∞	2.1E-01	2.1E-01	0.0E+00	∞	2.1E-01	2.1E-01
PGSA_LRE	-∞	∞	1.8E-01	1.8E-01	-∞	∞	2.1E-01	2.1E-01	-∞	∞	2.1E-01	2.1E-01
PHEt6	-∞	∞	3.7E-02	3.7E-02	-∞	∞	4.3E-02	4.3E-02	-∞	∞	4.3E-02	4.3E-02
PHETRS	0.0E+00	∞	3.7E-02	3.7E-02	0.0E+00	∞	4.3E-02	4.3E-02	0.0E+00	∞	4.3E-02	4.3E-02
PIabc	0.0E+00	∞	0.0E+00	-1.0E-13	0.0E+00	∞	0.0E+00	-1.1E-12	0.0E+00	∞	0.0E+00	6.3E-13
PIt6	-∞	∞	5.7E-01	5.7E-01	-∞	∞	6.5E-01	6.5E-01	-∞	∞	6.5E-01	6.5E-01
PKL	-∞	∞	1.7E+01	1.7E+01	-∞	∞	1.3E+01	1.3E+01	-∞	∞	1.3E+01	1.3E+01
PMANM	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
PMEVK	0.0E+00	∞	1.0E-03	1.0E-03	0.0E+00	∞	1.1E-03	1.1E-03	0.0E+00	∞	1.1E-03	1.1E-03
PMPK	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
PNS1	0.0E+00	∞	0.0E+00	4.6E-14	0.0E+00	∞	0.0E+00	-8.0E-13	0.0E+00	∞	0.0E+00	1.3E-13
PNS2	0.0E+00	∞	0.0E+00	4.6E-14	0.0E+00	∞	0.0E+00	-8.0E-13	0.0E+00	∞	0.0E+00	1.3E-13
PNS3	0.0E+00	∞	0.0E+00	4.6E-14	0.0E+00	∞	0.0E+00	-8.0E-13	0.0E+00	∞	0.0E+00	1.3E-13
PNS4	0.0E+00	∞	0.0E+00	4.6E-14	0.0E+00	∞	0.0E+00	-8.0E-13	0.0E+00	∞	0.0E+00	1.3E-13
PNTK	0.0E+00	∞	9.1E-05	9.1E-05	0.0E+00	∞	1.0E-04	1.0E-04	0.0E+00	∞	1.0E-04	1.0E-04
PNTOt2	-∞	∞	9.1E-05	9.1E-05	-∞	∞	1.0E-04	1.0E-04	-∞	∞	1.0E-04	1.0E-04
PPA	0.0E+00	∞	2.4E+00	2.4E+00	0.0E+00	∞	2.7E+00	2.7E+00	0.0E+00	∞	2.7E+00	2.7E+00
PPBNGS	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
PPCDC	0.0E+00	∞	9.1E-05	9.1E-05	0.0E+00	∞	1.0E-04	1.0E-04	0.0E+00	∞	1.0E-04	1.0E-04
PPDt1	-∞	∞	0.0E+00	8.2E-04	-∞	∞	1.1E+01	2.2E+01	-∞	∞	2.2E+01	2.2E+01
PPIK	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞
PPM	-∞	∞	-6.7E-02	-6.7E-02	-∞	∞	-7.6E-02	-7.6E-02	-∞	∞	-7.6E-02	-7.6E-02
PPM2	-∞	∞	0.0E+00	2.1E-14	-∞	∞	0.0E+00	-4.2E-13	-∞	∞	0.0E+00	2.1E-13
PPN13D	-∞	∞	-8.2E-04	0.0E+00	-∞	∞	-2.2E+01	-1.1E+01	-∞	∞	-2.2E+01	-2.2E+01
PPNCL	0.0E+00	∞	9.1E-05	9.1E-05	0.0E+00	∞	1.0E-04	1.0E-04	0.0E+00	∞	1.0E-04	1.0E-04
PRAGS	0.0E+00	∞	8.5E-02	8.5E-02	0.0E+00	∞	9.6E-02	9.6E-02	0.0E+00	∞	9.6E-02	9.6E-02
PRAIS	0.0E+00	∞	8.5E-02	8.5E-02	0.0E+00	∞	9.6E-02	9.6E-02	0.0E+00	∞	9.6E-02	9.6E-02
PRASCS	-∞	∞	8.5E-02	8.5E-02	-∞	∞	9.6E-02	9.6E-02	-∞	∞	9.6E-02	9.6E-02
PRFGS	0.0E+00	∞	8.5E-02	8.5E-02	0.0E+00	∞	9.6E-02	9.6E-02	0.0E+00	∞	9.6E-02	9.6E-02
PROt6	-∞	∞	-4.4E-01	-4.4E-01	-∞	∞	-5.9E-01	-5.9E-01	-∞	∞	-5.9E-01	-5.9E-01
PROTRS	0.0E+00	∞	4.4E-02	4.4E-02	0.0E+00	∞	5.0E-02	5.0E-02	0.0E+00	∞	5.0E-02	5.0E-02
PROTS_LRE_v6.0	0.0E+00	∞	1.1E+00	1.1E+00	0.0E+00	∞	1.3E+00	1.3E+00	0.0E+00	∞	1.3E+00	1.3E+00
PRPPS	-∞	∞	1.1E-01	1.1E-01	-∞	∞	1.3E-01	1.3E-01	-∞	∞	1.3E-01	1.3E-01
PSUDS	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞
PTAr	-∞	∞	-1.7E+01	-1.7E+01	-∞	∞	-1.3E+01	-1.8E+00	-∞	∞	-2.0E+00	-1.8E+00
PTPAT	-∞	∞	-∞	9.1E-05	-∞	∞	-∞	1.0E-04	-∞	∞	-∞	1.0E-04
PTPATi	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞
PTRCabc	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
PUNP1	-∞	∞	-4.6E-14	8.5E-14	-∞	∞	8.0E-13	-4.6E-12	-∞	∞	-1.2E-13	1.2E-12
PUNP2	-∞	∞	-1.1E-14	0.0E+00	-∞	∞	2.1E-13	0.0E+00	-∞	∞	-4.6E-14	0.0E+00
PUNP3	-∞	∞	-2.3E-14	3.2E-14	-∞	∞	7.9E-13	-4.2E-13	-∞	∞	-1.2E-13	9.9E-14
PUNP4	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
PUNP5	-∞	∞	-4.6E-14	-4.0E-13	-∞	∞	8.0E-13	-5.1E-12	-∞	∞	-1.3E-13	1.6E-12
PUNP6	-∞	∞	0.0E+00	1.1E-14	-∞	∞	0.0E+00	-2.1E-13	-∞	∞	0.0E+00	4.6E-14
PUNP7	-∞	∞	-4.6E-14	-3.2E-14	-∞	∞	8.0E-13	-4.0E-13	-∞	∞	-1.3E-13	7.8E-14
PYDAMK	0.0E+00	∞	0.0E+00	-8.7E-15	0.0E+00	∞	0.0E+00	-7.5E-13	0.0E+00	∞	0.0E+00	1.6E-13
PYDAMt	-∞	∞	4.5E-07	4.5E-07	-∞	∞	5.1E-07	5.1E-07	-∞	∞	5.1E-07	5.1E-07
PYDXK	0.0E+00	∞	4.5E-07	4.5E-07	0.0E+00	∞	5.1E-07	5.1E-07	0.0E+00	∞	5.1E-07	5.1E-07
PYDXPP	0.0E+00	∞	0.0E+00	-1.6E-14	0.0E+00	∞	0.0E+00	-8.1E-13	0.0E+00	∞	0.0E+00	1.5E-13
PYK	0.0E+00	∞	5.4E+00	5.4E+00	0.0E+00	∞	3.7E+00	1.5E+01	0.0E+00	∞	3.7E+00	4.0E+00
PYNP1	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞
PYNP1r	-∞	∞	-∞	0.0E+00	-∞	∞	-∞	0.0E+00	-∞	∞	-∞	0.0E+00
PYNP2	-∞	∞	-6.7E-02	-6.7E-02	-∞	∞	-7.6E-02	-7.6E-02	-∞	∞	-7.6E-02	-7.6E-02
PYNP3	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
PYNP4	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
PYRNS1	0.0E+00	∞	0.0E+00	4.6E-14	0.0E+00	∞	0.0E+00	-8.0E-13	0.0E+00	∞	0.0E+00	1.3E-13
PYRt2	-∞	∞	-1.7E-13	0.0E+00	-∞	∞	1.6E-12	0.0E+00	-∞	∞	-3.6E-13	0.0E+00

Reaction	Simulation A				Simulation B				Simulation C			
	LB	UB	Min	Max	LB	UB	Min	Max	LB	UB	Min	Max
PYRZAM	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
QUIND	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
R05218	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
R05220	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
R05223	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
R05225	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
R05226	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
RAFGH	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
RBFK	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
RBFSa	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
RBK	0.0E+00	∞	2.2E-02	2.2E-02	0.0E+00	∞	2.5E-02	2.5E-02	0.0E+00	∞	2.5E-02	2.5E-02
RBLK1	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
RBLK2	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
RBP4E	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
RBT5PDHy	-∞	∞	-1.6E-01	-1.6E-01	-∞	∞	-1.8E-01	-1.8E-01	-∞	∞	-1.8E-01	-1.8E-01
RBZP	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
RHC	0.0E+00	∞	2.2E-02	2.2E-02	0.0E+00	∞	2.5E-02	2.5E-02	0.0E+00	∞	2.5E-02	2.5E-02
RIBFLVt2	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
RNAS_LRE	0.0E+00	∞	1.3E-01	1.3E-01	0.0E+00	∞	1.4E-01	1.4E-01	0.0E+00	∞	1.4E-01	1.4E-01
RNDR1	0.0E+00	∞	0.0E+00	7.8E-03	0.0E+00	∞	0.0E+00	8.9E-03	0.0E+00	∞	0.0E+00	8.9E-03
RNDR2	0.0E+00	∞	0.0E+00	6.1E-03	0.0E+00	∞	0.0E+00	7.0E-03	0.0E+00	∞	0.0E+00	7.0E-03
RNDR3	0.0E+00	∞	0.0E+00	1.4E-02	0.0E+00	∞	0.0E+00	1.6E-02	0.0E+00	∞	0.0E+00	1.6E-02
RNDR4	0.0E+00	∞	0.0E+00	7.8E-03	0.0E+00	∞	0.0E+00	8.9E-03	0.0E+00	∞	0.0E+00	8.9E-03
RNTR1	0.0E+00	∞	0.0E+00	7.8E-03	0.0E+00	∞	0.0E+00	8.9E-03	0.0E+00	∞	0.0E+00	8.9E-03
RNTR2	0.0E+00	∞	0.0E+00	6.1E-03	0.0E+00	∞	0.0E+00	7.0E-03	0.0E+00	∞	0.0E+00	7.0E-03
RNTR3	0.0E+00	∞	0.0E+00	1.4E-02	0.0E+00	∞	0.0E+00	1.6E-02	0.0E+00	∞	0.0E+00	1.6E-02
RNTR4	0.0E+00	∞	0.0E+00	7.8E-03	0.0E+00	∞	0.0E+00	8.9E-03	0.0E+00	∞	0.0E+00	8.9E-03
RPE	-∞	∞	1.7E+01	1.7E+01	-∞	∞	1.3E+01	1.3E+01	-∞	∞	1.3E+01	1.3E+01
RPI	-∞	∞	-1.6E-01	-1.6E-01	-∞	∞	-1.8E-01	-1.8E-01	-∞	∞	-1.8E-01	-1.8E-01
SBTpts	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
SDPDS	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
SECYSL	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
SERAT	-∞	∞	2.2E-02	2.2E-02	-∞	∞	2.5E-02	2.5E-02	-∞	∞	2.5E-02	2.5E-02
SERD_L	0.0E+00	∞	3.1E-01	3.1E-01	0.0E+00	∞	3.3E-01	3.3E-01	0.0E+00	∞	3.3E-01	3.3E-01
SERt3	0.0E+00	∞	0.0E+00	-3.7E-14	0.0E+00	∞	0.0E+00	-1.1E-12	0.0E+00	∞	0.0E+00	2.2E-13
SERt6	-∞	∞	5.7E-01	5.7E-01	-∞	∞	6.3E-01	6.3E-01	-∞	∞	6.3E-01	6.3E-01
SERTRS	0.0E+00	∞	6.2E-02	6.2E-02	0.0E+00	∞	7.0E-02	7.0E-02	0.0E+00	∞	7.0E-02	7.0E-02
SHCHD	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
SHCHD2	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
SPMDabc	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
SRCHCOC	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
SSALx	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
SSALy	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
SUCBZL	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
SUCpts	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
TAPGL4	0.0E+00	∞	6.2E-03	6.2E-03	0.0E+00	∞	7.1E-03	7.1E-03	0.0E+00	∞	7.1E-03	7.1E-03
TDMAT6	0.0E+00	∞	8.7E-02	8.7E-02	0.0E+00	∞	9.8E-02	9.8E-02	0.0E+00	∞	9.8E-02	9.8E-02
TDPDRE	-∞	∞	1.2E-01	1.2E-01	-∞	∞	1.3E-01	1.3E-01	-∞	∞	1.3E-01	1.3E-01
TDPDRR	-∞	∞	-1.2E-01	-1.2E-01	-∞	∞	-1.3E-01	-1.3E-01	-∞	∞	-1.3E-01	-1.3E-01
TDPGDH	0.0E+00	∞	1.2E-01	1.2E-01	0.0E+00	∞	1.3E-01	1.3E-01	0.0E+00	∞	1.3E-01	1.3E-01
THDPS	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
THFGLUS	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
THMabc	0.0E+00	∞	4.5E-06	4.5E-06	0.0E+00	∞	5.1E-06	5.1E-06	0.0E+00	∞	5.1E-06	5.1E-06
THMDt2r	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00	-∞	∞	0.0E+00	0.0E+00
THRPDC	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
THRt2	0.0E+00	∞	7.0E-02	7.0E-02	0.0E+00	∞	8.0E-02	8.0E-02	0.0E+00	∞	8.0E-02	8.0E-02
THRt3	0.0E+00	∞	0.0E+00	-3.7E-14	0.0E+00	∞	0.0E+00	-1.1E-12	0.0E+00	∞	0.0E+00	2.2E-13
THRTRS	0.0E+00	∞	7.0E-02	7.0E-02	0.0E+00	∞	8.0E-02	8.0E-02	0.0E+00	∞	8.0E-02	8.0E-02

Reaction	Simulation A				Simulation B				Simulation C			
	LB	UB	Min	Max	LB	UB	Min	Max	LB	UB	Min	Max
THZPSN	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
THZPSN2	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
TMDK1	0.0E+00	∞	0.0E+00	-1.5E-14	0.0E+00	∞	0.0E+00	-8.1E-13	0.0E+00	∞	0.0E+00	1.7E-13
TMDK2	0.0E+00	∞	0.0E+00	-1.8E-14	0.0E+00	∞	0.0E+00	-7.0E-13	0.0E+00	∞	0.0E+00	1.7E-13
TMDPK	0.0E+00	∞	4.5E-06	4.5E-06	0.0E+00	∞	5.1E-06	5.1E-06	0.0E+00	∞	5.1E-06	5.1E-06
TMDS	0.0E+00	∞	7.8E-03	7.8E-03	0.0E+00	∞	8.9E-03	8.9E-03	0.0E+00	∞	8.9E-03	8.9E-03
TMPPP	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
TPI	-∞	∞	6.3E+00	6.3E+00	-∞	∞	4.7E+00	1.6E+01	-∞	∞	4.7E+00	5.0E+00
TRDR	0.0E+00	∞	2.8E-02	2.8E-02	0.0E+00	∞	3.2E-02	3.2E-02	0.0E+00	∞	3.2E-02	3.2E-02
TREpts	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
TRPt6	-∞	∞	6.6E-03	6.6E-03	-∞	∞	7.5E-03	7.5E-03	-∞	∞	7.5E-03	7.5E-03
TRPTRS	0.0E+00	∞	6.6E-03	6.6E-03	0.0E+00	∞	7.5E-03	7.5E-03	0.0E+00	∞	7.5E-03	7.5E-03
TYRt6	-∞	∞	3.1E-02	3.1E-02	-∞	∞	3.5E-02	3.5E-02	-∞	∞	3.5E-02	3.5E-02
TYRTRS	0.0E+00	∞	3.1E-02	3.1E-02	0.0E+00	∞	3.5E-02	3.5E-02	0.0E+00	∞	3.5E-02	3.5E-02
UAAGDS	0.0E+00	∞	6.6E-02	6.6E-02	0.0E+00	∞	7.5E-02	7.5E-02	0.0E+00	∞	7.5E-02	7.5E-02
UAAGLS1	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
UAG2E	-∞	∞	6.2E-03	6.2E-03	-∞	∞	7.1E-03	7.1E-03	-∞	∞	7.1E-03	7.1E-03
UAG2EMA	-∞	∞	0.0E+00	1.2E-15	-∞	∞	0.0E+00	-2.3E-13	-∞	∞	0.0E+00	1.4E-13
UAGCVT	0.0E+00	∞	6.6E-02	6.6E-02	0.0E+00	∞	7.5E-02	7.5E-02	0.0E+00	∞	7.5E-02	7.5E-02
UAGDP	0.0E+00	∞	1.4E-01	1.4E-01	0.0E+00	∞	1.6E-01	1.6E-01	0.0E+00	∞	1.6E-01	1.6E-01
UAGPT5	0.0E+00	∞	6.6E-02	6.6E-02	0.0E+00	∞	7.5E-02	7.5E-02	0.0E+00	∞	7.5E-02	7.5E-02
UAMAGS	0.0E+00	∞	6.6E-02	6.6E-02	0.0E+00	∞	7.5E-02	7.5E-02	0.0E+00	∞	7.5E-02	7.5E-02
UAMAS	0.0E+00	∞	6.6E-02	6.6E-02	0.0E+00	∞	7.5E-02	7.5E-02	0.0E+00	∞	7.5E-02	7.5E-02
UAPGR	0.0E+00	∞	6.6E-02	6.6E-02	0.0E+00	∞	7.5E-02	7.5E-02	0.0E+00	∞	7.5E-02	7.5E-02
UDCPDP	0.0E+00	∞	6.6E-02	6.6E-02	0.0E+00	∞	7.5E-02	7.5E-02	0.0E+00	∞	7.5E-02	7.5E-02
UDCPDPS	0.0E+00	∞	9.1E-05	9.1E-05	0.0E+00	∞	1.0E-04	1.0E-04	0.0E+00	∞	1.0E-04	1.0E-04
UDCPK	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
UDPDPS	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
UDPG4E	-∞	∞	5.9E-02	5.9E-02	-∞	∞	6.7E-02	6.7E-02	-∞	∞	6.7E-02	6.7E-02
UDPGALM	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
UGLDDS1	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
UGLDDS2	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
UGLDDS3	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
UGMDDS2	0.0E+00	∞	6.6E-02	6.6E-02	0.0E+00	∞	7.5E-02	7.5E-02	0.0E+00	∞	7.5E-02	7.5E-02
UNAGAMAMT	0.0E+00	∞	6.2E-03	6.2E-03	0.0E+00	∞	7.1E-03	7.1E-03	0.0E+00	∞	7.1E-03	7.1E-03
UPP3MT	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
UPP3S	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
UPPRT	0.0E+00	∞	0.0E+00	-6.4E-14	0.0E+00	∞	0.0E+00	-8.2E-13	0.0E+00	∞	0.0E+00	1.3E-13
URAt2	0.0E+00	∞	6.7E-02	6.7E-02	0.0E+00	∞	7.6E-02	7.6E-02	0.0E+00	∞	7.6E-02	7.6E-02
URIDK1	0.0E+00	∞	2.0E-01	2.0E-01	0.0E+00	∞	2.3E-01	2.3E-01	0.0E+00	∞	2.3E-01	2.3E-01
URIDK2	0.0E+00	∞	0.0E+00	-7.4E-15	0.0E+00	∞	0.0E+00	-4.1E-13	0.0E+00	∞	0.0E+00	8.5E-14
URIK1	0.0E+00	∞	0.0E+00	6.7E-02	0.0E+00	∞	0.0E+00	7.6E-02	0.0E+00	∞	0.0E+00	7.6E-02
URIK2	0.0E+00	∞	0.0E+00	6.7E-02	0.0E+00	∞	0.0E+00	7.6E-02	0.0E+00	∞	0.0E+00	7.6E-02
URIK3	0.0E+00	∞	0.0E+00	6.7E-02	0.0E+00	∞	0.0E+00	7.6E-02	0.0E+00	∞	0.0E+00	7.6E-02
VALt2	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞	0.0E+00	∞
VALt6	-∞	∞	-∞	6.6E-02	-∞	∞	-∞	7.5E-02	-∞	∞	-∞	7.5E-02
VALTRS	0.0E+00	∞	6.6E-02	6.6E-02	0.0E+00	∞	7.5E-02	7.5E-02	0.0E+00	∞	7.5E-02	7.5E-02
XANt2	0.0E+00	∞	0.0E+00	-1.7E-14	0.0E+00	∞	0.0E+00	-4.1E-13	0.0E+00	∞	0.0E+00	9.5E-14
XPPT	0.0E+00	∞	0.0E+00	-3.2E-14	0.0E+00	∞	0.0E+00	-4.0E-13	0.0E+00	∞	0.0E+00	7.8E-14
XYLK	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00	0.0E+00	∞	0.0E+00	0.0E+00
YUMPS	-∞	∞	-∞	0.0E+00	-∞	∞	-∞	0.0E+00	-∞	∞	-∞	0.0E+00

Note: LB, lower boundary constrain; UB, upper boundary constraint; Min, minimum flux within optimal solution; Max, maximum flux within optimal solution.

Content of supplementary material S3 of Chapter 4:
cDNA microarray experiments

Table 4.S3.1. Complete list of transcripts from *L. reuteri* that are differentially expressed in response to glycerol[a]. Comparisons were established to identify mid-log (exp) and early-stationary (sta) specific responses, and to detect the effect of glycerol in the differential response between mid-log and early-stationary growth phases (sta-exp).

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_0001	Chromosomal replication initiator protein dnaA	-	-	1.16	0.00	1.81	0.00
Lreu_0003	Hypothetical cytosolic protein	-	-	-2.18	0.00	-2.24	0.00
Lreu_0004	DNA replication and repair protein recF	-	-	-1.35	0.00	-1.22	0.03
Lreu_0007	SSU ribosomal protein S6P	-	-	-	-	0.81	0.04
Lreu_0008	Phage single-strand DNA binding protein	-	-	-	-	0.95	0.05
Lreu_0010	Phosphoesterase, DHH family protein	-	-	1.63	0.05	1.78	0.03
Lreu_0011	LSU ribosomal protein L9P	-	-	1.57	0.00	1.65	0.01
Lreu_0012	Replicative DNA helicase (EC 3.6.1.-)	-	-	1.56	0.00	2.32	0.00
Lreu_0015	Hypothetical protein	-0.63	0.02	-	-	-	-
Lreu_0023	Zn-dependent hydrolase (beta-lactamase superfamily)	-	-	-	-	0.90	0.04
Lreu_0030	1,3-propanediol dehydrogenase (EC 1.1.1.202)	1.43	0.00	1.52	0.00	1.69	0.00
Lreu_0035	Hypothetical exported protein	-	-	0.98	0.00	1.08	0.04
Lreu_0041		-	-	1.24	0.02	-	-
Lreu_0044	Aromatic amino acid aminotransferase (EC 2.6.1.57) / Acetyldiaminopimelate aminotransferase (EC 2.6.1.-)	-	-	1.37	0.03	-	-
Lreu_0045	ATP-dependent endopeptidase clp ATP-binding subunit clpL	-0.64	0.03	-0.78	0.00	-0.98	0.02
Lreu_0049	ATP-dependent nuclease subunit B	-	-	1.48	0.02	1.79	0.04
Lreu_0053	Sucrose transport protein	-2.23	0.00	-	-	-	-
Lreu_0054	maltose phosphorylase (EC 2.4.1.8)	-0.76	0.01	-	-	-	-
Lreu_0055	Beta-phosphoglucomutase (EC 5.4.2.6) / Glucose-1-phosphate phosphodismutase (EC 2.7.1.41)	-0.81	0.00	-	-	-	-
Lreu_0059	Two component system histidine kinase (EC 2.7.3.-)	-	-	0.73	0.00	-	-
Lreu_0061		-	-	1.87	0.00	-	-
Lreu_0063	Glutamine amidotransferase, class I	-	-	1.37	0.00	-	-
Lreu_0064	Aldo/keto reductase family	-	-	-0.75	0.01	-1.32	0.01
Lreu_0067	NADH oxidase H2O-forming (EC 1.6.-.-)	-	-	-2.47	0.00	-2.47	0.01
Lreu_0077		-	-	0.73	0.01	1.22	0.02
Lreu_0079		-	-	0.77	0.02	-	-
Lreu_0083	Oligo-1,6-glucosidase (EC 3.2.1.10)	-1.64	0.02	3.34	0.00	2.48	0.05
Lreu_0085	Carbamoyl-phosphate synthase large chain (EC 6.3.5.5)	-	-	-1.04	0.01	-	-
Lreu_0086	Carbamoyl-phosphate synthase small chain (EC 6.3.5.5)	-	-	-	-	-3.09	0.02
Lreu_0088	Transcriptional regulator, LacI family	-3.24	0.00	1.43	0.01	-	-
Lreu_0089	tRNA (Uracil-5-) -methyltransferase (EC 2.1.1.35)	-	-	1.55	0.02	-	-
Lreu_0090	NADPH-dependent FMN reductase family protein	-0.68	0.01	-1.36	0.00	-1.49	0.01
Lreu_0091	Oxidoreductase (EC 1.1.1.-)	-0.72	0.01	-1.24	0.00	-1.39	0.01
Lreu_0092	Iron-sulfur cluster assembly/repair protein ApbE	-0.63	0.02	-1.25	0.00	-1.56	0.01
Lreu_0098	Glutamine-binding protein / Glutamine transport system permease protein glnP	-	-	1.82	0.00	3.19	0.00
Lreu_0099	Glutamine transport ATP-binding protein glnQ	-	-	1.31	0.02	1.85	0.03
Lreu_0102	Inosine-uridine preferring nucleoside hydrolase (EC 3.2.2.1)	-1.33	0.00	-	-	-	-
Lreu_0103	3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157)	-2.72	0.00	-0.63	0.03	-	-
Lreu_0104	Methyltransferase gidB (EC 2.1.-.-)	-	-	1.36	0.00	1.47	0.03

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_0106	Chromosome partitioning protein parA	-	-	1.04	0.00	1.07	0.02
Lreu_0107	Stage 0 sporulation protein J	-	-	0.95	0.01	-	-
Lreu_0108	Hypothetical cytosolic protein	-	-	1.95	0.00	2.22	0.02
Lreu_0111	Deoxyribose-phosphate aldolase (EC 4.1.2.4)	-3.17	0.00	2.40	0.00	2.74	0.00
Lreu_0112	Phosphopentomutase (EC 5.4.2.7)	-3.26	0.00	2.17	0.00	2.57	0.00
Lreu_0113	Pyrimidine-nucleoside phosphorylase (EC 2.4.2.2)	-	-	-	-	1.95	0.03
Lreu_0114	Purine nucleoside phosphorylase II (EC 2.4.2.1)	-3.15	0.00	2.25	0.00	2.46	0.00
Lreu_0119	Branched-chain amino acid transport protein azlC	-	-	1.52	0.00	1.91	0.01
Lreu_0123	Aspartate carbamoyltransferase (EC 2.1.3.2)	1.61	0.00	-2.51	0.00	-5.74	0.00
Lreu_0124	Dihydroorotase (EC 3.5.2.3)	1.54	0.00	-3.07	0.00	-6.19	0.00
Lreu_0125	Dihydroorotate dehydrogenase, catalytic subunit (EC 1.3.3.1)	1.47	0.01	-2.90	0.00	-6.07	0.00
Lreu_0126	Orotidine 5'-phosphate decarboxylase (EC 4.1.1.23)	1.28	0.01	-3.22	0.00	-6.37	0.00
Lreu_0127	Orotate phosphoribosyltransferase (EC 2.4.2.10)	1.31	0.01	-2.75	0.00	-5.80	0.00
Lreu_0129	Cyclopropane-fatty-acyl-phospholipid synthase (EC 2.1.1.79)	-	-	0.68	0.01	-	-
Lreu_0133	Alpha-acetolactate decarboxylase (EC 4.1.1.5)	0.92	0.00	1.33	0.00	-	-
Lreu_0137	Phosphoribosylamidoimidazole-succinocarboxamide synthase (EC 6.3.2.6)	-	-	2.45	0.00	2.88	0.00
Lreu_0138	Phosphoribosylformylglycinamidine synthase, purS component (EC 6.3.5.3)	-	-	2.97	0.00	3.66	0.00
Lreu_0139	Phosphoribosylformylglycinamidine synthase (EC 6.3.5.3)	-	-	2.12	0.00	2.52	0.00
Lreu_0140	Phosphoribosylformylglycinamidine synthase (EC 6.3.5.3)	-	-	2.28	0.00	2.91	0.00
Lreu_0141	Amidophosphoribosyltransferase (EC 2.4.2.14)	-0.59	0.02	2.66	0.00	3.40	0.00
Lreu_0142	Phosphoribosylformylglycinamidine cyclo-ligase (EC 6.3.3.1)	-	-	1.90	0.00	2.65	0.00
Lreu_0143	Phosphoribosylglycinamide formyltransferase (EC 2.1.2.2)	-	-	1.50	0.00	2.15	0.00
Lreu_0144	Phosphoribosylaminoimidazolecarboxamide formyltransferase (EC 2.1.2.3) / IMP cyclohydrolase (EC 3.5.4.10)	-0.61	0.02	1.17	0.00	1.88	0.00
Lreu_0146	Phosphoglycerate mutase (EC 5.4.2.1)	-	-	0.89	0.00	1.00	0.02
Lreu_0156	Pyrazinamidase (EC 3.5.1.-) / Nicotinamidase (EC 3.5.1.19)	0.59	0.03	-	-	-	-
Lreu_0157	ADP-ribosylglycohydrolase (EC 3.2.-.-)	0.71	0.02	-	-	-	-
Lreu_0158	Purine-cytosine permease	0.77	0.03	-	-	-	-
Lreu_0159	Ribokinase (EC 2.7.1.15)	0.80	0.04	-	-	-	-
Lreu_0161	Transporter, MFS superfamily	-	-	2.40	0.00	2.88	0.00
Lreu_0162	3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100)	-	-	-1.68	0.00	-1.85	0.01
Lreu_0163		-	-	-2.73	0.00	-2.82	0.00
Lreu_0164	Quinate/shikimate dehydrogenase [Pyrroloquinoline-quinone] (EC 1.1.99.25)	-	-	-0.59	0.02	-1.17	0.01
Lreu_0165	Hypothetical protein	-	-	-1.69	0.00	-2.51	0.00
Lreu_0166	Tyrosine transporter	0.94	0.05	-	-	-	-
Lreu_0169	Alpha/beta hydrolase fold protein	-	-	1.10	0.01	-	-
Lreu_0170		-1.52	0.01	1.65	0.01	-	-
Lreu_0172	Oxidoreductase (EC 1.1.1.-)	-	-	0.67	0.02	-	-
Lreu_0173	Phosphatidylglycerophosphatase B (EC 3.1.3.27)	-0.96	0.00	-	-	-	-
Lreu_0174	DNA/RNA helicase (DEAD/DEAH box family)	-0.82	0.03	-	-	-	-
Lreu_0176	Pyridine nucleotide-disulphide oxidoreductase family protein (EC 1.-.-.-)	-	-	2.14	0.00	1.84	0.01
Lreu_0177	Hypothetical membrane spanning protein	-1.09	0.02	-4.93	0.00	-5.18	0.00
Lreu_0180	Lead, cadmium, zinc and mercury transporting ATPase (EC 3.6.3.-)	-	-	-1.12	0.05	-1.23	0.04
Lreu_0181	Phosphopentomutase (EC 5.4.2.7)	-	-	-0.82	0.02	-	-

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_0183	Rrf2 family protein	-	-	1.02	0.00	-	-
Lreu_0185	Glycine betaine transport system permease protein	-	-	-0.71	0.02	-1.01	0.04
Lreu_0186	Glycine betaine transport ATP-binding protein	-	-	-	-	-0.71	0.02
Lreu_0187	Glycine betaine transport system permease protein	-	-	-0.84	0.01	-1.12	0.02
Lreu_0190	Succinyl-diaminopimelate desuccinylase (EC 3.5.1.18)	1.22	0.01	-	-	-	-
Lreu_0191	ABC transporter permease protein	1.05	0.00	-	-	-	-
Lreu_0192	ABC transporter ATP-binding protein	1.16	0.00	-	-	-	-
Lreu_0193	ABC transporter substrate-binding protein	1.32	0.00	-	-	-	-
Lreu_0194	L-2-hydroxyisocaproate dehydrogenase (EC 1.1.1.-)	0.94	0.02	1.85	0.01	-	-
Lreu_0198	Amino acid permease	0.78	0.02	3.04	0.00	2.50	0.00
Lreu_0199	4-aminobutyrate aminotransferase (EC 2.6.1.19)	0.70	0.01	2.52	0.00	1.97	0.00
Lreu_0200	Na+/H+ antiporter napA	-	-	1.07	0.03	-	-
Lreu_0202	Nucleoside diphosphate kinase (EC 2.7.4.6)	-1.54	0.00	1.76	0.00	1.39	0.01
Lreu_0203	Adenine-specific methyltransferase (EC 2.1.1.72)	-	-	1.63	0.01	-	-
Lreu_0206	3-hydroxy-3-methylglutaryl-coenzyme A reductase (EC 1.1.1.34) / 3-hydroxy-3-methylglutaryl-coenzyme A reductase (EC 1.1.1.88)	-	-	1.14	0.02	1.56	0.05
Lreu_0210	Methionyl-tRNA synthetase (EC 6.1.1.10) / Protein secretion chaperonin CsaA	-	-	2.63	0.00	2.70	0.00
Lreu_0211	DNase, TatD family (EC 3.1.-.-)	-	-	2.16	0.00	2.12	0.00
Lreu_0212	Ribonuclease M5 (EC 3.1.26.8)	-	-	1.68	0.00	1.74	0.01
Lreu_0213	Dimethyladenosine transferase (EC 2.1.1.-)	-	-	1.94	0.00	2.04	0.00
Lreu_0214	VEG protein	-	-	1.53	0.00	1.49	0.02
Lreu_0219	Pur operon repressor	-	-	2.70	0.00	2.80	0.00
Lreu_0220	Glucosamine-1-phosphate acetyltransferase (EC 2.3.1.157) / UDP-N-acetylglucosamine pyrophosphorylase (EC 2.7.7.23)	-	-	2.34	0.00	2.50	0.00
Lreu_0221	Ribose-phosphate pyrophosphokinase (EC 2.7.6.1)	-	-	-0.72	0.00	-0.76	0.03
Lreu_0223	Hydrolase (HAD superfamily)	-	-	-2.60	0.00	-2.54	0.00
Lreu_0224	dGTP triphosphohydrolase	-	-	-2.69	0.00	-2.68	0.00
Lreu_0225	Lipoate-protein ligase A (EC 6.3.2.-)	-	-	-2.19	0.00	-2.39	0.00
Lreu_0226	Transposase	-	-	-0.99	0.00	-1.27	0.02
Lreu_0227	Transposase	-	-	-1.33	0.01	-1.41	0.02
Lreu_0229	DNA-directed RNA polymerase delta chain (EC 2.7.7.6)	0.79	0.01	-	-	-	-
Lreu_0232	UDP-N-acetylglucosamine 1-carboxyvinyltransferase (EC 2.5.1.7)	-	-	1.78	0.00	1.88	0.04
Lreu_0233	LSU ribosomal protein L31P	-	-	1.95	0.00	1.78	0.02
Lreu_0238	Fructose-bisphosphate aldolase (EC 4.1.2.13)	-	-	3.73	0.00	3.96	0.00
Lreu_0239	Phosphoglycerate mutase family protein	-	-	3.69	0.00	3.67	0.00
Lreu_0240	Protein tyrosine phosphatase (EC 3.1.3.48)	-	-	-1.80	0.00	-2.54	0.00
Lreu_0241	LemA protein	-	-	-	-	-0.66	0.02
Lreu_0242	Endopeptidase htpX (EC 3.4.24.-)	-	-	-0.85	0.00	-1.01	0.02
Lreu_0252	D-alanine-activating enzyme (EC 6.3.2.-)	-	-	1.08	0.00	0.98	0.02
Lreu_0253	Hypothetical protein	-	-	1.49	0.00	1.43	0.00
Lreu_0254	Holo-[acyl-carrier protein] synthase (EC 2.7.8.7)	-	-	0.61	0.03	-	-
Lreu_0259	2-oxo-hepta-3-ene-1,7-dioic acid hydratase (EC 4.2.-.-)	0.90	0.04	0.92	0.04	-	-
Lreu_0260	CBS domain containing protein	-	-	1.34	0.02	-	-
Lreu_0263	Heat shock protein 15	-	-	-	-	0.85	0.03

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_0264	Cell division protein DIVIC	-	-	-0.99	0.00	-	-
Lreu_0268	Cell division protein ftsH (EC 3.4.24.-)	-	-	1.30	0.00	1.37	0.01
Lreu_0276		-	-	-1.69	0.00	-1.67	0.02
Lreu_0282	Aspartate racemase (EC 5.1.1.13)	-	-	1.08	0.05	-	-
Lreu_0283	Transcriptional regulator, GntR family	-	-	0.72	0.03	-	-
Lreu_0284	Nicotinate phosphoribosyltransferase (EC 2.4.2.11)	-	-	1.51	0.03	1.57	0.05
Lreu_0285	NH(3)-dependent NAD(+) synthetase (EC 6.3.5.1)	-	-	1.44	0.03	-	-
Lreu_0286	Transcription accessory protein (S1 RNA binding domain)	-	-	1.00	0.00	1.48	0.02
Lreu_0288	Beta-galactosidase large subunit (EC 3.2.1.23)	-2.82	0.00	-	-	0.86	0.05
Lreu_0289	Beta-galactosidase small subunit (EC 3.2.1.23)	-2.75	0.00	-	-	-	-
Lreu_0293	Cystathionine beta-lyase (EC 4.4.1.8) / Cystathionine gamma-lyase (EC 4.4.1.1)	-	-	1.77	0.00	1.99	0.00
Lreu_0294	Cystine transport system permease protein	-	-	2.55	0.00	3.04	0.00
Lreu_0295	Cystine transport ATP-binding protein	-1.05	0.04	2.25	0.00	-	-
Lreu_0296	Cystine-binding protein	-	-	1.65	0.01	-	-
Lreu_0297	Aminopeptidase C (EC 3.4.22.40)	-	-	-0.95	0.00	-0.94	0.05
Lreu_0298	Ribose 5-phosphate isomerase (EC 5.3.1.6)	-	-	-1.19	0.00	-1.19	0.02
Lreu_0299	Acetyltransferase (EC 2.3.1.-)	-	-	-1.20	0.00	-1.29	0.01
Lreu_0303	Cysteinyl-tRNA synthetase (EC 6.1.1.16)	-	-	0.99	0.00	-	-
Lreu_0304	Hypothetical protein	-	-	0.68	0.01	-	-
Lreu_0313	LSU ribosomal protein L10P	-	-	0.67	0.02	1.26	0.02
Lreu_0315		-	-	-1.79	0.00	-2.32	0.01
Lreu_0317	Large-conductance mechanosensitive channel	-0.84	0.03	-0.93	0.03	-1.18	0.05
Lreu_0318	Transcriptional regulator, PadR family	-	-	-1.31	0.00	-1.10	0.04
Lreu_0319	Hypothetical protein	-	-	-1.14	0.00	-0.97	0.04
Lreu_0320		-	-	-1.16	0.01	-	-
Lreu_0321	Alcohol dehydrogenase (EC 1.1.1.1) / Acetaldehyde dehydrogenase [acetylating] (EC 1.2.1.10)	-2.01	0.00	-6.18	0.00	-7.18	0.00
Lreu_0322	Ribonucleoside-diphosphate reductase beta chain (EC 1.17.4.1)	-	-	1.20	0.00	-	-
Lreu_0324	Glutaredoxin	-	-	1.63	0.00	1.89	0.01
Lreu_0327		-	-	-0.88	0.00	-	-
Lreu_0328	Recombination protein recR	-	-	-1.01	0.00	-0.71	0.05
Lreu_0329		-	-	-1.06	0.00	-	-
Lreu_0330	Thymidylate kinase (EC 2.7.4.9)	-	-	-1.06	0.00	-	-
Lreu_0331	Hypothetical cytosolic protein	-	-	-1.04	0.02	-	-
Lreu_0332	DNA polymerase III, delta' subunit (EC 2.7.7.7)	-	-	-1.33	0.00	-0.95	0.02
Lreu_0333	Initiation-control protein	-	-	-1.25	0.00	-	-
Lreu_0334	Tetrapyrrole (Corrin/Porphyrin) methylase family protein	-	-	-1.11	0.00	-	-
Lreu_0335	Acyl-[acyl-carrier-protein] hydrolase (EC 3.1.2.14)	-	-	-1.14	0.01	-	-
Lreu_0339	UDP-glucose 4-epimerase (EC 5.1.3.2)	-	-	-0.61	0.01	-0.96	0.03
Lreu_0341	Non-proteolytic protein, peptidase family M22	-	-	0.78	0.01	-	-
Lreu_0345	Gamma-glutamyl phosphate reductase (EC 1.2.1.41)	-	-	0.64	0.02	-	-
Lreu_0346	Glutamate 5-kinase (EC 2.7.2.11)	-	-	0.93	0.05	-	-
Lreu_0347	Carbon-nitrogen hydrolase family protein	1.19	0.00	-	-	-	-
Lreu_0348	Aspartate aminotransferase (EC 2.6.1.1)	0.99	0.00	-0.79	0.05	-1.35	0.05

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_0353	10 kDa chaperonin GROES	-	-	0.85	0.00	-	-
Lreu_0354	60 kDa chaperonin GROEL	-	-	0.86	0.01	-	-
Lreu_0356	Amino acid permease	-	-	0.84	0.05	-	-
Lreu_0357	Amino acid permease	-	-	1.38	0.02	2.16	0.01
Lreu_0363	COMF operon protein 3	-	-	-1.39	0.03	-	-
Lreu_0364	Ribosome-associated factor Y	-	-	-0.78	0.00	-1.40	0.00
Lreu_0372	UTP--glucose-1-phosphate uridylyltransferase (EC 2.7.7.9)	-	-	0.91	0.00	-	-
Lreu_0373	Hypothetical protein	-1.32	0.00	-	-	-	-
Lreu_0375	Cysteine-rich domain of 2-hydroxy-acid oxidase GlcF homolog	-1.58	0.00	-1.16	0.00	-1.49	0.02
Lreu_0377	Succinyl-diaminopimelate desuccinylase (EC 3.5.1.18)	-	-	1.52	0.02	-	-
Lreu_0379	Acyl-CoA hydrolase (EC 3.1.2.20)	-	-	-	-	-0.88	0.04
Lreu_0381	Hydrolase (HAD superfamily)	1.14	0.01	-	-	-	-
Lreu_0382		-	-	-1.24	0.00	-2.47	0.00
Lreu_0387	Hypothetical membrane associated protein	-	-	0.83	0.02	-	-
Lreu_0392	Protein translocase subunit secG	-	-	1.29	0.04	-	-
Lreu_0393	Carboxylesterase (EC 3.1.1.1)	-	-	1.31	0.00	-	-
Lreu_0394	Exoribonuclease II (EC 3.1.13.1)	-	-	0.91	0.01	0.90	0.02
Lreu_0395	SsrA-binding protein	-	-	0.83	0.01	-	-
Lreu_0396		-	-	2.28	0.00	2.23	0.00
Lreu_0397	Uracil-DNA glycosylase (EC 3.2.2.-)	-1.04	0.02	1.77	0.00	2.26	0.01
Lreu_0398	Phosphate acetyltransferase (EC 2.3.1.8)	-1.22	0.00	-0.99	0.01	-1.18	0.03
Lreu_0399	ATP/GTP hydrolase	-	-	1.38	0.00	1.41	0.04
Lreu_0400	Acetyltransferase (EC 2.3.1.-)	-	-	0.72	0.04	-	-
Lreu_0401	Hypothetical cytosolic protein	-	-	1.63	0.04	-	-
Lreu_0402	DNA polymerase III, epsilon chain (EC 2.7.7.7)	-	-	1.83	0.00	1.58	0.03
Lreu_0415	Peptidoglycan binding protein	-	-	3.00	0.03	-	-
Lreu_0417	Two-component sensor kinase yclK (EC 2.7.3.-)	-	-	0.76	0.04	-	-
Lreu_0418	Glucose uptake protein homolog	-1.20	0.00	1.76	0.00	2.16	0.00
Lreu_0419	O-acetyltransferase (EC 2.3.1.-)	-1.90	0.00	1.40	0.01	-	-
Lreu_0420	Glucose-6-phosphate isomerase (EC 5.3.1.9)	-0.96	0.00	-	-	-	-
Lreu_0425	Ornithine carbamoyltransferase (EC 2.1.3.3)	1.21	0.00	-	-	-	-
Lreu_0426	Carbamate kinase (EC 2.7.2.2)	1.29	0.00	-	-	-	-
Lreu_0429	Copper-transporting ATPase (EC 3.6.3.10)	-	-	-1.54	0.00	-1.90	0.00
Lreu_0430	Copper-transporting ATPase (EC 3.6.3.10)	-	-	-1.54	0.00	-1.79	0.01
Lreu_0431	Copper-exporting ATPase (EC 3.6.3.4)	-	-	-0.94	0.00	-1.19	0.01
Lreu_0432		-0.65	0.01	-0.93	0.00	-1.41	0.01
Lreu_0438		-	-	-1.38	0.02	-	-
Lreu_0439		-	-	-1.12	0.01	-1.28	0.05
Lreu_0441	Transposase	-	-	-0.66	0.02	-	-
Lreu_0442	Transposase	-	-	-1.42	0.01	-	-
Lreu_0443		-	-	-5.03	0.00	-5.08	0.00
Lreu_0445	Arginine deiminase (EC 3.5.3.6)	-	-	-0.80	0.00	-0.72	0.01
Lreu_0447	Arginine/ornithine antiporter	-0.89	0.05	-1.29	0.00	-1.23	0.02

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_0448	Arginine/ornithine antiporter	-0.93	0.03	-	-	-	-
Lreu_0455	Serine hydroxymethyltransferase (EC 2.1.2.1)	-	-	-0.90	0.04	-0.86	0.01
Lreu_0456	Uracil phosphoribosyltransferase (EC 2.4.2.9)	-	-	-	-	-1.49	0.02
Lreu_0460	Uracil permease	1.06	0.02	-1.53	0.00	-3.17	0.00
Lreu_0461	ATP synthase A chain (EC 3.6.3.14)	-	-	-0.84	0.01	-	-
Lreu_0462	ATP synthase C chain (EC 3.6.3.14)	-	-	-0.95	0.00	-1.24	0.01
Lreu_0463	ATP synthase B chain (EC 3.6.3.14)	-	-	-0.98	0.00	-1.26	0.02
Lreu_0464	ATP synthase delta chain (EC 3.6.3.14)	-	-	-0.99	0.00	-1.31	0.01
Lreu_0465	ATP synthase alpha chain (EC 3.6.3.14)	-	-	-0.99	0.00	-1.25	0.02
Lreu_0466	ATP synthase gamma chain (EC 3.6.3.14)	-	-	-0.99	0.00	-1.22	0.01
Lreu_0467	ATP synthase beta chain (EC 3.6.3.14)	-	-	-1.02	0.00	-1.30	0.01
Lreu_0468	ATP synthase epsilon chain (EC 3.6.3.14)	-	-	-0.69	0.03	-1.26	0.01
Lreu_0473		-	-	-0.63	0.04	-	-
Lreu_0474	Rod shape-determining protein rodA	-	-	-0.73	0.03	-	-
Lreu_0477	D-alanine--D-alanine ligase (EC 6.3.2.4)	-	-	-	-	1.47	0.03
Lreu_0479	Arabinose-proton symporter	-3.21	0.01	-1.62	0.01	-	-
Lreu_0480	L-ribulokinase (EC 2.7.1.16)	-4.12	0.00	-1.68	0.02	-	-
Lreu_0481	L-ribulose-5-phosphate 4-epimerase (EC 5.1.3.4)	-3.36	0.00	-1.67	0.00	-	-
Lreu_0482	L-arabinose isomerase (EC 5.3.1.4)	-2.96	0.00	-1.82	0.00	-	-
Lreu_0483	Bacterial transferase family (hexapeptide motif)	-	-	-1.43	0.03	-	-
Lreu_0486	Universal stress protein family	-	-	-2.08	0.00	-2.91	0.00
Lreu_0492	Integral membrane protein	-	-	-1.45	0.05	-	-
Lreu_0503	Thiamine biosynthesis protein thiI	-	-	-	-	1.22	0.04
Lreu_0505	Cytochrome d ubiquinol oxidase subunit I (EC 1.10.3.-)	-0.66	0.01	-2.40	0.00	-2.48	0.00
Lreu_0506	Cytochrome d ubiquinol oxidase subunit II (EC 1.10.3.-)	-0.72	0.01	-2.31	0.00	-2.38	0.00
Lreu_0507	Transport ATP-binding protein cydD	-	-	-1.38	0.02	-	-
Lreu_0508	Transport ATP-binding protein cydC	-0.59	0.03	-1.36	0.00	-0.77	0.03
Lreu_0510	Folylpolyglutamate synthase (EC 6.3.2.17) / Dihydrofolate synthase (EC 6.3.2.12)	0.97	0.00	-	-	-	-
Lreu_0511	DNA repair protein radC	0.90	0.01	-	-	-	-
Lreu_0513	Rod shape-determining protein mreC	-	-	1.05	0.01	1.28	0.04
Lreu_0514	Rod shape-determining protein mreD	-	-	-	-	0.91	0.03
Lreu_0515	Histidine transport system permease protein hisM	1.40	0.01	-	-	-	-
Lreu_0517	Histidine-binding protein	1.43	0.02	-	-	-	-
Lreu_0520	Transcriptional regulator	-	-	0.80	0.01	1.27	0.02
Lreu_0524	Hydrolase (HAD superfamily)	-	-	0.94	0.03	-	-
Lreu_0529	Queuine tRNA-ribosyltransferase (EC 2.4.2.29)	-	-	-	-	0.89	0.05
Lreu_0530	Protein translocase subunit YajC	-	-	1.22	0.00	1.33	0.01
Lreu_0533	Alanyl-tRNA synthetase (EC 6.1.1.7)	-	-	1.55	0.00	1.63	0.02
Lreu_0534	Hypothetical cytosolic protein	-	-	1.75	0.00	1.56	0.00
Lreu_0535	Endonuclease involved in recombination	-	-	2.12	0.00	1.86	0.00
Lreu_0536	Hypothetical cytosolic protein	-	-	2.42	0.00	2.05	0.00
Lreu_0537	CvpA family membrane protein	-	-	-	-	0.80	0.05
Lreu_0539	Thioredoxin	-	-	0.67	0.04	-	-

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_0542	Xanthosine triphosphate pyrophosphatase (EC 3.6.1.-)	-	-	2.00	0.00	2.33	0.01
Lreu_0543	Phosphoesterase family protein	-	-	1.65	0.01	2.24	0.02
Lreu_0544	CBS domain containing protein	-	-	1.35	0.01	1.84	0.02
Lreu_0548		-	-	-0.75	0.01	-0.95	0.04
Lreu_0550	Catabolite control protein A	-	-	0.79	0.01	1.51	0.01
Lreu_0551		-	-	-	-	0.93	0.05
Lreu_0553	ComG operon protein 1	-	-	-1.10	0.04	-	-
Lreu_0558	Hypothetical protein	-	-	-1.63	0.05	-	-
Lreu_0559	Adenine-specific methyltransferase (EC 2.1.1.72)	-	-	1.02	0.00	0.93	0.03
Lreu_0560	Acetate kinase (EC 2.7.2.1)	-	-	0.70	0.01	-	-
Lreu_0561	Phosphohydrolase	-	-	1.79	0.04	-	-
Lreu_0562	Hypothetical cytosolic protein	-	-	1.62	0.00	1.49	0.05
Lreu_0568	Negative regulator of genetic competence mecA	-	-	-1.19	0.00	-1.51	0.01
Lreu_0569	Putative competence protein/transcription factor	1.09	0.02	-	-	-	-
Lreu_0584	Cell division protein mraZ	-	-	2.76	0.00	3.07	0.00
Lreu_0585	S-adenosyl-methyltransferase mraW (EC 2.1.1.-)	-	-	2.18	0.00	2.56	0.00
Lreu_0586	Cell division protein ftsL	-	-	2.37	0.00	2.81	0.00
Lreu_0588	Phospho-N-acetylmuramoyl-pentapeptide-transferase (EC 2.7.8.13)	-	-	-	-	1.25	0.04
Lreu_0591	Cell-division initiation protein DivIB	-	-	1.04	0.03	1.68	0.03
Lreu_0592	Cell division protein ftsA	-	-	1.91	0.00	1.85	0.00
Lreu_0594	Hypothetical cytosolic protein	-	-	1.69	0.00	1.77	0.00
Lreu_0595	Integral membrane protein	-	-	1.40	0.00	1.55	0.02
Lreu_0596	RNA binding protein	-	-	0.98	0.00	1.09	0.02
Lreu_0597	Cell division initiation protein DivIVA	-	-	1.33	0.00	1.31	0.01
Lreu_0599	Cold shock protein	-	-	0.70	0.03	-	-
Lreu_0600	ADP-ribose pyrophosphatase (EC 3.6.1.13)	-	-	1.22	0.00	1.03	0.03
Lreu_0601		-	-	0.97	0.01	-	-
Lreu_0602	5'-methylthioadenosine nucleosidase (EC 3.2.2.16) / S-adenosylhomocysteine nucleosidase (EC 3.2.2.9)	-	-	0.84	0.01	-	-
Lreu_0605	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase (EC 2.1.1.61)	-	-	1.49	0.00	1.36	0.02
Lreu_0606	Phosphoglycerate mutase family protein	-	-	1.21	0.00	-	-
Lreu_0607	Tetratricopeptide repeat family protein	-	-	1.08	0.00	-	-
Lreu_0610	Diaminopimelate epimerase (EC 5.1.1.7)	1.10	0.01	1.69	0.00	1.21	0.04
Lreu_0611	Aspartokinase (EC 2.7.2.4)	1.02	0.01	-	-	-	-
Lreu_0612	Diaminopimelate decarboxylase (EC 4.1.1.20)	1.36	0.00	-	-	-	-
Lreu_0613	Tetrahydrodipicolinate N-acetyltransferase (EC 2.3.1.89)	1.25	0.01	-0.69	0.01	-	-
Lreu_0614	Putative N-acetyldiaminopimelate deacetylase (EC 3.5.1.47)	1.22	0.01	-0.82	0.02	-	-
Lreu_0615	Dihydrodipicolinate synthase (EC 4.2.1.52)	1.19	0.00	-1.09	0.00	-	-
Lreu_0616	Dihydrodipicolinate reductase (EC 1.3.1.26)	1.06	0.05	-0.83	0.05	-	-
Lreu_0617	Aromatic amino acid aminotransferase (EC 2.6.1.57) / Acetyldiaminopimelate aminotransferase (EC 2.6.1.-)	1.36	0.00	-0.99	0.03	-	-
Lreu_0618	Aspartate-semialdehyde dehydrogenase (EC 1.2.1.11)	-	-	-0.63	0.02	-	-
Lreu_0622	Glycerate kinase (EC 2.7.1.31)	-	-	-1.50	0.00	-1.58	0.02
Lreu_0625	Cell wall-associated glycosyl hydrolase	-0.89	0.01	-	-	-	-
Lreu_0629	Hypothetical exported protein	-	-	-2.37	0.00	-3.13	0.01

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_0631	Pyruvate dehydrogenase E1 component alpha subunit (EC 1.2.4.1)	-3.17	0.00	-	-	-	-
Lreu_0632	Pyruvate dehydrogenase E1 component beta subunit (EC 1.2.4.1)	-3.26	0.00	-	-	-	-
Lreu_0633	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.12)	-3.36	0.00	-	-	-	-
Lreu_0634	Dihydrolipoamide dehydrogenase (EC 1.8.1.4)	-3.28	0.00	-	-	-	-
Lreu_0639	Hypothetical cytosolic protein	-	-	-	-	0.60	0.03
Lreu_0640	Methyltransferase (EC 2.1.1.-)	-	-	-	-	1.77	0.04
Lreu_0642	ATP-dependent endopeptidase Lon (EC 3.4.21.53)	-	-	0.78	0.04	-	-
Lreu_0647	SSU ribosomal protein S20P	0.85	0.03	-	-	-	-
Lreu_0652	Trigger factor, ppiase (EC 5.2.1.8)	-	-	1.52	0.00	1.42	0.01
Lreu_0659	Ribonuclease Z (EC 3.1.26.11)	-	-	1.14	0.01	1.16	0.05
Lreu_0669	Phage Terminase Small Subunit	-	-	-0.91	0.05	-	-
Lreu_0674	Phage protein	-	-	-	-	-1.85	0.04
Lreu_0676	Hydroxymethylglutaryl-CoA synthase (EC 2.3.3.10)	-	-	0.61	0.04	-	-
Lreu_0677	LexA repressor (EC 3.4.21.88)	-	-	0.67	0.03	-	-
Lreu_0678	Hypothetical cytosolic protein	-	-	-1.01	0.00	-1.40	0.01
Lreu_0679	Hypothetical cytosolic protein	0.74	0.00	-0.72	0.01	-1.32	0.01
Lreu_0690	Undecaprenyl pyrophosphate synthetase (EC 2.5.1.31)	-	-	1.63	0.00	-	-
Lreu_0691	Phosphatidate cytidylyltransferase (EC 2.7.7.41)	-	-	1.73	0.00	2.00	0.01
Lreu_0692	Membrane endopeptidase, M50 family	-	-	1.43	0.00	1.85	0.01
Lreu_0693	Prolyl-tRNA synthetase (EC 6.1.1.15)	-	-	1.04	0.05	1.49	0.03
Lreu_0695	Hypothetical cytosolic protein	-	-	-0.62	0.02	-	-
Lreu_0698	LSU ribosomal protein L7AE	-	-	-0.62	0.04	-	-
Lreu_0701	tRNA pseudouridine synthase B (EC 4.2.1.70)	-	-	-0.76	0.03	-	-
Lreu_0703		1.19	0.02	-	-	-	-
Lreu_0704	Heat-inducible transcription repressor hrcA	-	-	1.86	0.00	1.35	0.02
Lreu_0705	GrpE protein	0.59	0.03	1.60	0.00	1.31	0.03
Lreu_0706	Chaperone protein dnaK	-	-	1.62	0.00	1.22	0.05
Lreu_0707	Chaperone protein dnaJ	0.60	0.03	-	-	-	-
Lreu_0710	Hypothetical protein	-	-	-0.78	0.05	-	-
Lreu_0711		-	-	-1.09	0.01	-1.43	0.02
Lreu_0712		-	-	-0.89	0.00	-1.10	0.02
Lreu_0714	Dipeptidase A (EC 3.4.13.-)	-1.01	0.03	-	-	-	-
Lreu_0715	Malate dehydrogenase (EC 1.1.1.37)	-	-	1.12	0.02	-	-
Lreu_0726	Arginine transport ATP-binding protein artP	-	-	1.90	0.00	-	-
Lreu_0727	Arginine-binding protein / Arginine transport system permease protein artQ	-5.10	0.00	2.34	0.00	-	-
Lreu_0731	Argininosuccinate synthase (EC 6.3.4.5)	-6.32	0.00	1.18	0.02	-	-
Lreu_0732	Argininosuccinate lyase (EC 4.3.2.1)	-5.98	0.00	-	-	-	-
Lreu_0735	PhoH protein	-	-	0.60	0.05	-	-
Lreu_0736	Hypothetical metal-binding protein	-	-	0.67	0.02	-	-
Lreu_0741	Glycyl-tRNA synthetase beta chain (EC 6.1.1.14)	-	-	0.64	0.05	-	-
Lreu_0742	DNA primase (EC 2.7.7.-)	-	-	1.63	0.01	2.18	0.01
Lreu_0743	RNA polymerase sigma factor rpoD	-	-	1.13	0.00	1.38	0.02
Lreu_0744	Aromatic amino acid aminotransferase (EC 2.6.1.57) / Acetyldiaminopimelate aminotransferase (EC 2.6.1.-)	-	-	1.71	0.00	1.62	0.01

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_0749		0.82	0.00	-	-	-	-
Lreu_0750	DNA polymerase III alpha subunit (EC 2.7.7.7)	-	-	-1.74	0.00	-2.00	0.02
Lreu_0758	Riboflavin transporter	-	-	3.67	0.00	3.57	0.00
Lreu_0760	ATP-dependent DNA helicase recQ (EC 3.6.1.-)	-	-	1.07	0.03	1.30	0.04
Lreu_0761	Peptidoglycan-specific endopeptidase, M23 family	-	-	1.88	0.00	2.21	0.00
Lreu_0763	SSU ribosomal protein S1P	-	-	-	-	1.59	0.05
Lreu_0765	DNA-binding protein HU	-	-	-1.57	0.00	-2.32	0.01
Lreu_0772	Fatty acid-binding protein, DegV family	-	-	0.69	0.01	-	-
Lreu_0775	Hypothetical cytosolic protein	-	-	0.60	0.01	0.79	0.05
Lreu_0787	Transcriptional regulator	-	-	1.09	0.00	-	-
Lreu_0789	Phosphinothricin N-acetyltransferase (EC 2.3.1.-)	-	-	0.97	0.02	-	-
Lreu_0790	General stress protein, Gls24 family	-0.76	0.03	-1.48	0.00	-1.95	0.01
Lreu_0792		-	-	-1.31	0.00	-1.81	0.01
Lreu_0801	Transcriptional regulator, Cro/CI family	-	-	0.80	0.01	-	-
Lreu_0802		-	-	-1.79	0.00	-2.40	0.00
Lreu_0803	Phage antirepressor protein	-	-	-2.20	0.00	-2.94	0.00
Lreu_0806		-	-	-2.32	0.00	-2.77	0.00
Lreu_0811	RecT protein	-	-	-2.60	0.00	-3.22	0.01
Lreu_0812	Phage protein	-0.60	0.03	-2.57	0.00	-3.15	0.00
Lreu_0813	Phage replication protein	-0.62	0.04	-2.54	0.00	-3.26	0.00
Lreu_0814		-	-	-2.58	0.00	-3.35	0.00
Lreu_0815		-0.64	0.05	-2.62	0.00	-3.24	0.00
Lreu_0816		-	-	-2.52	0.00	-3.01	0.00
Lreu_0821		-	-	-2.51	0.00	-3.13	0.00
Lreu_0822		-	-	-2.30	0.00	-2.59	0.00
Lreu_0823		-	-	-2.25	0.00	-2.68	0.00
Lreu_0827		-	-	-2.42	0.00	-2.94	0.00
Lreu_0829		-	-	-2.30	0.00	-2.78	0.00
Lreu_0830		-	-	-2.02	0.00	-2.64	0.00
Lreu_0831		-	-	-1.77	0.00	-2.34	0.01
Lreu_0832		-	-	-1.68	0.00	-2.10	0.00
Lreu_0833	Terminase large subunit	-	-	-1.79	0.00	-2.32	0.00
Lreu_0834	Portal protein	-	-	-1.84	0.00	-2.49	0.00
Lreu_0835	Phage protein	-	-	-1.78	0.00	-2.38	0.00
Lreu_0836		-	-	-1.91	0.00	-2.21	0.00
Lreu_0837		-	-	-1.82	0.00	-2.58	0.00
Lreu_0838	Major capsid protein	-	-	-1.77	0.00	-2.46	0.00
Lreu_0839	Phage protein	-	-	-1.87	0.00	-2.73	0.00
Lreu_0840		-	-	-1.86	0.00	-2.64	0.00
Lreu_0841	Phage protein	-	-	-1.90	0.00	-2.61	0.00
Lreu_0842		-	-	-2.08	0.00	-2.83	0.00
Lreu_0843	Phage protein	-	-	-1.91	0.00	-2.78	0.00
Lreu_0844	Phage protein	-	-	-1.83	0.00	-2.76	0.00

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_0845		-	-	-1.98	0.00	-2.74	0.00
Lreu_0846	Phage protein	-	-	-1.75	0.00	-2.37	0.00
Lreu_0847		-	-	-1.73	0.00	-2.40	0.00
Lreu_0848	Phage protein	-	-	-1.62	0.00	-2.35	0.00
Lreu_0849	Phage protein	-	-	-1.25	0.00	-1.89	0.00
Lreu_0850		-	-	-1.80	0.00	-2.41	0.00
Lreu_0851		-	-	-1.98	0.00	-2.67	0.00
Lreu_0852		-	-	-1.66	0.03	-	-
Lreu_0854		-	-	-1.83	0.00	-2.60	0.00
Lreu_0856		-	-	-1.25	0.00	-2.09	0.00
Lreu_0857		-	-	-1.68	0.01	-2.34	0.01
Lreu_0864		-	-	-1.06	0.00	-1.05	0.03
Lreu_0866	Amidase family protein	-1.53	0.00	1.87	0.01	2.13	0.03
Lreu_0867		-	-	-0.89	0.00	-1.43	0.01
Lreu_0868	Integral membrane protein	-	-	-1.59	0.00	-2.23	0.00
Lreu_0877	Inosine-uridine preferring nucleoside hydrolase (EC 3.2.2.1)	-1.95	0.00	-	-	-	-
Lreu_0878	Diaminohydroxyphosphoribosylaminopyrimidine deaminase (EC 3.5.4.26) / 5-amino-6-(5-phosphoribosylamino)uracil reductase (EC 1.1.1.193)	-	-	4.36	0.00	4.89	0.00
Lreu_0879	Riboflavin synthase alpha chain (EC 2.5.1.9)	-	-	3.25	0.00	3.70	0.00
Lreu_0880	GTP cyclohydrolase II (EC 3.5.4.25) / 3,4-dihydroxy-2-butanone-4-phosphate synthase (EC 4.1.2.-)	-	-	3.96	0.00	4.46	0.00
Lreu_0885	RNA polymerase ECF-type sigma factor	-	-	1.59	0.00	-	-
Lreu_0889		-	-	-2.37	0.00	-2.63	0.00
Lreu_0890	Ferrous iron transport protein B	-	-	-2.31	0.00	-2.23	0.02
Lreu_0891	ATP-dependent transporter sufC	-1.22	0.00	-1.24	0.00	-1.02	0.02
Lreu_0892	SufD protein	-1.28	0.00	-	-	-	-
Lreu_0893	Cysteine desulfurase (EC 2.8.1.7) / Selenocysteine lyase (EC 4.4.1.16)	-1.27	0.00	-0.75	0.02	-	-
Lreu_0894	IscU protein	-1.32	0.00	-0.72	0.05	-	-
Lreu_0895	ABC transporter-associated protein sufB	-1.15	0.03	-	-	-	-
Lreu_0896	Hypothetical cytosolic protein	-0.62	0.01	-0.84	0.05	-	-
Lreu_0897	Transcriptional regulator, Xre family	-0.72	0.01	-	-	-	-
Lreu_0898	Two-component response regulator	-	-	-0.84	0.01	-	-
Lreu_0910	Alpha-galactosidase (EC 3.2.1.22)	-3.11	0.00	-	-	-	-
Lreu_0920	Multimodular transpeptidase-transglycosylase PBP 1A	-	-	0.95	0.01	0.99	0.05
Lreu_0922	Hypothetical cytosolic protein	-	-	-1.41	0.00	-1.51	0.01
Lreu_0923	Cell division initiation protein DivIVA	-	-	0.96	0.00	0.87	0.05
Lreu_0926	Ribonuclease HI (EC 3.1.26.4) / Cell wall enzyme EBSB	-	-	-1.33	0.01	-1.39	0.04
Lreu_0931	Carbamoyl-phosphate synthase large chain (EC 6.3.5.5)	-0.67	0.02	-0.94	0.02	-	-
Lreu_0932	Fibronectin-binding protein / Fibrinogen-binding protein	-	-	1.21	0.01	1.43	0.04
Lreu_0933	Fatty acid-binding protein, DegV family	-	-	1.25	0.01	1.35	0.02
Lreu_0936	Lipopolysaccharide 1,2-glucosyltransferase (EC 2.4.1.58)	-	-	-0.99	0.01	-1.13	0.05
Lreu_0940	Hypothetical protein	0.67	0.03	-	-	-	-
Lreu_0941	ACT domain-containing protein	0.91	0.02	1.51	0.00	1.52	0.03
Lreu_0950	2-polyprenylphenol 6-hydroxylase accessory protein ubiB	-	-	-1.89	0.00	-1.88	0.01

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_0951	EpiH/GdmH-related protein	-	-	-1.84	0.00	-1.81	0.02
Lreu_0952	Lactoylglutathione lyase family protein	-	-	0.74	0.02	-	-
Lreu_0954	CrcB family protein	-	-	-0.83	0.00	-0.78	0.03
Lreu_0959		-	-	-0.77	0.02	-	-
Lreu_0968	putative acyltransferases and hydrolases with the alpha/beta hydrolase fold	-	-	-0.98	0.02	-	-
Lreu_0970	nucleoside deoxyribosyltransferase (EC 2.4.2.6)	-	-	-0.98	0.00	-1.35	0.01
Lreu_0972	Hypothetical protein	-	-	0.96	0.01	-	-
Lreu_0978	Lipase (EC 3.1.1.3)	-	-	-1.39	0.01	-	-
Lreu_0981	Enoyl-[acyl-carrier protein] reductase (NADH) (EC 1.3.1.9)	-	-	-0.78	0.05	-	-
Lreu_0991	3-oxoacyl-[acyl-carrier-protein] synthase III (EC 2.3.1.41)	-	-	0.68	0.05	-	-
Lreu_0992	Transcriptional regulator, MarR family	-	-	0.91	0.01	-	-
Lreu_0999	Transporter, Drug/Metabolite Exporter family	-	-	-2.34	0.00	-2.61	0.00
Lreu_1003	Transposase	-	-	-0.59	0.03	-	-
Lreu_1005		-	-	-3.61	0.00	-3.55	0.00
Lreu_1006		-	-	-3.04	0.00	-2.88	0.00
Lreu_1007	Transcription regulator, crp family	-	-	1.13	0.00	1.10	0.02
Lreu_1008		-	-	0.87	0.00	1.24	0.01
Lreu_1017	Alpha-glucosidase (EC 3.2.1.20)	-1.80	0.00	-	-	-	-
Lreu_1018	Phosphatidylglycerophosphatase B homolog	-	-	-1.06	0.00	-	-
Lreu_1029	5'-nucleotidase (EC 3.1.3.5)	-1.55	0.00	1.66	0.02	1.49	0.01
Lreu_1030		-	-	-2.35	0.00	-3.02	0.01
Lreu_1033	Lysozyme (EC 3.2.1.17)	-	-	-1.03	0.01	-	-
Lreu_1034	Phosphotyrosine-protein phosphatase (capsular polysaccharide biosynthesis) (EC 3.1.3.48)	-	-	-0.96	0.00	-	-
Lreu_1035	SWF/SNF family helicase	-	-	-1.35	0.00	-	-
Lreu_1036	SWF/SNF family helicase	-	-	-0.92	0.01	-	-
Lreu_1058		-	-	-1.63	0.00	-2.15	0.00
Lreu_1061	Transposase	-	-	-0.99	0.00	-1.27	0.02
Lreu_1078	Bis(5'-nucleosyl)-tetraphosphatase (symmetrical) (EC 3.6.1.41)	-	-	-1.59	0.00	-1.81	0.00
Lreu_1081	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase (EC 2.1.1.14)	1.37	0.03	-	-	-	-
Lreu_1082	Polyketide cyclase family protein	1.34	0.03	-	-	-	-
Lreu_1084	Farnesyl pyrophosphate synthetase (EC 2.5.1.1) / Geranyltranstransferase (EC 2.5.1.10) / Farnesyltransferase (EC 2.5.1.29) / Heptaprenyl diphosphate synthase component II (EC 2.5.1.30)	-	-	0.82	0.02	-	-
Lreu_1085	Beta-galactosidase (EC 3.2.1.23)	-	-	-1.27	0.01	-	-
Lreu_1086	Lactose permease	-1.65	0.01	-1.33	0.02	-	-
Lreu_1087	Transcriptional regulator, LacI family	-	-	0.59	0.04	-	-
Lreu_1090	Exodeoxyribonuclease III (EC 3.1.11.2)	-	-	0.92	0.02	-	-
Lreu_1091	Transcriptional regulator, Cro/CI family	-	-	0.88	0.04	-	-
Lreu_1092		-	-	-	-	-1.39	0.01
Lreu_1095		-	-	-2.62	0.00	-3.16	0.02
Lreu_1096		-	-	-2.33	0.00	-2.80	0.00
Lreu_1097		-	-	-2.03	0.00	-2.73	0.00
Lreu_1098		-	-	-1.25	0.00	-2.09	0.00
Lreu_1099		-	-	-1.83	0.00	-2.60	0.00

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_1100		-	-	-1.76	0.00	-2.46	0.00
Lreu_1101		-	-	-1.66	0.03	-	-
Lreu_1102		-	-	-1.98	0.00	-2.67	0.00
Lreu_1103		-	-	-1.92	0.00	-2.59	0.00
Lreu_1104	Phage protein	-	-	-1.97	0.00	-2.64	0.00
Lreu_1105		-	-	-1.99	0.00	-2.70	0.03
Lreu_1106	Phage protein	-0.64	0.05	-2.31	0.00	-2.84	0.01
Lreu_1107	Phage protein	-0.97	0.03	-2.10	0.02	-	-
Lreu_1108		-	-	-2.65	0.00	-3.12	0.01
Lreu_1109	Phage tail protein	-0.71	0.02	-2.65	0.00	-3.54	0.00
Lreu_1110	Phage tail protein	-0.73	0.01	-2.67	0.00	-3.68	0.00
Lreu_1111	Phage protein	-0.65	0.03	-2.27	0.00	-2.96	0.00
Lreu_1112		-0.80	0.01	-2.63	0.00	-3.47	0.00
Lreu_1113	Phage protein	-0.79	0.01	-2.58	0.00	-3.46	0.00
Lreu_1114	Phage protein	-0.86	0.01	-2.70	0.00	-3.61	0.00
Lreu_1115	ATP-dependent endopeptidase clp proteolytic subunit clpP (EC 3.4.21.92)	-0.95	0.01	-2.75	0.00	-3.72	0.00
Lreu_1116	Portal protein	-0.81	0.03	-3.09	0.00	-3.69	0.00
Lreu_1117	Terminase large subunit	-0.71	0.03	-2.63	0.00	-3.21	0.00
Lreu_1118		-	-	-2.57	0.00	-2.88	0.00
Lreu_1119		-	-	-2.02	0.02	-	-
Lreu_1120	Terminase small subunit	-0.82	0.02	-2.52	0.00	-3.21	0.00
Lreu_1121	Phage endonuclease	-	-	-2.42	0.00	-2.81	0.01
Lreu_1122		-	-	-1.89	0.00	-2.24	0.00
Lreu_1124		-	-	-1.63	0.00	-2.04	0.00
Lreu_1127		-	-	-2.35	0.00	-2.75	0.00
Lreu_1138	Phage protein	-0.60	0.03	-2.57	0.00	-3.15	0.00
Lreu_1139	RecT protein	-	-	-2.71	0.00	-3.08	0.01
Lreu_1140		-0.98	0.04	-3.38	0.00	-3.84	0.00
Lreu_1141		-	-	-1.43	0.01	-1.53	0.03
Lreu_1142	Phage antirepressor protein	-	-	-2.29	0.00	-3.05	0.00
Lreu_1143		-	-	-2.92	0.00	-3.80	0.00
Lreu_1144		-	-	-1.36	0.00	-1.71	0.00
Lreu_1145		-	-	-1.35	0.00	-1.60	0.01
Lreu_1175	DNA-directed RNA polymerase omega chain (EC 2.7.7.6)	-	-	1.05	0.01	-	-
Lreu_1176	Guanylate kinase (EC 2.7.4.8)	-	-	1.63	0.00	1.19	0.01
Lreu_1184	Methylenetetrahydrofolate dehydrogenase (NADP+) (EC 1.5.1.5) / Methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9)	-	-	-0.72	0.03	-	-
Lreu_1185	N utilization substance protein B	-	-	1.54	0.00	1.35	0.02
Lreu_1186	General stress protein, Gls24 family	-	-	1.16	0.00	-	-
Lreu_1191	hypothetical ribosome-associated protein	-	-	0.77	0.02	-	-
Lreu_1192	LSU ribosomal protein L21P	-	-	0.64	0.01	0.92	0.03
Lreu_1193	Transcriptional regulator, TetR family	-1.01	0.02	-2.47	0.00	-2.59	0.01
Lreu_1197	Hypothetical cytosolic protein	-	-	-1.28	0.02	-	-
Lreu_1198	Peptide methionine sulfoxide reductase msrA (EC 1.8.4.11)	0.62	0.02	-1.97	0.00	-2.53	0.00

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_1199	ABC transporter substrate-binding protein	1.22	0.03	-	-	-	-
Lreu_1201	Glutamyl-tRNA synthetase (EC 6.1.1.17)	-	-	-0.68	0.01	-0.92	0.05
Lreu_1202	Glutamine synthetase (EC 6.3.1.2)	-	-	-0.66	0.04	-	-
Lreu_1220	Hydrolase (HAD superfamily)	-	-	-2.88	0.00	-3.23	0.00
Lreu_1221	23S rRNA methyltransferase (EC 2.1.1.-)	-	-	-3.34	0.00	-3.44	0.00
Lreu_1226	Glutamate/gamma-aminobutyrate antiporter	-1.27	0.00	-	-	-	-
Lreu_1227	Transmembrane histidine kinase CsrS	-	-	2.10	0.03	2.43	0.04
Lreu_1228	Response regulator CsrR	-	-	2.35	0.00	2.62	0.00
Lreu_1243	Replication initiation and membrane attachment protein	-	-	0.98	0.02	0.99	0.04
Lreu_1248	Hypothetical membrane spanning protein	-	-	-2.74	0.01	-2.81	0.04
Lreu_1249	Integral membrane protein	-	-	0.99	0.01	-	-
Lreu_1251	tRNA binding domain protein	-	-	0.68	0.01	-	-
Lreu_1252	Thioredoxin	-	-	1.07	0.00	-	-
Lreu_1255	IolS protein	-1.64	0.02	-1.73	0.00	-1.70	0.00
Lreu_1256	Poly(Glycerol-phosphate) alpha-glucosyltransferase (EC 2.4.1.52)	-1.93	0.03	-2.20	0.00	-2.14	0.01
Lreu_1265	Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8)	-	-	0.63	0.04	-	-
Lreu_1268	Hypothetical cytosolic protein	-	-	0.72	0.03	-	-
Lreu_1270	Arginyl-tRNA synthetase (EC 6.1.1.19)	-1.06	0.04	2.91	0.00	3.31	0.00
Lreu_1277	Folylpolyglutamate synthase (EC 6.3.2.17) / Dihydrofolate synthase (EC 6.3.2.12)	-	-	0.71	0.02	-	-
Lreu_1278		-	-	0.79	0.02	-	-
Lreu_1286	Ribose operon repressor	-	-	-2.69	0.00	-2.53	0.00
Lreu_1287		-	-	-2.99	0.00	-3.28	0.00
Lreu_1290	Universal stress protein family	-	-	-0.76	0.01	-1.39	0.01
Lreu_1291	Xaa-His dipeptidase (EC 3.4.13.3)	-	-	1.73	0.01	1.55	0.04
Lreu_1294	Phage protein	0.87	0.03	-	-	-	-
Lreu_1295	Leucyl-tRNA synthetase (EC 6.1.1.4)	-	-	1.26	0.00	1.01	0.03
Lreu_1296	S-adenosylmethionine synthetase (EC 2.5.1.6)	-	-	1.31	0.00	1.40	0.02
Lreu_1298	Transposase	-	-	0.61	0.01	-	-
Lreu_1299	Multidrug resistance protein B	0.88	0.02	-	-	-	-
Lreu_1307	Small heat shock protein	-0.84	0.01	-	-	-	-
Lreu_1308	Aldo/keto reductase family protein	-	-	-1.89	0.00	-2.19	0.00
Lreu_1309	Dihydropyrimidine dehydrogenase [NADP+] beta subunit (EC 1.3.1.2)	-1.63	0.00	-3.59	0.00	-3.62	0.00
Lreu_1310	Dihydropyrimidine dehydrogenase [NADP+] alpha subunit (EC 1.3.1.2)	-1.85	0.00	-2.89	0.00	-2.92	0.00
Lreu_1311	Exodeoxyribonuclease III (EC 3.1.11.2)	-	-	-	-	-0.98	0.03
Lreu_1312	peptidoglycan binding protein (LysM domain)	-	-	2.98	0.02	-	-
Lreu_1313	Hypothetical protein	-	-	1.25	0.00	-	-
Lreu_1314	peptidoglycan binding protein (LysM domain)	-	-	3.32	0.02	-	-
Lreu_1318	Multidrug resistance protein B	-	-	-1.28	0.03	-	-
Lreu_1323	1,2-diacylglycerol 3-glucosyltransferase (EC 2.4.1.157)	-	-	-	-	1.74	0.04
Lreu_1325	Phosphocarrier protein HPr	-	-	1.03	0.00	-	-
Lreu_1327	ATP-dependent endopeptidase clp ATP-binding subunit clpE	-	-	1.03	0.00	-	-
Lreu_1329	Hypothetical membrane associated protein	-	-	-0.84	0.01	-1.16	0.02
Lreu_1348	Cell wall-associated glycosyl hydrolase	-1.76	0.00	-	-	-	-

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_1353	Hypothetical membrane spanning protein	-	-	-	-	1.50	0.05
Lreu_1358	Transposase	-	-	-1.42	0.01	-	-
Lreu_1363		-	-	1.22	0.02	-	-
Lreu_1364	Cell wall-associated glycosyl hydrolase	-	-	3.03	0.00	2.50	0.00
Lreu_1365	N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	-	-	0.81	0.05	-	-
Lreu_1367	Phage-associated cell wall hydrolase	-	-	2.41	0.00	2.11	0.01
Lreu_1368	Acyltransferase	-	-	1.33	0.04	-	-
Lreu_1369	Putative galactofuranosyltransferase (EC 2.4.1.-)	-	-	1.74	0.02	2.54	0.03
Lreu_1372		-	-	1.04	0.01	1.32	0.05
Lreu_1373		-	-	1.22	0.00	1.90	0.00
Lreu_1375	Undecaprenyl-phosphate beta-glucosephosphotransferase (EC 2.7.8.-)	-	-	1.08	0.01	-	-
Lreu_1376	Bactoprenol glucosyl transferase (EC 2.4.1.-)	-	-	1.41	0.00	1.98	0.01
Lreu_1378	Regulatory protein recX	-	-	1.69	0.01	2.12	0.01
Lreu_1381	Ribonuclease BN (EC 3.1.-.-)	-	-	1.98	0.00	1.87	0.03
Lreu_1382	Glutamate/gamma-aminobutyrate antiporter	-1.24	0.02	-	-	-	-
Lreu_1383	Arginine/ornithine antiporter	-1.50	0.02	-0.91	0.03	-0.99	0.04
Lreu_1384	Pyridoxal-dependent aminotransferase, arginine catabolism	-2.17	0.00	-	-	-0.95	0.05
Lreu_1388	Hypothetical protein	-	-	-	-	-2.06	0.04
Lreu_1389	UDP-N-acetylglucosamine 2-epimerase (EC 5.1.3.14)	-	-	-0.96	0.03	-	-
Lreu_1392	Hypothetical cytosolic protein	-	-	2.96	0.00	2.73	0.01
Lreu_1394	L-2-hydroxyisocaproate dehydrogenase (EC 1.1.1.-)	-	-	1.52	0.05	-	-
Lreu_1395		-	-	1.65	0.00	1.48	0.01
Lreu_1401	Transcriptional regulator	-0.68	0.03	-2.78	0.00	-3.14	0.00
Lreu_1402	Hypothetical membrane spanning protein	-0.65	0.04	-2.96	0.00	-3.27	0.00
Lreu_1407	O6-methylguanine-DNA methyltransferase (EC 2.1.1.63)	-	-	1.06	0.03	-	-
Lreu_1408	Nitrobenzoate nitroreductase (EC 1.6.6.-)	-	-	1.23	0.00	-	-
Lreu_1409		-	-	0.92	0.01	-	-
Lreu_1410	IAA acetyltransferase (EC 2.3.1.-)	-	-	1.10	0.01	-	-
Lreu_1416	DNA/RNA helicase (DEAD/DEAH box family)	-	-	-1.60	0.00	-1.50	0.02
Lreu_1417		-	-	-1.90	0.00	-2.08	0.02
Lreu_1422	Hypothetical cytosolic protein	-	-	-1.03	0.00	-1.15	0.02
Lreu_1423		-	-	-1.20	0.00	-1.28	0.04
Lreu_1426	Non-specific DNA-binding protein Dps / Iron-binding ferritin-like antioxidant protein / Ferroxidase (EC 1.16.3.1)	-	-	-1.55	0.00	-1.75	0.02
Lreu_1427	Heavy metal binding protein	-	-	-1.71	0.00	-2.17	0.01
Lreu_1433	Type I restriction-modification system specificity subunit	-	-	-	-	1.00	0.02
Lreu_1434	Integrase/recombinase (XerC/CodV family)	-	-	0.73	0.05	-	-
Lreu_1440	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B (EC 6.3.5.-)	-	-	1.37	0.00	1.34	0.02
Lreu_1441	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit A (EC 6.3.5.-)	-	-	1.37	0.00	1.38	0.01
Lreu_1442	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit C (EC 6.3.5.-)	-	-	1.45	0.01	-	-
Lreu_1443	Lipoprotein (pheromone precursor)	-	-	1.11	0.01	-	-
Lreu_1447	Xanthine phosphoribosyltransferase (EC 2.4.2.-)	-	-	1.21	0.00	-	-
Lreu_1448	Peptidoglycan hydrolase (3.2.1.-)	-	-	1.05	0.00	0.63	0.02
Lreu_1449	(R,R)-butanediol dehydrogenase (EC 1.1.1.4) / Acetoin dehydrogenase (EC 1.1.1.5)	-	-	-	-	-1.16	0.02

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_1451	LSU ribosomal protein L13P	-	-	0.60	0.03	-	-
Lreu_1455	Cobalt transport ATP-binding protein cbiO	-	-	1.85	0.01	-	-
Lreu_1456	LSU ribosomal protein L17P	-	-	0.76	0.02	1.08	0.04
Lreu_1457	DNA-directed RNA polymerase alpha chain (EC 2.7.7.6)	-	-	0.73	0.01	1.12	0.02
Lreu_1491	DNA-directed RNA polymerase beta' chain (EC 2.7.7.6)	-	-	1.15	0.01	-	-
Lreu_1492	DNA-directed RNA polymerase beta chain (EC 2.7.7.6)	-	-	1.86	0.00	1.93	0.01
Lreu_1493	Transcriptional regulator, TetR family	-	-	-0.88	0.02	-1.13	0.05
Lreu_1496	Alcohol dehydrogenase (EC 1.1.1.1)	-0.87	0.01	-2.22	0.00	-2.95	0.00
Lreu_1498	Deoxyguanosine kinase (EC 2.7.1.113)	-	-	-	-	1.60	0.03
Lreu_1500	Lysine-specific permease	2.02	0.00	-	-	-	-
Lreu_1505	Cold shock protein	0.93	0.05	-	-	-	-
Lreu_1508	Hypothetical membrane associated protein	1.01	0.00	-	-	-	-
Lreu_1509	Hypothetical membrane associated protein	0.97	0.01	-	-	-	-
Lreu_1510	Universal stress protein family	-	-	-0.63	0.05	-1.50	0.01
Lreu_1511	Glutamine transport ATP-binding protein glnQ	-	-	1.43	0.00	-	-
Lreu_1513	Glutamine transport system permease protein glnM	-	-	1.21	0.00	1.38	0.02
Lreu_1514	Glutamine transport system permease protein glnP	-	-	0.72	0.01	-	-
Lreu_1517		-	-	-1.99	0.00	-2.11	0.00
Lreu_1518	Transcriptional regulator, MerR family	-	-	-0.93	0.03	-	-
Lreu_1525	Multidrug resistance protein B	-	-	-1.12	0.03	-	-
Lreu_1529	2-oxoglutarate/malate translocator	-	-	-3.63	0.00	-3.96	0.00
Lreu_1531	Fumarate hydratase (EC 4.2.1.2)	-1.73	0.00	-4.15	0.00	-4.31	0.00
Lreu_1534	Peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8)	0.81	0.01	-	-	-	-
Lreu_1536		-	-	-0.80	0.02	-0.93	0.05
Lreu_1538	Aldose 1-epimerase (EC 5.1.3.3)	-2.83	0.00	-	-	-	-
Lreu_1539		1.47	0.04	-	-	-	-
Lreu_1542	Sucrose phosphorylase (EC 2.4.1.7)	-	-	-2.12	0.00	-2.14	0.03
Lreu_1544	Xaa-His dipeptidase (EC 3.4.13.3)	-2.32	0.02	-	-	-	-
Lreu_1545	Arginine/ornithine antiporter	-2.71	0.00	-	-	-	-
Lreu_1546	Dyp-type peroxidase family protein	-1.73	0.00	-	-	-	-
Lreu_1547	ABC transporter ATP-binding protein uup	-1.26	0.05	-	-	-	-
Lreu_1549	Endo-1,4-beta-xylanase (EC 3.2.1.8)	-	-	-0.66	0.05	-	-
Lreu_1552	IRON-SULFUR FLAVOPROTEIN	-	-	-1.08	0.03	-	-
Lreu_1554	Ferrochelatase (EC 4.99.1.1)	-	-	1.29	0.02	-	-
Lreu_1559	Myosin-crossreactive antigen	-1.86	0.00	-1.18	0.00	-1.17	0.03
Lreu_1560	Chromate reductase (EC 1.-.-.-) / NADPH-dependent FMN reductase (EC 1.5.1.-) / Oxygen-insensitive NADPH nitroreductase (EC 1.-.-.-)	-1.66	0.00	-	-	-	-
Lreu_1574	Acetyltransferase (EC 2.3.1.-)	-	-	0.78	0.02	-	-
Lreu_1581	Protein Translation Elongation Factor P (EF-P)	-	-	1.19	0.00	1.03	0.05
Lreu_1582	regulatory protein (pfoS/R)	1.27	0.03	-	-	-	-
Lreu_1584	Transposase	-	-	-0.67	0.05	-	-
Lreu_1585	ABC transporter substrate-binding protein	0.78	0.02	-	-	-	-
Lreu_1586	Autoinducer-2 production protein luxS / Ribosylhomocysteinase (EC 4.4.1.21)	0.89	0.01	2.07	0.00	2.27	0.01
Lreu_1587		-	-	0.83	0.04	-	-

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_1588	integral membrane protein	-	-	0.98	0.02	-	-
Lreu_1593	Transposase	-	-	1.08	0.04	-	-
Lreu_1594	Hypothetical protein	-	-	-	-	-1.25	0.02
Lreu_1608	Hypothetical protein	-	-	-0.95	0.02	-	-
Lreu_1613	Neopullulanase (EC 3.2.1.135) / Cyclomaltodextrinase (EC 3.2.1.54) / Maltogenic alpha-amylase (EC 3.2.1.133)	-2.09	0.00	-	-	-	-
Lreu_1614	Sucrose transport protein	-1.95	0.00	-	-	-	-
Lreu_1617	GMP synthase [glutamine-hydrolyzing] (EC 6.3.5.2)	0.59	0.03	1.52	0.00	-	-
Lreu_1618	Hypothetical ATPase	-1.47	0.00	0.76	0.02	-	-
Lreu_1619	Pantothenate kinase (EC 2.7.1.33)	-0.78	0.01	-	-	-	-
Lreu_1622	NADH oxidase H2O-forming (EC 1.6.-.-)	-	-	-2.10	0.00	-1.80	0.01
Lreu_1623	Transposase	-	-	-1.33	0.01	-1.41	0.02
Lreu_1624	Transposase	-	-	-0.99	0.00	-1.27	0.02
Lreu_1625	ATP-dependent DNA helicase rep (EC 3.6.1.-)	-	-	0.61	0.00	0.68	0.01
Lreu_1627	Phosphoglycerate mutase (EC 5.4.2.1)	-	-	1.51	0.00	1.48	0.01
Lreu_1628	GTP-binding protein hflX	-	-	1.22	0.00	1.22	0.05
Lreu_1629	Integral membrane protein	-1.85	0.00	-	-	-	-
Lreu_1630	Hypothetical protein	-1.44	0.00	-	-	-	-
Lreu_1631	D-2-hydroxyacid dehydrogenase (EC 1.1.1.-)	-2.10	0.00	-	-	-	-
Lreu_1632	Phage infection protein	-	-	-1.03	0.05	-	-
Lreu_1640	Amino acid permease	-	-	1.98	0.00	-	-
Lreu_1646	Spermidine/putrescine transport ATP-binding protein potA	-	-	1.34	0.00	1.43	0.04
Lreu_1650	Non-specific DNA-binding protein Dps / Iron-binding ferritin-like antioxidant protein / Ferroxidase (EC 1.16.3.1)	-2.25	0.00	-	-	-	-
Lreu_1652	Phosphoglycolate phosphatase (EC 3.1.3.18)	0.67	0.04	1.33	0.01	-	-
Lreu_1657		-	-	-0.97	0.02	-1.11	0.04
Lreu_1658	Inosine-uridine preferring nucleoside hydrolase (EC 3.2.2.1)	-0.87	0.01	-	-	-	-
Lreu_1663		-	-	-1.24	0.02	-	-
Lreu_1664	Hypothetical membrane spanning protein	-	-	-2.68	0.00	-2.97	0.00
Lreu_1665	Na+/H+ antiporter nhaP	-	-	-1.56	0.00	-1.77	0.01
Lreu_1666	Hypothetical membrane spanning protein	0.81	0.04	-	-	-	-
Lreu_1677	Lipoate-protein ligase A (EC 6.3.2.-)	-2.39	0.00	-	-	-	-
Lreu_1678	Universal stress protein family	-	-	-0.92	0.00	-1.16	0.02
Lreu_1679	Translation elongation and release factors (GTPases)	-	-	-1.07	0.00	-1.14	0.03
Lreu_1681	Branched-chain amino acid transport system carrier protein	-	-	-0.63	0.05	-	-
Lreu_1684	cinnamoyl ester hydrolase (EC 3.1.1.-)	-	-	-2.07	0.00	-2.51	0.00
Lreu_1688	Transcriptional regulator	-	-	-0.83	0.00	-0.79	0.04
Lreu_1690	Multidrug/protein/lipid ABC transporter family, ATP-binding and permease protein	-	-	-1.42	0.03	-	-
Lreu_1692	D-2-hydroxyacid dehydrogenase (EC 1.1.1.-)	-1.32	0.00	-1.60	0.00	-2.16	0.00
Lreu_1695	Nicotinate-nucleotide--dimethylbenzimidazole phosphoribosyltransferase (EC 2.4.2.21)	-1.09	0.00	0.79	0.04	1.31	0.03
Lreu_1696		-1.28	0.00	1.68	0.00	2.53	0.00
Lreu_1697	Alpha-ribazole-5'-phosphate phosphatase (EC 3.1.3.73)	-0.93	0.02	1.50	0.04	2.14	0.01
Lreu_1698	Adenosylcobinamide-GDP ribazoletransferase (EC 2.7.8.26)	-0.80	0.03	1.26	0.01	1.79	0.02
Lreu_1699	Adenosylcobinamide kinase (EC 2.7.1.156) / Adenosylcobinamide-phosphate guanylyltransferase (EC 2.7.7.62)	-0.71	0.04	1.42	0.01	1.82	0.03

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_1700	Glutamate-1-semialdehyde 2,1-aminomutase (EC 5.4.3.8)	-	-	1.08	0.02	1.87	0.01
Lreu_1701	Delta-aminolevulinic acid dehydratase (EC 4.2.1.24)	-	-	1.09	0.00	1.75	0.01
Lreu_1702	Porphobilinogen deaminase (EC 2.5.1.61)	-	-	1.07	0.01	2.08	0.01
Lreu_1704	Precorrin-2 dehydrogenase (EC 1.3.1.76) / Sirohydrochlorin ferrochelatase (EC 4.99.1.4)	-	-	-	-	1.24	0.03
Lreu_1706	Cobalt transport ATP-binding protein cbiO	-	-	-	-	1.57	0.02
Lreu_1707	Cobalt transport protein cbiQ	-	-	-	-	1.66	0.01
Lreu_1708	Cobalt transport protein cbiN	-	-	-	-	1.52	0.01
Lreu_1709	CbiM protein	-	-	-	-	1.45	0.02
Lreu_1710	Precorrin-2 C20-methyltransferase (EC 2.1.1.130)	-	-	-	-	1.72	0.01
Lreu_1711	Sirohydrochlorin cobaltochelatase (EC 4.99.1.3)	-	-	-	-	1.62	0.01
Lreu_1712	Uroporphyrin-III C-methyltransferase (EC 2.1.1.107) / Uroporphyrinogen-III synthase (EC 4.2.1.75)	-	-	-	-	1.79	0.01
Lreu_1713	Precorrin-6X reductase (EC 1.3.1.54)	-	-	-	-	1.51	0.01
Lreu_1716	Precorrin-4 C11-methyltransferase (EC 2.1.1.133)	-	-	-	-	1.17	0.02
Lreu_1717	Precorrin-6Y C5,15-methyltransferase [decarboxylating] subunit CbiT (EC 2.1.1.132)	-	-	-	-	1.54	0.01
Lreu_1718	Precorrin-6Y C5,15-methyltransferase [decarboxylating] subunit CbiE (EC 2.1.1.132)	-	-	-	-	1.38	0.01
Lreu_1720	Precorrin-8X methylmutase (EC 5.4.1.2)	-	-	-	-	1.39	0.01
Lreu_1721	Adenosylcobinamide-phosphate synthase (EC 6.3.1.10)	-	-	0.80	0.01	1.57	0.01
Lreu_1722	Cobyrinic acid a,c-diamide synthase (EC 6.3.1.-)	-	-	1.25	0.00	1.89	0.00
Lreu_1723	Threonine-phosphate decarboxylase (EC 4.1.1.81)	-	-	1.45	0.00	1.86	0.01
Lreu_1726	Oxidoreductase (EC 1.1.1.-)	-	-	1.10	0.00	1.53	0.01
Lreu_1727	Flavodoxin	-1.98	0.00	0.75	0.02	1.39	0.01
Lreu_1728	Propanediol utilization protein pduV	-1.86	0.00	-	-	-	-
Lreu_1729		0.83	0.02	1.19	0.01	2.06	0.01
Lreu_1730	Phosphoglycerate mutase family protein	-	-	1.56	0.00	2.20	0.01
Lreu_1731	Protein tyrosine phosphatase (EC 3.1.3.48)	1.15	0.00	1.40	0.00	1.73	0.00
Lreu_1732	Ethanolamine utilization protein eutS	0.90	0.00	0.82	0.00	1.02	0.02
Lreu_1734	Propanol dehydrogenase (EC 1.1.1.-)	0.90	0.00	0.62	0.01	0.90	0.03
Lreu_1735	Propionaldehyde dehydrogenase [CoA-acylating] (EC 1.2.1.-)	0.81	0.00	-	-	0.82	0.04
Lreu_1736	Protein glcG	0.86	0.00	-	-	-	-
Lreu_1737	Cobalamin adenosyltransferase family protein	0.82	0.00	-	-	0.80	0.05
Lreu_1738	Ethanolamine utilization protein eutN	0.88	0.00	0.71	0.02	0.99	0.03
Lreu_1739		0.80	0.02	0.69	0.03	-	-
Lreu_1740	PduL	0.73	0.01	-	-	-	-
Lreu_1741	Propanediol utilization protein pduA	0.81	0.00	0.66	0.02	0.95	0.04
Lreu_1742		0.76	0.01	0.64	0.02	-	-
Lreu_1743		0.84	0.00	0.60	0.01	0.96	0.03
Lreu_1744	Glycerol dehydratase reactivation factor large subunit	0.74	0.01	-	-	0.84	0.03
Lreu_1745	Diol dehydratase gamma subunit (EC 4.2.1.28)	0.80	0.00	0.73	0.01	1.00	0.03
Lreu_1746	Diol dehydratase beta subunit (EC 4.2.1.28)	-	-	0.72	0.03	-	-
Lreu_1747	Diol dehydratase large subunit (EC 4.2.1.28)	0.71	0.01	0.88	0.00	1.18	0.02
Lreu_1748	Propanediol utilization protein pduB	0.72	0.00	0.86	0.00	0.99	0.02
Lreu_1749	Propanediol utilization protein pduA	0.81	0.00	0.78	0.01	1.08	0.03
Lreu_1751	Ethanolamine utilization protein eutJ	-	-	1.19	0.01	1.82	0.04

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_1752	Glycerol uptake facilitator protein	-	-	0.66	0.02	0.92	0.04
Lreu_1754	Hypothetical cytosolic protein	-	-	-1.47	0.00	-1.79	0.01
Lreu_1755	Hypothetical membrane spanning protein	-	-	-1.82	0.00	-1.98	0.01
Lreu_1762	NrdI protein	-	-	1.72	0.00	1.35	0.02
Lreu_1763	PhnB protein	-	-	0.70	0.02	-	-
Lreu_1768	Lactose permease	-3.68	0.00	-	-	-	-
Lreu_1769	Universal stress protein family	-	-	-2.28	0.00	-2.84	0.00
Lreu_1771	Proton/sodium-glutamate symport protein	0.73	0.02	-	-	-	-
Lreu_1775	Ebg operon repressor	-1.15	0.00	-	-	-	-
Lreu_1776	Galactose-1-phosphate uridylyltransferase (EC 2.7.7.10)	-2.22	0.00	-	-	-	-
Lreu_1777	Galactokinase (EC 2.7.1.6)	-	-	-	-	0.72	0.05
Lreu_1778		-	-	-1.56	0.00	-1.84	0.01
Lreu_1779	Protein tyrosine phosphatase (EC 3.1.3.48)	-	-	-0.83	0.02	-1.23	0.02
Lreu_1781		-	-	-0.66	0.02	-0.95	0.04
Lreu_1783		-	-	0.87	0.04	-	-
Lreu_1785	Nicotinamide mononucleotide transporter	0.78	0.05	-	-	-	-
Lreu_1789	ABC transporter ATP-binding protein	-	-	-1.00	0.02	-	-
Lreu_1790	ABC transporter permease protein	-	-	-1.60	0.01	-	-
Lreu_1791	Homoserine O-succinyltransferase (EC 2.3.1.46)	-	-	0.99	0.01	-	-
Lreu_1802	Hypothetical cytosolic protein	-	-	0.93	0.03	-	-
Lreu_1808		-	-	-1.95	0.00	-2.53	0.00
Lreu_1812	Peptidoglycan hydrolase (3.2.1.-)	-	-	-1.35	0.00	-1.72	0.01
Lreu_1813	Hypothetical membrane spanning protein	-	-	-2.29	0.00	-2.41	0.01
Lreu_1814	ABC transporter ATP-binding protein	0.84	0.04	-	-	-	-
Lreu_1816	NlpC/P60 family protein	-	-	1.09	0.02	1.15	0.02
Lreu_1825	Ribosomal large subunit pseudouridine synthase D (EC 4.2.1.70)	-	-	0.70	0.00	0.85	0.02
Lreu_1826	Magnesium and cobalt transport protein corA	-	-	1.82	0.00	1.76	0.04
Lreu_1828	Xanthine permease	-	-	1.14	0.00	1.48	0.01
Lreu_1829	Tyrosine transporter	-	-	-0.95	0.02	-	-
Lreu_1830	Histidyl-tRNA synthetase (EC 6.1.1.21)	-	-	-1.03	0.01	-	-
Lreu_1837	Phosphoglycerate mutase family protein	-0.91	0.05	-0.72	0.02	-	-
Lreu_1840	Glycerol dehydrogenase (EC 1.1.1.6)	-1.74	0.00	-3.42	0.00	-3.60	0.00
Lreu_1848	Histidyl-tRNA synthetase (EC 6.1.1.21)	-	-	-	-	0.92	0.04
Lreu_1853	Phage-associated cell wall hydrolase	-	-	2.34	0.00	-	-
Lreu_1854	Transcriptional regulator, ArsR family	-	-	-1.05	0.00	-1.36	0.01
Lreu_1855		-	-	-1.44	0.02	-1.96	0.01
Lreu_1857	ABC transporter ATP-binding protein	-	-	0.95	0.01	-	-
Lreu_1860	Mannitol 2-dehydrogenase (EC 1.1.1.67)	-	-	-1.97	0.05	-	-
Lreu_1866	Amino acid permease	-	-	2.14	0.00	3.32	0.00
Lreu_1867	2-dehydropantoate 2-reductase (EC 1.1.1.169)	1.53	0.00	-	-	-	-
Lreu_1868	Transcriptional regulator, Cro/CI family	-	-	-1.69	0.00	-2.24	0.00
Lreu_1870	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase (EC 2.1.1.14)	1.45	0.03	-	-	-	-
Lreu_1872	Hypothetical protein	-0.69	0.01	-	-	0.78	0.04

Locus	Product[b]	exp		sta		sta-exp	
		M[c]	p[d]	M[c]	p[d]	M[c]	p[d]
Lreu_1873	Hypothetical protein	-1.10	0.01	-	-	-	-
Lreu_1874	Acetyltransferase, GNAT family	-	-	2.85	0.04	-	-
Lreu_1875	Putative NAD-dependent dehydrogenase	-	-	4.89	0.00	4.63	0.00
Lreu_1877	Nitroreductase family protein	-	-	3.58	0.00	2.88	0.00
Lreu_1878	Transcriptional regulator, MarR family	-	-	2.71	0.00	2.46	0.01
Lreu_1882	Putative NAD-dependent dehydrogenase	-	-	1.54	0.00	0.62	0.04
Lreu_1883	Aminopeptidase N (EC 3.4.11.15)	-	-	2.54	0.00	2.29	0.01
Lreu_1884	Hypothetical membrane spanning protein	-	-	3.42	0.00	3.33	0.00
Lreu_1886	Asparaginyl-tRNA synthetase (EC 6.1.1.22)	-	-	-1.15	0.00	-1.25	0.01
Lreu_1893	GMP synthase [glutamine-hydrolyzing] (EC 6.3.5.2)	-	-	1.08	0.00	-	-
Lreu_1894	Hypothetical cytosolic protein	-	-	2.18	0.01	2.03	0.04
Lreu_1897	Transcriptional regulator, TetR family	-	-	-1.05	0.01	-1.28	0.03
Lreu_1899	Transcriptional regulators, LysR family	-	-	-1.14	0.00	-1.26	0.04
Lreu_1902	Aminotransferase class I family	0.99	0.01	1.49	0.03	-	-
Lreu_1904	2-oxo-hepta-3-ene-1,7-dioic acid hydratase (EC 4.2.-.-)	0.90	0.05	1.05	0.05	1.00	0.05
Lreu_1907	Transcriptional regulators, LysR family	-	-	-1.02	0.04	-1.38	0.05
Lreu_1911	Hypothetical cytosolic protein	-	-	-1.26	0.01	-1.41	0.04
Lreu_1918	Bacteriocin processing peptidase (EC 3.4.22.-) / Bacteriocin export ABC transporter	-	-	2.32	0.04	4.27	0.03
Lreu_1923		-1.56	0.02	-	-	-	-
Lreu_1924	Beta-lactamase family protein	-	-	-0.84	0.01	-1.13	0.03
Lreu_1926	Membrane protein, MgtC/SapB family	1.22	0.01	-	-	-	-
Lreu_1927	Hypothetical cytosolic protein	0.85	0.01	-	-	-	-
Lreu_1932	Exoenzymes regulatory protein aepA precursor	-1.72	0.00	1.67	0.00	1.49	0.01
Lreu_1934	Phosphinothricin N-acetyltransferase (EC 2.3.1.-)	-	-	1.65	0.02	-	-
Lreu_1935	Signal peptidase I (EC 3.4.21.89)	-	-	1.97	0.01	-	-
Lreu_1937	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--lysine ligase (EC 6.3.2.7)	-	-	-	-	1.02	0.05
Lreu_1938	Asparagine synthetase [glutamine-hydrolyzing] (EC 6.3.5.4)	-	-	-	-	2.37	0.01
Lreu_1943	Ribonuclease P protein component (EC 3.1.26.5)	-	-	1.40	0.01	2.08	0.02
Lreu_1944	LSU ribosomal protein L34P	-	-	-	-	0.70	0.04

[a] Transcripts were considered for analysis if p-value < 0.05 and M > |0.585|.

[b] Annotation retrieved from ERGO (http://ergo.integratedgenomics.com/ERGO) (1).

[c] M, $\log_2$(intensity of signal with glycerol/intensity of signal without glycerol).

[d] p, p-value.

Table 4.S3.2. Distribution of transcriptomic data through out the different categories of clusters of orthologous groups[a] (2).

		upregulated with glycerol (%)	downregulated with glycerol (%)	regulated with glycerol (%)
C - Energy production and conversion (59)	exp	8.47	25.42	33.90
	sta	18.64	28.81	47.46
	mid-sta	8.47	25.42	33.90
D - Cell cycle control, cell division, chromosome partitioning (17)	exp	0.00	0.00	0.00
	sta	23.53	11.76	35.29
	mid-sta	23.53	5.88	29.41
E - Amino acid transport and metabolism (130)	exp	14.62	10.77	25.38
	sta	29.23	13.08	42.31
	mid-sta	15.38	9.23	24.62
F - Nucleotide transport and metabolism (75)	exp	10.67	14.67	25.33
	sta	29.33	14.67	44.00
	mid-sta	25.33	13.33	38.67
G - Carbohydrate transport and metabolism (80)	exp	1.25	23.75	25.00
	sta	18.75	12.50	31.25
	mid-sta	17.50	3.75	21.25
H - Coenzyme transport and metabolism (71)	exp	4.23	8.45	12.68
	sta	28.17	4.23	32.39
	mid-sta	33.80	2.82	36.62
I - Lipid transport and metabolism (34)	exp	0.00	5.88	5.88
	sta	17.65	14.71	32.35
	mid-sta	5.88	5.88	11.76
J - Translation, ribosomal structure and biogenesis (139)	exp	0.72	1.44	2.16
	sta	17.27	5.76	23.02
	mid-sta	17.27	3.60	20.86
K - Transcription (93)	exp	3.23	6.45	9.68
	sta	22.58	15.05	37.63
	mid-sta	10.75	15.05	25.81
L - Replication,	exp	0.57	1.70	2.27

		upregulated with glycerol (%)	downregulated with glycerol (%)	regulated with glycerol (%)
recombination and repair (176)	sta	10.23	11.36	21.59
	mid-sta	8.52	9.66	18.18
M - Cell wall/membrane/envelope biogenesis (80)	exp	1.25	6.25	7.50
	sta	26.25	10.00	36.25
	mid-sta	22.50	6.25	28.75
N - Cell motility (1)	exp	0.00	0.00	0.00
	sta	0.00	0.00	0.00
	mid-sta	0.00	0.00	0.00
O - Posttranslational modification, protein turnover, chaperones (42)	exp	11.90	14.29	26.19
	sta	30.95	16.67	47.62
	mid-sta	11.90	16.67	28.57
P - Inorganic ion transport and metabolism (58)	exp	12.07	3.45	15.52
	sta	6.90	13.79	20.69
	mid-sta	10.34	10.34	20.69
Q - Secondary metabolites biosynthesis, transport and catabolism (26)	exp	50.00	0.00	50.00
	sta	53.85	0.00	53.85
	mid-sta	38.46	0.00	38.46
R - General function prediction only (235)	exp	3.83	7.23	11.06
	sta	13.19	15.32	28.51
	mid-sta	5.53	10.21	15.74
S - Function unknown (149)	exp	6.04	8.72	14.77
	sta	16.78	19.46	36.24
	mid-sta	12.08	16.11	28.19
T - Signal transduction mechanisms (53)	exp	7.55	3.77	11.32
	sta	24.53	24.53	49.06
	mid-sta	13.21	22.64	35.85
U - Intracellular trafficking, secretion, and vesicular transport (23)	exp	0.00	4.35	4.35
	sta	26.09	13.04	39.13
	mid-sta	13.04	8.70	21.74
V - Defense mechanisms (34)	exp	0.00	5.88	5.88

	upregulated with glycerol (%)	downregulated with glycerol (%)	regulated with glycerol (%)
sta	2.94	20.59	23.53
mid-sta	2.94	11.76	14.71

[a] COG assignment retrieved from ERGO (http://ergo.integratedgenomics.com/ERGO) (1). Note: number between brackets refers to the total number of genes of *L. reuteri* associated to COG.

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CHAPTER 5

PocR Regulates Glycerol Reduction, Assembly of Metabolosomes and Vitamin B_{12} Biosynthesis in *Lactobacillus reuteri* JCM1112 as in Otherwise Unrelated Enteric Bacteria

Abstract

Lactobacillus reuteri has been shown to encode a vitamin B_{12} biosynthesis pathway that is phylogenetically related to the one present in some representatives of γ-Proteobacteria, such as *Salmonella* and *Yersinia*. Here we present evidence supporting that the similarities between these otherwise unrelated organisms extend to their regulatory mechanisms.

In collaboration with Douwe Molenaar, Bas Teusink, Willem M. de Vos and Jeroen Hugenholtz

Manuscript in preparation.

PocR Regulates Glycerol Reduction, Assembly of Metabolosomes and B_{12} Synthesis in *L. reuteri* as in Otherwise Unrelated Enteric Bacteria

Lactobacillus reuteri is a heterofermentative lactic acid bacterium colonizing the gastro-intestinal tract (GI-tract) of various mammals, including humans (12). It is able to reduce glycerol to 1,3-propanediol in a two-step enzymatic conversion, yielding NAD^+ (8). The first reaction, glycerol dehydratase (EC 4.1.2.30), converts glycerol to 3-hydroxypropanaldehyde (reuterin) requiring the presence of B_{12} as a co-factor (4). Reuterin is a potent antimicrobial, bestowing *L. reuteri* with an important growth advantage over other residents of the GI-tract, such as Gram-negative enteric bacteria (3).

Recently, we have shown that *L. reuteri* CRL1098 encodes the complete machinery necessary for *de novo* synthesis of vitamin B_{12} in a single chromosomal gene cluster (9). Unexpectedly, this cluster was shown to be very similar to that present in various representatives of *Salmonella* and stands out against the canonical phylogeny deduced from 16S RNA sequences. Complete genome sequence analysis of the type strain of *L. reuteri* (http://www.jgi.doe.gov/) revealed that the region immediately upstream of the vitamin B_{12} biosynthesis cluster also maintains a gene order similar to that of *Salmonella*. Moreover, the functionality of this upstream region was demonstrated to match that of *Salmonella* as it encodes the assembly machinery of metabolosomes and the several subunits of a large diol dehydratase that can metabolize both glycerol and 1,2-propanediol (11). Within this cluster resides a gene (*lreu_1750*) predicted to encode a putative transcription factor of the AraC type family, containing a typical helix-turn-helix- domain. Based on its conserved genomic context, this gene has been suggested to encode a homolog of PocR, a regulatory protein of the propanediol utilization (*pdu*) and B_{12} biosynthesis clusters of enteric bacteria (8, 11). However, detailed comparisons between Lreu_1750 and PocR revealed only limited similarities in amino acid sequence (Fig. 5.S1, supplementary material 5S). Moreover, genes that code for products very similar to PocR of enteric bacteria have been found in other B_{12}-producing *Bacilli*, such as representatives of *Listeria* and *Clostridium.* The closest homolog of Lreu_1750 present in the complete genomes is found in *L. brevis* ATCC367 (GI:116334199). *L. brevis* is one of the few other lactobacilli for which the B_{12}-dependent production of 1,3-propanediol has been demonstrated (4), although it does not have the capability to synthesize B_{12}. The predicted products of *lreu_1750* and its homolog in *L. brevis* are approximately 60 amino acid residues longer in the C-terminus in comparison to PocR from *S. typhimurium* LT2, which could affect its functionality.

Here we provide the first experimental evidence to support the functional assignment of Lreu_1750. This was achieved by overexpression of *lreu_1750* and assessing its impact on central carbon metabolism and vitamin B_{12} biosynthesis. In addition, we characterize the genome-wide transcriptional response of the overexpression in comparison to the wild-type leading to the identification of the PocR regulon.

Construction of a strain overexpressing lreu_1750. Gene *lreu_1750* was overexpressed constitutively under control of the *pepN* promoter in a similar fashion as previously described (10). Chromosomal DNA of *L. reuteri* JCM1112 was isolated according to standard procedures (7). A fragment containing *lreu_1750* was amplified using Herculase II DNA polymerase (Stratagene, La Jolla, USA), and primers P180 and P181 (Table 5.1). After digestion with *Kpn*I, the modified amplicon was purified with the JETPURE PCR Product Purification Kit (GENOMED, Bad Oeynhausen, Germany) and cloned in pNZ7021 making use of the *Kpn*I and *Pml*I restriction sites directly downstream of the *pepN* promoter. The resulting plasmid, termed pNZ7748, was used directly from the ligation reactions to transform *L. lactis* NZ9000 by electroporation (13). Subsequently, pNZ7748 was purified from *L. lactis* as previously described (7) and, after verification of its structure, used to transform *L. reuteri* also by electroporation (12).

Table 5.1. Strains, plasmids and primers used in this study.

Materials	Relevant features	Source or reference
Strains		
L. reuteri JCM1112	Type strain, synonymous to ATCC 23272, DSM 20016 and F275. Human isolate.	Japanese Collection of Microorganisms (Riken, Japan)
L. lactis NZ9000	MG1363 *pepN:nisRK*, cloning host.	NIZO culture collection (Ede, Netherlands)
L. delbrueckii NIZO235	*L. delbrueckii* subsp. *lactis* ATCC 7830. Vitamin B_{12} assay indicator strain.	*idem*
Plasmids		
pNZ7021	Cm^R, pNZ8148 derivative with the nisin promoter replaced by the *pepN* promoter	(13)
pNZ7748	Cm^R, pNZ7021 derivative harbouring Lreu_1750 downstream of the *pepN* promoter.	this study
Primers	***5' – 3'***	***Application***
P180	AAAAGGTACCGTAGGCGAAATTCAAATGTACG	Amplification of Lreu_1750 and addition of *Kpn*I site
P181	GAATAAATAAGAGGCTGGGCAC	Amplification of Lre231
P182	ATGAACTCTATTCAGGAATTG	Control of pNZ7748

The physiological effects of the overexpression of *lreu_1750* were studied by cultivating *Lb reuteri* pNZ7748 (*lreu_1750* overexpression) and *Lb reuteri* pNZ7021 (empty plasmid) in chemically defined medium (CDM) in pH-controlled batch fermentations in the presence or absence of glycerol carried out as described previously (8). At different time points, samples were taken for transcriptome, supernatant and B_{12} analysis (Fig. 5.1).

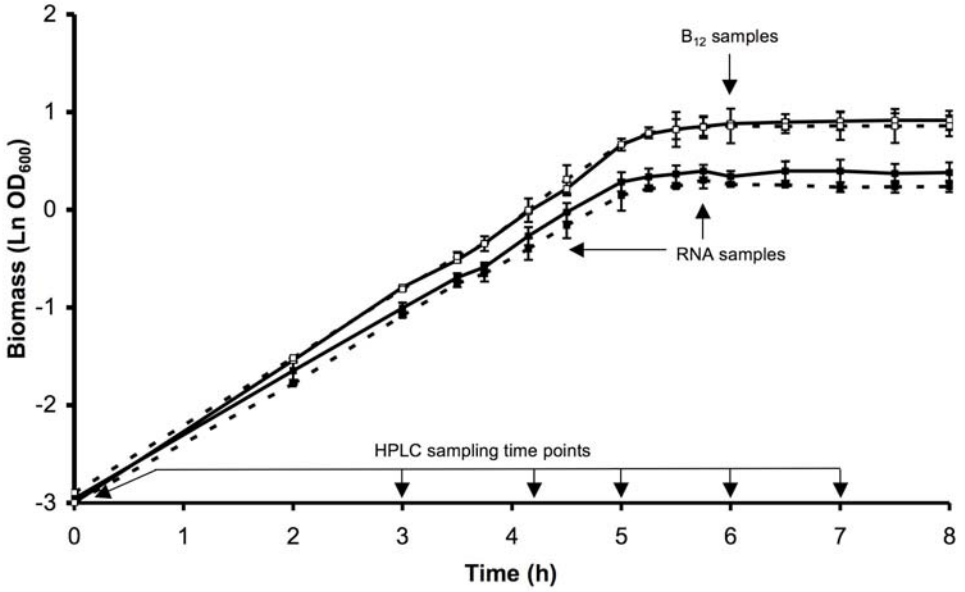

Figure 5.1. Biomass formation and sampling scheme in pH-controlled batch fermentations of CDM in the presence (white squares) or absence (black square) of glycerol by *L. reuteri* transformed with pNZ7021 (solid lines) or pNZ7748 (dashed lines).

Phenotypic characterization of overexpressing of lreu_1750. In order to investigate the involvement of Lreu_1750 in regulating glycerol reduction and B_{12} biosynthesis, we determined the levels of B_{12} production and the extracellular concentration of main fermentation substrates and products, as described elsewhere (8). In CDM with glycerol we observed a clear enhancement in the shift from ethanol to acetate formation in the overexpressing strain. Ethanol production dropped sharply to about 0.2 molecules formed per molecule of glucose consumed. This resulted in a 22.5% increase of 1,3-propanediol production, which, as previously noted (8), is produced on a 2:1 molar ratio with acetate to assure the regeneration of reducing equivalents (Fig. 5.2c. and d.). In

contrast, when glycerol was not added to CDM, there was hardly any distinctive phenotype attribuTable 5.to the overexpression of *lreu_1750* (Fig. 5.2a. and b), except for a slight impairment of μ_{max} (< 10%). The involvement of Lreu_1750 in the regulation of vitamin B_{12} synthesis is supported by the 40-45% increase in vitamin B_{12} production that accompanies its overexpression (Fig. 5.3). This increase was obtained regardless of the addition of glycerol, and did not alter the approximately five-fold higher B_{12} production observed in the presence of glycerol.

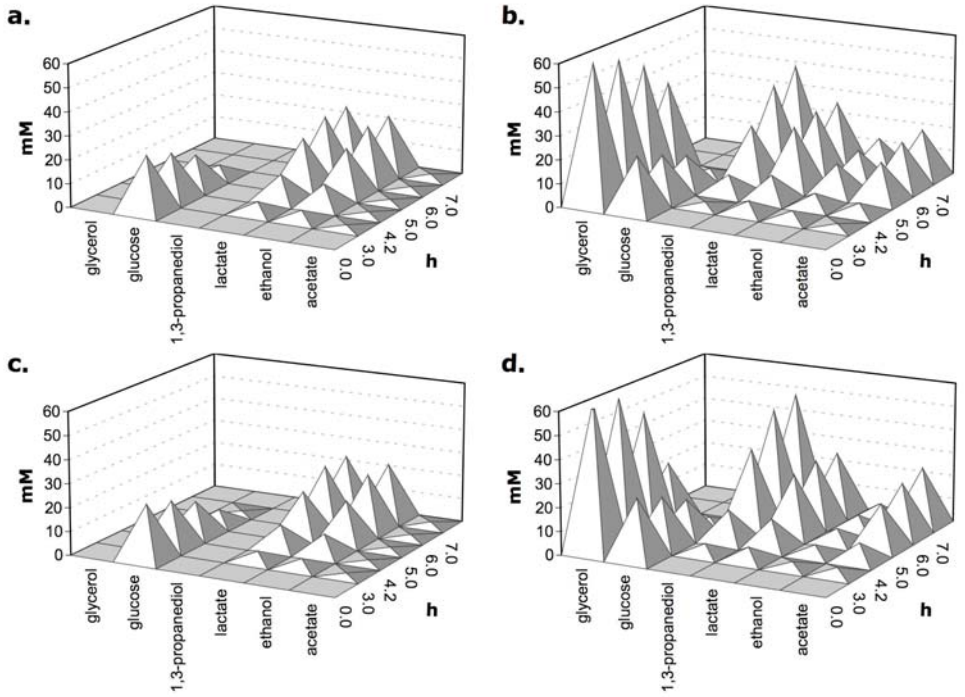

Figure 5.2. Substrate consumption and product formation by different constructs of *L. reuteri* in CDM and in CDM with 0.5% glycerol (v/v). a. *L. reuteri* pNZ7021 in CDM; b. *L. reuteri* pNZ7021 in CDM with glycerol; c. *L. reuteri* pNZ7748 in CDM; d. *L. reuteri* pNZ7748 in CDM with glycerol.

Transcriptional response of L. reuteri to the overexpression of Lreu_1750. The transcriptomes of cells transformed with pNZ7748 and pNZ7021 were compared using cDNA microarrays as previously detailed (8) using a hybridization scheme comprising 17 arrays in a loop-design (supplementary material 5S). The custom probe design of the Agilent 11K microarray platform (Agilent Technologies, Santa Clara, CA, USA) used is available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under

accession number GPL6856, and the data obtained were deposited in the same repository (accession number pending). We compared the response of *L. reuteri* to overexpressing *lreu_1750* in CDM and in CDM supplemented with glycerol. However, since there is a large overlap between the two (more than 70%), and since glycerol has been shown to induce the expression of *lreu_1750*, reducing the effect of the overexpression, only the data from CDM without glycerol will be discussed. Nonetheless, the complete list of differentially regulated genes is available in Table 5.S1 (supplementary material 5S).

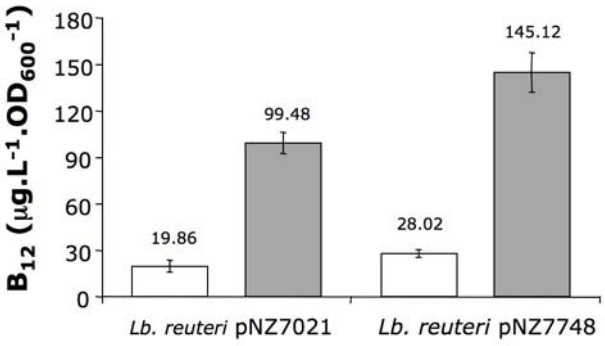

Figure 5.3. B_{12} production by *L. reuteri* pNZ7021 (empty plasmid) and *L. reuteri* pNZ7748 (*lreu_1750* overexpression) in CDM (white bars) and in CDM supplemented with glycerol (grey bars).

The most striking feature of overexpressing *lreu_1750* is that only 120 genes (approximately 6% of the genome) are differentially regulated, of which, all but two are up-regulated. Three functional classes were overrepresented, namely the ones related to coenzymes, secondary metabolites and energy production (Table 5.S2, supplementary material 5S). A closer inspection of the list of differentially regulated genes shows that *lreu_1750* is clearly involved in the regulation of the same processes that have been linked to PocR in *Salmonella* (1). These include the activation of the *pdu* cluster flanking *lreu_1750*, encoding the several subunits of the diol dehydratase and the metabolosome-assembly proteins, along with the up-regulation of the two operons of the B_{12} biosynthesis cluster (9). Besides these genes stretching from *lreu_1695* to *lreu_1752*, which are expected to be regulated by PocR by homology with *Salmonella* (2), we found two genes within the same operon with unknown function (Lreu_0429 and 0430), which were ~3.5-fold up-regulated (Table 5.2). A closer look at their sequence indicates that these are most likely subunits of an ATPase transporter (5), which is in accordance with annotations from ERGO (6). We speculate that these are cobalt-transporters, rather then being involved in the

transport of copper, based on the fact that cobalt availability can limit the synthesis of B_{12}. We also found genes related to sugar uptake and carbon metabolism to be regulated. These are presumably related to the up-regulation of *lreu_0088* (transcription factor of LacI family), but could also be a consequence of a slight drop in growth rate caused by the overexpression (Fig. 5.1). A considerable number of enzymes involved in recombination and DNA repair proteins were also up-regulated, but this has most likely to do with the homologous region to the genome of *L. reuteri* present in plasmid pNZ7748 and not in pNZ7021.

Table 5.2. Selection of loci associated to PocR and not within its flanking region.[a.]

Locus	Function	M	*p*	Accession number
Lreu_0088	Transcriptional regulator, LacI family	1.11	0.03	gi|148543330
Lreu_0103	3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157)	0.89	0.03	gi|148543342
Lreu_0429	Putative cobalt-transporting ATPase[b.]	1.85	0.00	gi|148543665
Lreu_0430	Putative cobalt-transporting ATPase[b.]	1.76	0.00	gi|148543666
Lreu_0479	Arabinose-proton symporter	1.67	0.00	gi|148543714
Lreu_0631	Pyruvate dehydrogenase alpha subunit (EC 1.2.4.1)	0.88	0.02	gi|148543863
Lreu_0632	Pyruvate dehydrogenase beta subunit (EC 1.2.4.1)	0.86	0.04	gi|148543864
Lreu_0633	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.12)	0.81	0.03	gi|148543865
Lreu_0910	Alpha-galactosidase (EC 3.2.1.22)	0.90	0.03	gi|148544139
Lreu_1007	Transcription regulator, Crp family	-0.82	0.04	gi|148544234
Lreu_1531	Fumarate hydratase (EC 4.2.1.2)	1.09	0.05	gi|148544743
Lreu_1768	Lactose permease	0.97	0.05	gi|148544974
Lreu_1832	Histidine decarboxylase (EC 4.1.1.22)	-1.67	0.03	gi|148545038

[a.] Genes predicted to encode phage-related proteins, recombinases, mobile elements, DNA repair and general or unknown functions were omitted (for full list, please see supplementary material, Table 5.S1.1)
[b.] Annotated as hypothetical protein in GenBank and as copper-transporting ATPase (EC 3.6.3.10) in ERGO database (6).

Final remarks. In this study, we have provided experimental evidence for the annotation of Lreu_1750 as PocR and identified the genes associated to the PocR regulon in *L. reuteri* by global transcriptional analysis of the impacts of its overexpression. Furthermore, we have demonstrated its stimulatory effects in B_{12} and 1,3-propanediol productions, for which we observed a 40-45% and 22.5% increase, respectively. The findings reported here could be applied to improving the yields of these two industrially relevant compounds.

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Content of supplementary material of Chapter 5

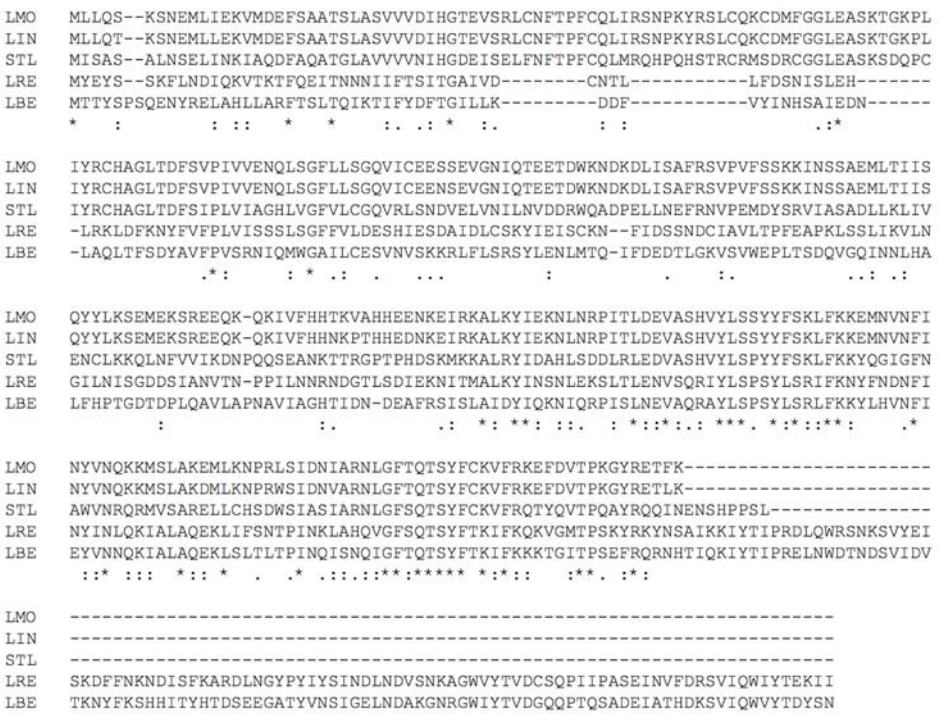

Figure 5.S1. Amino acid sequence alignment of Lreu_1750 and its closest homolog in *Listeria monocytogenes* (LMO), *Listeria innocua* (LIN), *Salmonella typhimurium* LT2 (STL) and *Lb. brevis* (LEB). Obtained using ClustalW with default settings (3).

Hybridization Scheme of cDNA microarray experiments

The following samples were hybridized per array labeled with cyanine3 and cyanine5, respectively: sta-F6 and sta-F5, sta-F7 and sta-F8, sta-F5 and sta-F7, sta-F8 and sta-F6, sta-F3 and sta-F4, sta-F1 and sta-F3, sta-F2 and sta-F1, sta-F4 and sta-F2, exp-F3 and exp-F4, exp-F1 and exp-F3, exp-F2 and exp-F1, exp-F4 and exp-F2, exp-F4 and sta-F4, sta-F3 and exp-F3, sta-F2 and sta-F8, sta-F4 and sta-F6, exp-F2 and sta-F2. Here, F1 and F5 represent completely independent biological duplicates of *L. reuteri* pNZ7021 cultured in the absence of glycerol; F2 and F6 represent completely independent biological duplicates of *L. reuteri* pNZ7748 cultured in the absence of glycerol; F3 and F7 represent completely independent biological duplicates of *L. reuteri* pNZ7021 cultured in the presence of glycerol; and F4 and F8 represent completely independent biological duplicates of *L. reuteri* pNZ7748 cultured in the presence of glycerol The prefix exp- and sta- stand for cells harvested at mid logarithmic and early stationary growth phases, respectively.

Table 5.S1. Complete list of genes from *L. reuteri* that are differentially expressed by the overexpression of *lreu_1750*[a]. Comparisons were established for cells cultured in CDM and in CDM supplemented with glycerol.

Locus	Function[b]	CDM		CDMglycerol		COG	Accession number
		M[c]	p[d]	M[c]	p[d]		
Lreu_0539	Thioredoxin			0.97	0.02	C	gi|148543774
Lreu_0631	Pyruvate dehydrogenase E1 component alpha subunit (EC 1.2.4.1)	0.88	0.02	-1.53	0.00	C	gi|148543863
Lreu_0632	Pyruvate dehydrogenase E1 component beta subunit (EC 1.2.4.1)	0.86	0.04	-1.66	0.00	C	gi|148543864
Lreu_0633	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.12)	0.81	0.03	-1.44	0.00	C	gi|148543865
Lreu_0634	Dihydrolipoamide dehydrogenase (EC 1.8.1.4)			-1.17	0.02	C	gi|148543866
Lreu_1531	Fumarate hydratase (EC 4.2.1.2)	1.09	0.05			C	gi|148544743
Lreu_1727	Flavodoxin	0.92	0.01			C	gi|148544933
Lreu_1734	Propanol dehydrogenase (EC 1.1.1.-)	1.86	0.00			C	gi|148544940
Lreu_1735	Propionaldehyde dehydrogenase [CoA-acylating] (EC 1.2.1.-)	1.94	0.00			C	gi|148544941
Lreu_0098	Glutamine-binding protein / Glutamine transport system permease protein glnP			1.23	0.00	E	gi|148543337
Lreu_1544	Xaa-His dipeptidase (EC 3.4.13.3)			-0.94	0.01	E	gi|148544756
Lreu_1728	Propanediol utilization protein pduV			-1.05	0.02	E	gi|148544934
Lreu_1732	Ethanolamine utilization protein eutS	1.87	0.00			E	gi|148544938
Lreu_1748	Propanediol utilization protein pduB	2.22	0.00			E	gi|148544954
Lreu_1751	Ethanolamine utilization protein eutJ	0.87	0.03			E	gi|148544957
Lreu_0111	Deoxyribose-phosphate aldolase (EC 4.1.2.4)			-1.90	0.00	F	gi|148543350
Lreu_0114	Purine nucleoside phosphorylase II (EC 2.4.2.1)			-1.75	0.00	F	gi|148543353
Lreu_0112	Phosphopentomutase (EC 5.4.2.7)			-1.89	0.00	G	gi|148543351
Lreu_0288	Beta-galactosidase large subunit (EC 3.2.1.23)			-1.27	0.00	G	gi|148543525
Lreu_0289	Beta-galactosidase small subunit (EC 3.2.1.23)			-1.21	0.00	G	gi|148543526
Lreu_0418	Glucose uptake protein homolog			-0.87	0.03	G	gi|148543654
Lreu_0910	Alpha-galactosidase (EC 3.2.1.22)	0.90	0.03			G	gi|148544139
Lreu_1017	Alpha-glucosidase (EC 3.2.1.20)			-1.64	0.00	G	gi|148544244
Lreu_1538	Aldose 1-epimerase (EC 5.1.3.3)			-0.89	0.02	G	gi|148544750
Lreu_1752	Glycerol uptake facilitator protein	0.93	0.02			G	gi|148544958
Lreu_1768	Lactose permease	0.97	0.05	-1.49	0.00	G	gi|148544974
Lreu_1776	Galactose-1-phosphate uridylyltransferase (EC 2.7.7.10)			-0.97	0.04	G	gi|148544982
Lreu_0155	Nicotinate phosphoribosyltransferase (EC 2.4.2.11)			0.98	0.04	H	gi|148543394
Lreu_1677	Lipoate-protein ligase A (EC 6.3.2.-)			-1.17	0.01	H	gi|148544883
Lreu_1705	Adenosylcobyric acid synthase (glutamine-hydrolysing) (EC 6.3.5.10)			0.96	0.03	H	gi|148544911
Lreu_1710	Precorrin-2 C20-methyltransferase (EC 2.1.1.130)			0.96	0.01	H	gi|148544916
Lreu_1711	Sirohydrochlorin cobaltochelatase (EC 4.99.1.3)	0.83	0.05	1.03	0.01	H	gi|148544917
Lreu_1712	Uroporphyrin-III C-methyltransferase (EC 2.1.1.107) / Uroporphyrinogen-III synthase (EC 4.2.1.75)	0.86	0.03	0.82	0.05	H	gi|148544918
Lreu_1713	Precorrin-6X reductase (EC 1.3.1.54)	0.84	0.04			H	gi|148544919
Lreu_1714	Precorrin-3B C17-methyltransferase (EC 2.1.1.131)	0.93	0.05			H	gi|148544920
Lreu_1716	Precorrin-4 C11-methyltransferase (EC 2.1.1.133)	0.98	0.02			H	gi|148544922
Lreu_1717	Precorrin-6Y C5,15-methyltransferase [decarboxylating] subunit CbiT (EC 2.1.1.132)	1.04	0.02			H	gi|148544923
Lreu_1718	Precorrin-6Y C5,15-methyltransferase [decarboxylating] subunit CbiE (EC 2.1.1.132)	1.10	0.02			H	gi|148544924
Lreu_0103	3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157)	0.89	0.03			I	gi|148543342
Lreu_0088	Transcriptional regulator, LacI family	1.11	0.03			K	gi|148543330
Lreu_0801	Transcriptional regulator, Cro/CI family			0.81	0.04	K	gi|148544031
Lreu_0831		1.21	0.01	1.77	0.00	K	gi|148544061
Lreu_1109	Phage tail protein	2.29	0.00	1.48	0.00	K	gi|148544333
Lreu_1111	Phage protein	2.00	0.00	1.44	0.01	K	gi|148544335

Locus	Function[b]	CDM		CDMglycerol		COG	Accession number
		M[c]	p[d]	M[c]	p[d]		
Lreu_1112		2.18	0.00	1.69	0.00	K	gi|148544336
Lreu_1750	Transcriptional regulator, AraC family	3.11	0.00	3.58	0.00	K	gi|148544956
Lreu_1796	Transcriptional regulator			0.84	0.05	K	gi|148545002
Lreu_0383	Excinuclease ABC subunit B	1.05	0.05			L	gi|148543619
Lreu_0384	Excinuclease ABC subunit A	0.94	0.03			L	gi|148543620
Lreu_0523	RecA protein			1.18	0.02	L	gi|148543758
Lreu_0796	Site-specific recombinase	1.32	0.04			L	gi|148544026
Lreu_0811	RecT protein	1.95	0.00	1.73	0.00	L	gi|148544041
Lreu_1120	Terminase small subunit	2.28	0.00	1.46	0.00	L	gi|148544344
Lreu_1139	RecT protein	1.76	0.02	1.55	0.03	L	gi|148544363
Lreu_1114	Phage protein	2.15	0.00	1.61	0.00	M	gi|148544338
Lreu_0539	Thioredoxin			0.97	0.02	O	gi|148543774
Lreu_0891	ATP-dependent transporter sufC			-0.81	0.03	O	gi|148544121
Lreu_0216	Manganese-binding protein			-1.68	0.05	P	gi|148543453
Lreu_0217	Manganese transport system ATP-binding protein			-1.44	0.02	P	gi|148543454
Lreu_1650	Non-specific DNA-binding protein Dps / Iron-binding ferritin-like antioxidant protein / Ferroxidase (EC 1.16.3.1)	1.08	0.02	0.83	0.04	P	gi|148544857
Lreu_1706	Cobalt transport ATP-binding protein cbiO			1.06	0.03	P	gi|148544912
Lreu_1707	Cobalt transport protein cbiQ			1.14	0.03	P	gi|148544913
Lreu_1708	Cobalt transport protein cbiN			1.14	0.03	P	gi|148544914
Lreu_1709	CbiM protein	0.81	0.04	1.04	0.01	P	gi|148544915
Lreu_0156	Pyrazinamidase (EC 3.5.1.-) / Nicotinamidase (EC 3.5.1.19)			1.04	0.04	Q	gi|148543395
Lreu_1738	Ethanolamine utilization protein eutN	2.05	0.00			Q	gi|148544944
Lreu_1740	PduL	2.17	0.00			Q	gi|148544946
Lreu_1741	Propanediol utilization protein pduA	2.18	0.00			Q	gi|148544947
Lreu_1742		2.23	0.00			Q	gi|148544948
Lreu_1743		2.29	0.00			Q	gi|148544949
Lreu_1744	Glycerol dehydratase reactivation factor large subunit	2.18	0.00			Q	gi|148544950
Lreu_1745	Diol dehydratase gamma subunit (EC 4.2.1.28)	2.20	0.00			Q	gi|148544951
Lreu_1747	Diol dehydratase large subunit (EC 4.2.1.28)	2.23	0.00			Q	gi|148544953
Lreu_1749	Propanediol utilization protein pduA	2.18	0.00			Q	gi|148544955
Lreu_0419	O-acetyltransferase (EC 2.3.1.-)			-0.93	0.04	R	gi|148543655
Lreu_0479	Arabinose-proton symporter	1.67	0.00			R	gi|148543714
Lreu_0833	Terminase large subunit	1.30	0.01	1.67	0.00	R	gi|148544063
Lreu_1117	Terminase large subunit	2.13	0.00	1.37	0.01	R	gi|148544341
Lreu_1795	ATPase associated with chromosome architecture/replication	1.35	0.01			R	gi|148545001
Lreu_0429	Copper-transporting ATPase (EC 3.6.3.10)	1.85	0.00	0.87	0.03	S	gi|148543665
Lreu_0430	Copper-transporting ATPase (EC 3.6.3.10)	1.76	0.00	0.86	0.04	S	gi|148543666
Lreu_0803	Phage antirepressor protein	1.60	0.00	1.70	0.00	S	gi|148544033
Lreu_0838	Major capsid protein	1.16	0.04	1.65	0.01	S	gi|148544068
Lreu_0846	Phage protein	1.47	0.00	1.50	0.00	S	gi|148544076
Lreu_1104	Phage protein	1.92	0.00	1.30	0.02	S	gi|148544328
Lreu_1106	Phage protein	2.50	0.00	1.32	0.02	S	gi|148544330
Lreu_1110	Phage tail protein	2.41	0.00	1.47	0.00	S	gi|148544334
Lreu_1116	Portal protein	2.17	0.00	1.32	0.01	S	gi|148544340
Lreu_1142	Phage antirepressor protein	1.62	0.00	1.70	0.00	S	gi|148544366
Lreu_1545	Arginine/ornithine antiporter			-1.57	0.00	S	gi|148544757
Lreu_1629	Integral membrane protein	1.53	0.00	0.83	0.04	S	gi|148544836
Lreu_1736	Protein glcG	2.09	0.00			S	gi|148544942
Lreu_1737	Cobalamin adenosyltransferase family protein	2.17	0.00			S	gi|148544943
Lreu_0240	Protein tyrosine phosphatase (EC 3.1.3.48)			0.85	0.03	T	gi|148543477

Locus	Function[b]	CDM		CDMglycerol		COG	Accession number
		M[c]	p[d]	M[c]	p[d]		
Lreu_0677	LexA repressor (EC 3.4.21.88)	1.29	0.00	1.46	0.00	T	gi|148543909
Lreu_0834	Portal protein	1.33	0.01	1.68	0.00	T	gi|148544064
Lreu_1007	Transcription regulator, crp family	-0.82	0.04			T	gi|148544234
Lreu_1731	Protein tyrosine phosphatase (EC 3.1.3.48)	1.88	0.00			T	gi|148544937
Lreu_1115	ATP-dependent endopeptidase clp proteolytic subunit clpP (EC 3.4.21.92)	2.22	0.00	1.66	0.00	U	gi|148544339
Lreu_0812	Phage protein	1.96	0.00	1.65	0.01	V	gi|148544042
Lreu_1121	Phage endonuclease	1.88	0.01	1.37	0.02	V	gi|148544345
Lreu_1138	Phage protein	1.96	0.00	1.65	0.01	V	gi|148544362
Lreu_0178		0.92	0.02	1.38	0.00		gi|148543415
Lreu_0802		1.52	0.00	1.76	0.00		gi|148544032
Lreu_0806		1.84	0.00	1.73	0.00		gi|148544036
Lreu_0813	Phage replication protein	1.97	0.00	1.56	0.00		gi|148544043
Lreu_0814		2.03	0.00	1.57	0.00		gi|148544044
Lreu_0815		1.98	0.00	1.58	0.01		gi|148544045
Lreu_0816		1.92	0.00	1.82	0.00		gi|148544046
Lreu_0821		1.91	0.00	1.73	0.00		gi|148544051
Lreu_0822		1.74	0.00	1.58	0.00		gi|148544052
Lreu_0823		1.73	0.00	1.70	0.00		gi|148544053
Lreu_0827		1.82	0.00	1.95	0.00		gi|148544057
Lreu_0829		1.10	0.01	1.91	0.00		gi|148544059
Lreu_0830		0.99	0.02	1.79	0.00		gi|148544060
Lreu_0832		1.22	0.01	1.80	0.00		gi|148544062
Lreu_0835	Phage protein	1.37	0.01	1.57	0.00		gi|148544065
Lreu_0836		1.43	0.00	1.44	0.00		gi|148544066
Lreu_0837		1.15	0.03	1.63	0.00		gi|148544067
Lreu_0839	Phage protein	1.26	0.03	1.66	0.01		gi|148544069
Lreu_0840		1.34	0.02	1.79	0.00		gi|148544070
Lreu_0841	Phage protein	1.18	0.04	1.67	0.01		gi|148544071
Lreu_0842		1.29	0.03	1.58	0.01		gi|148544072
Lreu_0843	Phage protein	1.32	0.01	1.64	0.00		gi|148544073
Lreu_0844	Phage protein	1.31	0.01	1.59	0.00		gi|148544074
Lreu_0845		1.51	0.01	1.49	0.01		gi|148544075
Lreu_0847		1.62	0.00	1.57	0.00		gi|148544077
Lreu_0848	Phage protein	1.58	0.00	1.44	0.00		gi|148544078
Lreu_0850		1.49	0.00	1.45	0.00		gi|148544080
Lreu_0851		1.44	0.00	1.55	0.00		gi|148544081
Lreu_0852		1.38	0.04				gi|148544082
Lreu_0854		1.35	0.00	1.56	0.00		gi|148544084
Lreu_0856		1.26	0.01	1.43	0.00		gi|148544086
Lreu_0857				1.17	0.01		gi|148544087
Lreu_1095		1.91	0.01	1.55	0.01		gi|148544319
Lreu_1096		1.96	0.00	1.34	0.00		gi|148544320
Lreu_1097		1.43	0.01	1.47	0.00		gi|148544321
Lreu_1098		1.26	0.01	1.43	0.00		gi|148544322
Lreu_1099		1.35	0.00	1.56	0.00		gi|148544323
Lreu_1100		1.32	0.01	1.43	0.00		gi|148544324
Lreu_1101		1.38	0.04				gi|148544325
Lreu_1102		1.44	0.00	1.55	0.00		gi|148544326
Lreu_1103		1.82	0.00	1.41	0.00		gi|148544327
Lreu_1105		2.11	0.05				gi|148544329

Locus	Function[b]	CDM		CDMglycerol		COG	Accession number
		M[c]	p[d]	M[c]	p[d]		
Lreu_1108		2.45	0.00	1.24	0.01		gi|148544332
Lreu_1113	Phage protein	2.20	0.00	1.66	0.00		gi|148544337
Lreu_1122		1.46	0.01	1.12	0.01		gi|148544346
Lreu_1127		1.68	0.00	1.69	0.00		gi|148544351
Lreu_1140		1.57	0.04	1.81	0.02		gi|148544364
Lreu_1143		1.78	0.00	1.73	0.00		gi|148544367
Lreu_1630	Hypothetical protein	1.08	0.01	0.89	0.01		gi|148544837
Lreu_1739		1.79	0.00				gi|148544945
Lreu_1832	Histidine decarboxylase (EC 4.1.1.22)	-1.67	0.03	-1.95	0.01		gi|148545038
Lreu_1838				1.16	0.02		gi|148545044
Lreu_1855				1.12	0.05		gi|148545060

[a] Transcripts were considered for analysis if *p*-value < 0.05 and M > |0.585|.
[b] Annotation retrieved from ERGO (http://ergo.integratedgenomics.com/ERGO) (1).
[c] M, $\log_2$(intensity of signal with glycerol/intensity of signal without glycerol).
[d] *p*, *p*-value.

Table 5.S2. Distribution of differentially expressed genes through out the several categories of clusters of orthologuous groups (COG) (2).

COG category		up-regulated when *lreu_1750* is overexpressed	down-regulated when *lreu_1750* is overexpressed	regulated *lreu_1750* is overexpressed
C - Energy production and conversion (59)	CDM	11.86	0.00	11.86
	CDMglycerol	1.69	6.78	8.47
D - Cell cycle control, cell division, chromosome partitioning (17)	CDM	0.00	0.00	0.00
	CDMglycerol	0.00	0.00	0.00
E - Amino acid transport and metabolism (130)	CDM	2.31	0.00	2.31
	CDMglycerol	0.77	1.54	2.31
F - Nucleotide transport and metabolism (75)	CDM	0.00	0.00	0.00
	CDMglycerol	0.00	2.67	2.67
G - Carbohydrate transport and metabolism (80)	CDM	3.75	0.00	3.75
	CDMglycerol	0.00	10.00	10.00
H - Coenzyme transport and metabolism (71)	CDM	9.86	0.00	9.86
	CDMglycerol	7.04	1.41	8.45
I - Lipid transport and metabolism (34)	CDM	2.94	0.00	2.94
	CDMglycerol	0.00	0.00	0.00
J - Translation, ribosomal structure and biogenesis (139)	CDM	0.00	0.00	0.00
	CDMglycerol	0.00	0.00	0.00
K - Transcription (93)	CDM	6.45	0.00	6.45
	CDMglycerol	7.53	0.00	7.53

COG category		up-regulated when *lreu_1750* is overexpressed	down-regulated when *lreu_1750* is overexpressed	regulated *lreu_1750* is overexpressed
L - Replication, recombination and repair (176)	CDM	3.41	0.00	3.41
	CDMglycerol	2.27	0.00	2.27
M - Cell wall/membrane/envelope biogenesis (80)	CDM	1.25	0.00	1.25
	CDMglycerol	1.25	0.00	1.25
N - Cell motility (1)	CDM	0.00	0.00	0.00
	CDMglycerol	0.00	0.00	0.00
O - Posttranslational modification, protein turnover, chaperones (42)	CDM	0.00	0.00	0.00
	CDMglycerol	2.38	2.38	4.76
P - Inorganic ion transport and metabolism (58)	CDM	3.45	0.00	3.45
	CDMglycerol	8.62	3.45	12.07
Q - Secondary metabolites biosynthesis, transport and catabolism (26)	CDM	34.62	0.00	34.62
	CDMglycerol	3.85	0.00	3.85
R - General function prediction only (235)	CDM	1.70	0.00	1.70
	CDMglycerol	0.85	0.43	1.28
S - Function unknown (149)	CDM	8.72	0.00	8.72
	CDMglycerol	7.38	0.67	8.05
T - Signal transduction mechanisms (53)	CDM	5.66	1.89	7.55
	CDMglycerol	5.66	0.00	5.66
U - Intracellular trafficking, secretion, and vesicular transpor (23)	CDM	4.35	0.00	4.35
	CDMglycerol	4.35	0.00	4.35
V - Defense mechanisms (34)	CDM	8.82	0.00	8.82
	CDMglycerol	8.82	0.00	8.82

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CHAPTER 6

EFFECT OF AMINO ACID AVAILABILITY ON VITAMIN B_{12} PRODUCTION IN *LACTOBACILLUS REUTERI*

Abstract

Recent functional genomics and genome-scale modeling approaches indicated that B_{12} production in *Lactobacillus reuteri* could be improved by medium optimization. Here we show that a series of systematic single amino acid omissions could significantly modulate the production of B_{12} from nearly undetectable levels (by isoleucine omission) to 20-fold higher than previously reported through omission of cysteine. We analyzed, by cDNA microarray experiments, the transcriptional response of *L. reuteri* to the medium lacking cysteine. These results supported the observed high B_{12} production and provided new avenues for future improvement of production of vitamin B_{12}.

In collaboration with Bas Teusink, Douwe Molenaar, Maurice van Heck, Sander Sieuwerts, Willem M. de Vos, Jeroen Hugenholtz
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Introduction

Vitamin B_{12} analogues (generally termed B_{12}) form a diverse subgroup of the tetrapyrroles, and consist of structurally complex molecules that contain a ring-contracted porphinoid with cobalt chelated at the core (5, 10). Synthesis of B_{12} is restricted to a few clades of bacteria and archaea, while vitamin B_{12} auxotrophies are widely spread in prokaryotes, protists and animals, including humans (11).

Vitamin B_{12} is a relevant compound from an anthropocentric perspective. It is an essential dietary compound (vitamin) with a recommended nutrient intake of 2.4 μg/day for healthy adults (4) and its deficiency has been associated with several pathologies, namely different forms of anemia and neurological dysfunction, amongst others (19). Additionally, it is also an industrially relevant compound since it is an essential co-factor in reactions incorporated in biotechnological processes, such as the production of 1,3-propanediol (2).

Production of vitamin B_{12} relies solely on microbial production, since chemical methods remain economically unviable due to the technical complexity of the synthesis process (8). *Pseudomonas denitrificans* and *Propionibacteria* subsp. are accounTable 6.for most of the industrially produced vitamin B_{12}, with a productivity as high as 300 $mg.L^{-1}$ (8).

Lactobacillus reuteri is a heterofermentative lactic acid bacterium that colonizes the gastro-intestinal tract of humans and other animals (23). It has been shown to contain a functionally active B_{12} biosynthetic gene cluster that encodes all the enzymes required for the synthesis of this important co-factor from 5-aminolevulinate (13, 14). Catalyzed by glycerol dehydratase (EC 4.2.1.30), a B_{12}-dependent reaction, *L. reuteri* is able to synthesize 3-hydroxypropanaldehyde (reuterin) (20). This compound with broad-spectrum antimicrobial activity (1) can be further reduced to 1,3-propanediol restoring NAD^+, if coupled to the oxidation of another carbon source.

We have recently studied the glucose and glycerol co-fermentation by *L. reuteri* using functional genomics and genome-scale modeling techniques. This enabled us to broaden our view of the physiological responses of *L. reuteri* to glycerol, evidencing strong implications in amino acid metabolism and B_{12} biosynthesis (12). Here, we have focused on these new targets and attempted to engineer the production of B_{12} in *L. reuteri* using physiological approaches. This led to the discovery that single amino acid omissions can significantly affect the production of B_{12} in *L. reuteri*. Hence, we studied the genome-wide transcriptional response of *L. reuteri* to the omission of cysteine in search of mechanistic insights and new leads for metabolic engineering.

Materials and Methods

Strains, media and culture conditions. We obtained *L. reuteri* JCM1112 (type-strain, human isolate) from the Japanese Collection of Microorganisms (Riken, Japan). It was cultured at 37ªC in an adapted version of chemically defined medium (CDM) from which vitamin B_{12} was omitted (22). When appropriate, 0.5% of glycerol was added. Cells were cultured under microaerophilic conditions (*i.e.* no stirring), unless stated otherwise. When mentioned, conditioned oxygen availability was ensured by cultivating L. reuteri in anaerobic jars filled with either O_2-free N_2, or a mixture of 95% N_2 and 5% CO_2 cultures.

Single omission growth experiments. We investigated for *L. reuteri*, the effect on B_{12} production and growth behavior, of omitting separately every single amino acid that enters in the composition of CDM. Parallel experiments were performed in the presence and absence of glycerol, omitting an amino acid per culture and using a culture without any omission as a reference. Independent biological duplos were carried out as described. An overnight culture of *L. reuteri* washed twice in 0.85 % NaCl (w/v) was used to inoculate all media combinations to an initial optical density at 600 nm (OD_{600}) of 0.05. Cultures were propagated in batch for five consecutive transfers in the corresponding media monitoring growth every 24 hours. After 48 hours, if we could not measure at least two doublings ($OD_{600} < 0.2$), the culture would be discarded and not used for further analysis. If the fourth transfer had an OD_{600} above 0.5 after 24 hours of incubation, it was used to inoculate 20 ml of the corresponding media. This last culture was used to determine the maximum specific growth rate (μ_{max}) and the B_{12} content.

Growth rate determination. L. reuteri JCM1112 was cultivated in the several variations of CDM, in 96 well microtiterplates (Greiner, Alphen a/d Rijn, The Netherlands). Different replicates were positioned on the plate using a checkerboard format distribution (3). Plates were incubated at 37°C in a Genios microtiterplate reader (Tecan, Zurich, Switzerland) set to monitor growth by measuring OD_{595} every 15 min. All measurements were independently performed at least twice in at least 8 biological replicates. μ_{max} was determined in a high-throughput fashion by in-house scripts that calculate μ for every five consecutive time points through out the growth curve and return its maximum value per well.

Coenzyme B_{12} determination. B_{12} production by *L. reuteri* in the different CDM variations was determined according to the Official Methods of Analysis of AOAC International, using the *L. delbrueckii* subsp. *lactis* ATCC 7830 vitamin B_{12} assay (6). Cell extracts for B_{12} analysis were prepared as described elsewhere (13).

Fermentation conditions. pH-controlled batch fermentations of *L. reuteri* in the presence or absence of cysteine were carried out in an experimental set-up consisting of

four vessels with a reaction volume of 400 ml. Prior to inoculation, the media vessels were gassed with O_2-free N_2 (15 ml/min) for 1 hour. Temperature was kept constant at 37°C and pH was fixed to 5.8 by titration with 5 M NaOH. Homogeneity was assured by continuously stirring throughout the whole fermentation. An exponentially growing culture in the corresponding media was used to inoculate the fermentors to an initial OD_{600} of 0.05. Periodic measurements of OD_{600} were used to monitor biomass formation. Samples for transcriptome analysis were harvested at mid-logarithmic growth phase (OD_{600} 1), and in early-stationary phase, 15 minutes after exponential growth ceased (Fig. 6.1). At this stage of the growth curve, we also sampled for B_{12} analysis (*idem*).

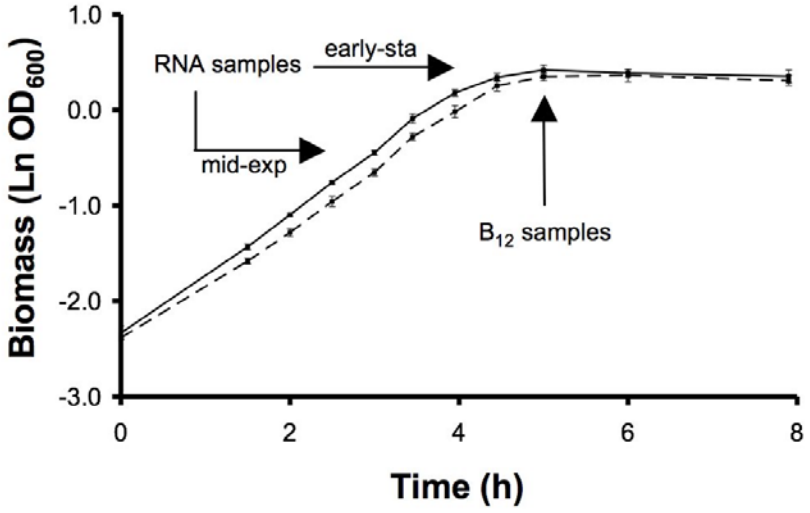

Figure 6.1. Biomass formation and sampling scheme in pH-controlled batch fermentations of CDM in the presence (solid line) or absence (dashed line) of cysteine.

Microarray design. Dedicated microarrays for *L. reuteri* JCM1112 were spotted in the high density Agilent 44K platform (Agilent Technologies, Santa Clara, CA, USA), based on the draft genome sequence of *L. reuteri* JCM1112 released by JGI (retrieved in 03/2006). We used the custom probe design covering 1700 (out of 1900) predicted coding regions (~90% coverage) that we had developed for the 11K format available at GEO (http://www.ncbi.nlm.nih.gov/geo) under accession number GPL6856, and had it printed four fold per array. Oligos were designed to probe the predicted coding regions, representing 82.9% with 24 probes (6 unique) or more, and only 3.9% with 12 (3 unique) or less.

RNA isolation. Cells harvested from cultures in the presence or absence of cysteine were sampled by rapid quenching using a cold methanol method (9). Extraction and purification of total RNA was carried out as previously described (12). RNA concentration was measured on a ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). The integrity of the mRNA species was confirmed on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). A threshold for the 23S/16S RNA ratio of 1.6 was set for the samples to be considered satisfactory. Only samples above were used for transcriptome analysis.

cDNA synthesis and labeling. For each sample, 20 μg of RNA were used for first strand cDNA synthesis using Superscript III reverse transcriptase (Invitrogen, Breda, The Netherlands) according to the recommendations of the manufacture. All samples of newly synthesized cDNA were purified and labeled with cyanine 3 and cyanine 5. Indirect labeling was executed resorting to the CyScribe first-strand cDNA labeling kit (Amersham, United Kingdom) following manufacturer's recommendations. cDNA concentration and labeling incorporation were verified using the ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA).

Microarray hybridization. We used 0.8 μg of labeled cDNA of each dye per hybridization. All samples were hybridized with each label at least once to facilitate the recognition of possible dye effects. A hybridization scheme consisting of a loop design comprising 16 microarrays was implemented to scrutinize the transcriptome of mid-logarithmic and early-stationary growth phase cells cultured in pH-controlled batch fermentations of CDM from which cysteine was omitted, or not. The following samples were hybridized per array labeled with cyanine3 and cyanine5, respectively: exp-F1 and exp-F2, exp-F2 and exp-F3, exp-F3 and exp-F4, exp-F4 and exp-F1, sta-F3 and sta-F4, sta-F4 and exp-F1, exp-F1 and exp-F3, exp-F3 and sta-F3, sta-F2 and sta-F1, sta-F3 and exp-F2, sta-F4 and sta-F3, sta-F1 and sta-F4, sta-F1 and exp-F1, exp-F2 and exp-F2, sta-F3 and exp-F3, exp-F1 and sta-F4. Here, the prefix exp- stands for cells harvested at mid logarithmic growth phase and sta- for cells harvested during early stationary. F1 and F3 represent totally independent biological duplicates cultured in CDM, and F2 and F4 represent also independent biological duplicates, but cultured in CDM lacking cysteine. The hybridizations were performed at 60°C for 17 h, after which, the slides were washed according to the recommendations of the manufacturer. Scanning took place immediately after drying the slides as described elsewhere (16).

Scanning and microarray data analysis. Slides were scanned with a ScanArray Express scanner (Perkin-Elmer) set to maximum resolution (5 μm). ImaGene (BioDiscovery) version 5.6 was used for image analysis, spot quantification and data

extraction. After normalizing the transcriptome data by local fitting of an M-A plot applying the loess algorithm (24) using the Limma package (17) in R (http://www.r-project.org), we analyzed the transcriptome data as described elsewhere (16). Statistical significance was tested from the deviation between biological duplicates by implementation of the eBayes function included in Limma (cross-probe variance estimation) and false discovery rate adjustment of the *p*-values (18). Two comparisons were established to characterize the impact in *L. reuteri* of removing cysteine (i) during exponential growth phase (mid-logarithmic phase response), and (ii) during early stationary growth phase (early-stationary phase response). Transcripts were considered for analysis if the *p*-value was smaller than 0.05 and the absolute Log_2 (ratio) greater than 0.585. The transcriptome data was visualized by projection on metabolic maps of the genome-scale model developed previously for *L. reuteri* (12).

Microarray accession numbers. The microarray platform developed in this study is available at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) (accession number pending). The microarray data obtained were deposited in the same repository (accession number pending).

Results

We have recently observed that cultivation of *L. reuteri* in a chemically defined medium (CDM) with glycerol has profound implications on B_{12} production and amino acid metabolism (12). Here, we followed these newly obtained leads and attempt to modulate the production of B_{12} by changing the amino acid composition of CDM and describing the phenotypic changes observed related to growth and to the production of this co-factor.

Single amino acid omissions and growth of L. reuteri. We found that eight amino acids could be omitted from CDM without completely inhibiting the growth of *L. reuteri.* These affected growth rate and final biomass formation to variable degrees (Table 6.1). The omission of Serine (Ser) profoundly reduced the specific growth rate and final optical density both in the absence or presence of glycerol. In CDM lacking isoleucine (Ile) the final biomass formation is much more affected than the growth rate. Omission of the other amino acids alanine (Ala), aspartate (Asp), cysteine (Cys), glycine (Gly), lysine (Lys), or proline (Pro) only slightly affected final biomass formation. The growth rates measured in all single amino acid omissions increased with the addition of glycerol, except for CDM lacking Gly, which was only mildly affected (6% reduction).

Single amino acid omissions and vitamin B_{12} production. L. reuteri JCM1112 produces approximately 20 $\mu g.L^{-1}.OD_{600}^{-1}$ of B_{12} in complete CDM without glycerol. If the latter is added, B_{12} production in *L. reuteri* increases about five fold to 100 $\mu g.L^{-1}.OD_{600}^{-1}$.

Table 6.1. Growth properties of *L. reuteri* in single amino acid deficient CDM supplemented or not with glycerol [a.]

Amino acid omission	Conc.[b.] (mM)	CDM		CDM + glycerol		Variation CDM to CDM + glycerol
		μ_{max} (h^{-1})	Final OD_{595}	μ_{max} (h^{-1})	Final OD_{595}	
None	n.a.	0.453 (100%)	1.21 (100%)	0.575 (100%)	1.29 (100%)	27%
Ala	2.69	0.368 (81%)	1.17 (97%)	0.519 (90%)	1.31 (102%)	41%
Asp	3.16	0.445 (98%)	1.20 (99%)	0.551 (96%)	1.31 (102%)	24%
Cys	0.83	0.387 (85%)	1.16 (96%)	0.512 (89%)	1.26 (98%)	32%
Gly	2.33	0.370 (82%)	1.17 (97%)	0.349 (61%)	1.21 (94%)	-6%
Ile	1.60	0.356 (79%)	0.41 (34%)	0.411 (72%)	0.46 (36%)	15%
Lys	2.68	0.433 (96%)	1.20 (99%)	0.540 (94%)	1.27 (98%)	25%
Pro	5.86	0.424 (94%)	1.19 (98%)	0.555 (97%)	1.28 (99%)	31%
Ser	3.24	0.106 (23%)	0.25 (21%)	0.131 (23%)	0.24 (19%)	24%

[a.] Values are expressed as average (% of same condition without any amino acid omission) of at least eight biological replicates. Experiment was repeated twice with similar results.
[b.] Concentration of amino acid in CDM when present (n.a., not applicable).

When B_{12} production was compared between *L. reuteri* cultures grown in complete, as opposed to single amino acid deficient-CDM, a great disparity of effects was clearly observed. (Table 6.2). The omission of Cys is the most remarkable since, even without the addition of glycerol, it leads to a 5-fold increase in B_{12} production relative to complete CDM. Furthermore, the boost in B_{12} synthesis can also be further incremented by the presence of glycerol, enabling the accumulation of stimulatory effects, resulting in over 350 $\mu g.L^{-1}.OD_{600}^{-1}$.

The increase in B_{12} production due to the addition of glycerol, although to varying proportions, was observed regardless of the amino acid omissions from the composition of CDM. When Ile is lacking, the largest stimulatory effect of glycerol is registered (20-fold). However, this is caused by the sharp reduction (greater than 10-fold) in B_{12} production to levels barely detectable when glycerol is absent, rather then by an increase of B_{12} production in CDM with glycerol.

Besides the effects already mention, in the absence of glycerol, *L. reuteri* produced higher amounts of vitamin B_{12} in CDM lacking Lys, Ala, Gly and Asp, whilst in its presence, only the omission of Ala and Asp had a positive effect.

Table 6.2. Vitamin B_{12} production by *L. reuteri* in single amino acid deficient CDM supplemented or not with glycerol [a.]

Amino acid omission	CDM	CDM + glycerol	Fold change from CDM to CDMglycerol	Fold change relative to reference condition[b.]
None	21.2 ± 3.6 (100%)	105.0 ± 17.0 (100%)	5	5
Ala	43.2 ± 4.7 (204%)	143.3 ± 10.9 (136%)	3	7
Asp	29.0 ± 4.8 (137%)	146.8 ± 10.4 (140%)	5	7
Cys	103.2 ± 13.0 (488%)	351.8 ± 28.4 (335%)	3	17
Gly	33.0 ± 3.3 (156%)	113.9 ± 14.1 (108%)	3	5
Ile	2.1 ± 0.4 (10%)	42.4 ± 5.8 (40%)	20	2
Lys	50.5 ± 5.1 (239%)	114.0 ± 22.5 (109%)	2	5
Pro	22.0 ± 4.7 (104%)	72.6 ± 11.9 (69%)	3	3
Ser	16.1 ± 3.6 (76%)	76.0 ± 10.4 (72%)	5	4

[a.] Values are expressed as average ± SD (% of same condition without any amino acid omission) of at least three technical replicates. Experiment was repeated twice with similar results.
[b.] Reference condition is CDM without glycerol added and without any amino acid omission.

Effect of oxygen on B_{12} production. Cysteine is known as a potent reducing agent. Omission of cysteine will lead to increased redox potential and oxygen availability in the growth medium. The concentration of oxygen has been reported to affect the production of B_{12} in *Propionibacteria* (7). For this reason, we decided to check whether the stimulatory effect caused by the omission of Cys, could be altered by varying oxygen availability. This was done by cultivating in parallel *L. reuteri* under microaerophilic conditions, and in an anaerobic vessel containing either pure N_2 or a mixture of 95% N_2 with 5% CO_2, and checking B_{12} production in all possible variations of CDM combining the presence and absence of glycerol and cysteine (Fig. 6.2). In 100% N_2, the production of B_{12} was slightly increased by about 30%. The only exception was the condition in which cysteine is absent

and glycerol is present, for which there is only a trend, but no significant increase (*p*-value > 0.05). Cultivation in the presence of 95% N_2 with 5% CO_2, caused the B_{12} content to increase slightly more, this time about 50%. The trend observed for the cultures that combine the absence of cysteine with the presence of glycerol is even more pronounced. Under this last condition B_{12} production was raised to 428 ± 40 $\mu g.L^{-1}.OD_{600}^{-1}$.

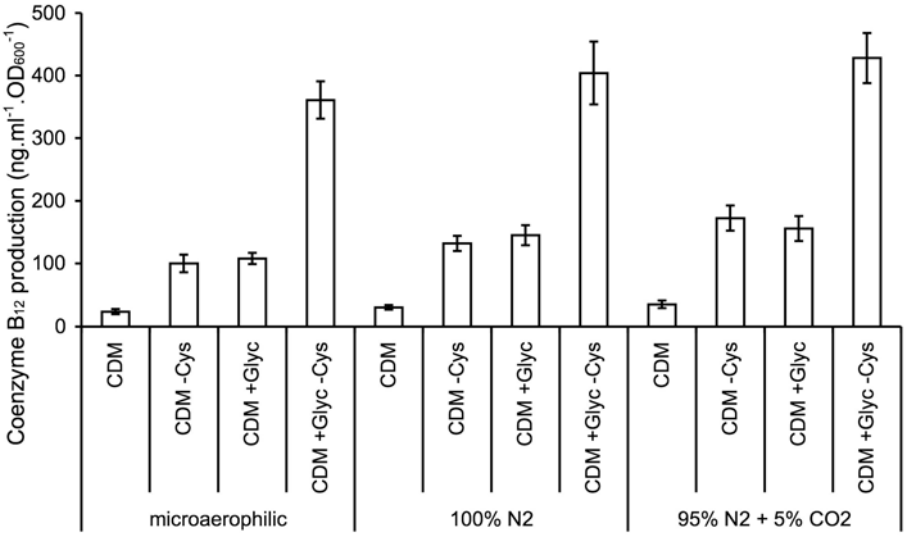

Figure 6.2. Vitamin B_{12} production by *L. reuteri* under different gas environments in variations of CDM. -Cys, cysteine omitted; +Glyc, supplemented with glycerol.

Transcriptomics of L. reuteri in absence of cysteine. To gain further insight on the stimulatory effect of Cys in B_{12} production, we studied the genome-wide transcriptional response of *L. reuteri* to cultivation in the absence of Cys, by developing and performing cDNA microarray experiments. We determined the specific responses in mid-exponential and early-stationary growth phases. The complete list of loci that were found to be differentially regulated is available in Table 6.S1 (supplementary material) and a small selection that is discussed in the tesxt is present at Table 6.3.

In mid-exponential phase, we found 140 genes differentially regulated, of which only 8 were found to be down-, while 132 were up-regulated. Concerning genes differently regulated in the stationary growth phase, we found 294 genes differentially regulated, 125 down- and 169 up-regulated. A total of 58 genes are shared between both data sets, while 82 were exclusively differentially regulated in exponential phase and 236 are specific for stationary phase.

Table 6.3. Selection of differentially regulated genes mentioned in the text (see Table 6.S1 for full list)

Locus	Product	M_{exp}	M_{sta}
Amino acid transport and metabolism			
Lreu_0190	Succinyl-diaminopimelate desuccinylase (EC 3.5.1.18)		-0.70
Lreu_0293	Cystathionine beta-lyase (EC 4.4.1.8) / Cystathionine gamma-lyase (EC 4.4.1.1)	1.72	
Lreu_0294	Cystine transport system permease protein	1.94	3.23
Lreu_0295	Cystine transport ATP-binding protein	2.26	3.16
Lreu_0348	Aspartate aminotransferase (EC 2.6.1.1)		-0.87
Lreu_0377	Succinyl-diaminopimelate desuccinylase (EC 3.5.1.18)		-1.24
Lreu_0425	Ornithine carbamoyltransferase (EC 2.1.3.3)	-3.38	-0.69
Lreu_0426	Carbamate kinase (EC 2.7.2.2)	-2.27	
Lreu_0445	Arginine deiminase (EC 3.5.3.6)	-2.09	
Lreu_0502	Cysteine desulfurase (EC 2.8.1.7)/Selenocysteine lyase (EC 4.4.1.16)		1.16
Lreu_0610	Diaminopimelate epimerase (EC 5.1.1.7)		-1.07
Lreu_0611	Aspartokinase (EC 2.7.2.4)		-1.51
Lreu_0612	Diaminopimelate decarboxylase (EC 4.1.1.20)		-0.70
Lreu_0613	Tetrahydrodipicolinate N-acetyltransferase (EC 2.3.1.89)		-0.65
Lreu_1553	Cysteine synthase (EC 2.5.1.47)	1.27	2.06
Lreu_1791	Homoserine O-succinyltransferase (EC 2.3.1.46)	0.80	1.27
Lreu_1792	Cysteine synthase (EC 2.5.1.47)	0.96	1.16
Nucleotide transport and metabolism			
Lreu_0123	Aspartate carbamoyltransferase (EC 2.1.3.2)		-5.32
Lreu_0124	Dihydroorotase (EC 3.5.2.3)		-5.36
Lreu_0125	Dihydroorotate dehydrogenase, catalytic subunit (EC 1.3.3.1)		-5.09
Lreu_0126	Orotidine 5'-phosphate decarboxylase (EC 4.1.1.23)		-4.91
Lreu_0127	Orotate phosphoribosyltransferase (EC 2.4.2.10)		-4.82
Coenzyme transport and metabolism			
Lreu_0510	Folylpolyglutamate synthase (EC 6.3.2.17)/Dihydrofolate synthase (EC 6.3.2.12)		-2.33
Lreu_0878	Diaminohydroxyphosphoribosylaminopyrimidine deaminase (EC 3.5.4.26) / 5-amino-6-(5-phosphoribosylamino)uracil reductase		-0.92
Lreu_0879	Riboflavin synthase alpha chain (EC 2.5.1.9)		-0.81
Lreu_0880	GTP cyclohydrolase II (EC 3.5.4.25) / 3,4-dihydroxy-2-butanone-4-phosphate synthase (EC 4.1.2.-)		-0.75
Lreu_1279	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase (EC 2.7.6.3)		-0.63
Lreu_1280	Dihydroneopterin aldolase (EC 4.1.2.25)		-0.73
Lreu_1710	Precorrin-2 C20-methyltransferase (EC 2.1.1.130)		0.71
Lreu_1711	Sirohydrochlorin cobaltochelatase (EC 4.99.1.3)		0.72

Locus	Product	M_{exp}	M_{sta}
Lreu_1712	Uroporphyrin-III C-methyltransferase (EC 2.1.1.107) / Uroporphyrinogen-III synthase (EC 4.2.1.75)		0.60
Posttranslational modification, protein turnover, chaperones			
Lreu_0324	Glutaredoxin		0.71
Lreu_0353	10 kDa chaperonin GROES		-0.83
Lreu_0354	60 kDa chaperonin GROEL		-0.84
Lreu_0376	Thioredoxin reductase (EC 1.8.1.9)	1.11	0.67
Lreu_0539	Thioredoxin	1.45	0.96
Inorganic ion transport and metabolism			
Lreu_1707	Cobalt transport protein cbiQ		0.85
Lreu_1708	Cobalt transport protein cbiN		0.63
Lreu_1709	CbiM protein		0.69

In order to facilitate the analysis of the transcriptome data, we examined the distribution of the genes found to be differentially regulated according to the functional class of the predicted encoded protein, using the COG classification system (21). The full distribution is available at Table 6.S2 (supplementary material 6S) and a selection is presented here based on its relevance for the topics here discussed (Fig. 6.4).

Not surprisingly, the COG category associated with amino acid transport and metabolism was found to be overrepresented both mid-exponential and stationary phase. The split within this category between up- and down-regulated is quite even. Specific attention was given to genes involved in cysteine metabolism. There are 2 genes (*lreu_1553* and *lreu_1792*) annotated in the chromosome of *L. reuteri* that are predicted to encode cysteine synthase (EC 2.5.1.47) and both are up-regulated in the absence of cysteine. The same holds for the genes (*lreu_0293-0295*, *lreu_0502*, *lreu_1791*) coding for enzymes involved in the metabolism of sulfur-containing compounds, such as cysteine desulfurase (EC 2.8.1.7), selenocysteine lyase (EC 4.4.1.16) and cystathionine beta- and gamma-lyases (respectively, EC 4.4.1.8 and EC 4.4.1.1), amongst others. Genes predicted to encode enzymes related to arginine, aspartate and ornithine tended to be down-regulated (*lreu_0425*, *lreu_0426* and *lreu_0445*). The latter have in common that they lead to the synthesis of carbomyl phosphate, a precursor of pyrimidines.

Harmoniously, genes associated with the COG category that includes proteins assigned to nucleotide transport and metabolism, are not only overrepresented, but also display a clear tendency to be down-regulated. Indeed, a closer look clearly shows that a complete cluster starting from *lreu_0123* to *lreu_0127* is drastically down-regulated in the absence of cysteine (M-value around -5).

Co-factors such as folate derivatives are important in the synthesis of DNA and RNA precursors. The folate biosynthesis gene cluster (*lreu_0510* and *lreu_1279-1280*, along with the one for riboflavin (*lreu_0878-880*) appear down-regulated when cysteine is omitted from CDM and account for roughly half of the regulated genes assigned to coenzyme transport and metabolism (Table 6.S1). As expected the B_{12} biosynthesis gene cluster was up-regulated.

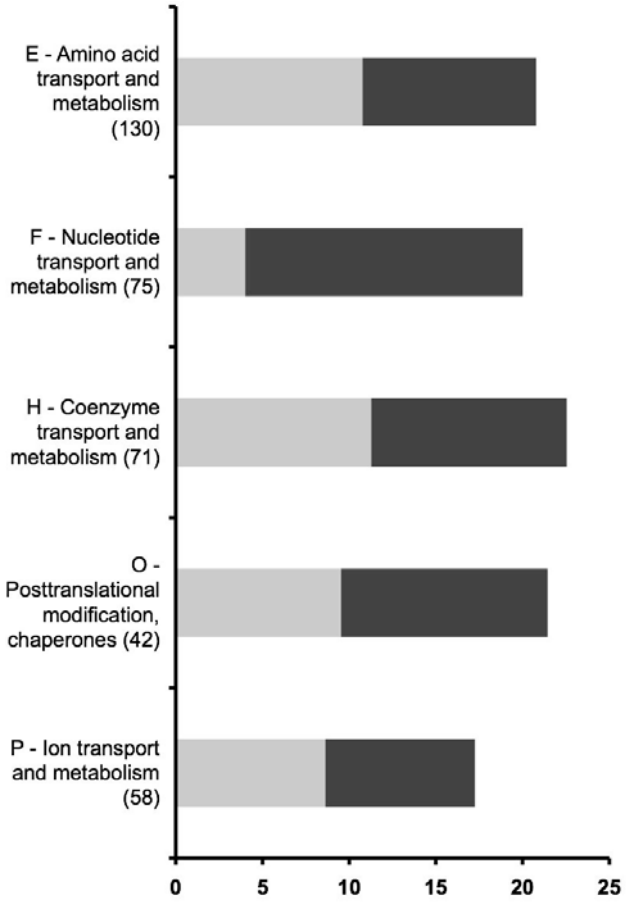

Figure 6.3. Distribution of transcripts whose expression was affected by glycerol during early-stationary growth phase throughout a selection of COGs (see Suplementary Material 1, Table 6.S2 for full distribution). Transcripts were considered to be differentially regulated for $M > |0.585|$ and p-value < 0.05. Light and dark grey bars represent proportion of up-regulated transcripts in the absence or presence of cysteine, respectively. Between brackets is the total number of genes from the genome of *L. reuteri* included in the corresponding COG.

An overrepresentation was observed for genes encoding proteins assigned to COG category related to posttranslational modification and chaperons. Again the split between up- and down- regulated appears quite even, implying that the absence of cysteine will not affect indiscriminately all chaperons. Amongst others, in the down-regulated group we find loci predicted to encode the co-chaperonin complex GroES and GroEL (*lreu_353-354*), while in the up-regulated we can find thioredoxin, thioredoxin reductase (EC 1.8.1.9) and glutaredoxin (*lreu_539*, *lreu_0376* and *lreu_0324*, respectively).

One final category is the ion transport and metabolism proteins. Within this category, most genes were down-regulated, which can be ascribed mainly to the down-regulation of unspecific ABC transporters. Highly relevant is the finding that the up-regulated group includes genes involved in the scavenging of cobalt from the environment (*lreu_1707-1709*).

Discussion

The average B_{12} content of an early-stationary culture of *L. reuteri* JCM1112 in CDM is approximately 20 $\mu g.L^{-1}.OD_{600}^{-1}$. This amount is increased about five fold if CDM is supplemented with glycerol. In these conditions, *L. reuteri* uses glycerol to recycle NAD+ via the conversion of glycerol to 3-hydroxypropanaldehyde, which is subsequently reduced to 1,3-propanediol (2). The co-fermentation of glucose and glycerol by *L. reuteri* has recently been reported to have a major effect on the amino acid metabolism (12). In this study, we have determined the effect on growth behavior and B_{12} production of individually omitting the amino acids present in CDM. This was carried out in parallel in the presence and absence of glycerol.

In this screening exercise, we have identified eight amino acids to be essential for *L. reuteri* to grow at least two generations in the first two days after inoculation. The well-established positive effect of glycerol on the growth rate and B_{12} production of *L. reuteri* cultivated in complete CDM was observed regardless of the omission of the single amino acids with the exception of glycine (Table 6.1). When glycine is omitted, the addition of glycerol to CDM still has a positive effect on B_{12} production (Table 6.2), but the effect on μ_{max} is lost. An explanation for this might reside in the fact that the consumption rate of this amino acid in the presence of glycerol is nearly twice as fast as the one measured in the absence of it (12). This is indicative of an increase demand for glycine in the presence of glycerol, which apparently cannot be met when *L. reuteri* has to rely solely on its own native production. This hypothesis is in agreement with the sharp decrease in μ_{max} observed in the absence of glycine and glycerol, both for *L. reuteri* and *L. plantarum* (22).

In the absence of glycerol, the single omission of several amino acids lead to important increments in B_{12} production, for instance aspartate 40%, glycine 60%, alanine

200% and lysine 240%. Any of these findings *per se* could lead to great improvements in B_{12} production processes, especially the omission of lysine since it has a very little impact on the growth behavior of *L. reuteri*. However, they are completely overshadowed by the 500% increase in B_{12} production caused by the omission of cysteine alone. Combined with the stimulatory effect of glycerol, a 17-fold increase in B_{12} production was achieved (Table 6.2). In the presence of glycerol, the omission of the other amino acids from CDM had relatively little effect on vitamin B_{12} production.

We have attempted to gain mechanistic insights and new clues on how to further increase the synthesis of B_{12} in *L. reuteri* by studying its genome-wide transcriptional response to the omission of cysteine. Due to the properties of cysteine as a reducing agent we decided to first carry out an exploratory experiment under controlled oxygen availability. When cultured anaerobically, *Lb reuteri* was the first organism reported to produce exclusively pseudovitamin B_{12}, presenting a great convenience if one attempts to purify it. Under tight anaerobic conditions, B_{12} production was increased in *L. reuteri* by little more than 30%. This could be anticipated, since *L. reuteri* has been shown to encode the oxygen-independent biosynthetic route towards B_{12} (14), and the initial step of the industrial production of B_{12} using *Propionibacteria* is carried out under strict anaerobic conditions (7). However, the increase caused by the absence of cysteine from the medium was not diminished under anaerobiosis, implying that this amino acid does indeed have other effects in the metabolism of *L. reuteri*. When we compare B_{12} production of cells grown anaerobically versus microaerophilically, combining the omission of cysteine with the addition of glycerol, it is clear that even though the trend is still there, the increase is not as high as expected. This suggests that after increasing 20-fold the production of B_{12} another bottleneck is encountered.

The transcriptome analysis of cells grown in the absence of cysteine in comparison to the ones grown in complete CDM confirms that indeed in *L. reuteri* cysteine is made from serine via cysteine synthase, and using sulfur groups derived from methionine. As mentioned, *L. reuteri* contains two copies of cysteine synthase genes (*lreu_1553* and *lreu_1792*), which are up-regulated in the absence of cysteine. We noticed that while the increased expression of the copy encoded by *lreu_1792* changes very little between the exponential and stationary data sets, its paralog, *lreu_1553* seems to be preferred in the later stages of growth. All pathways from amino acids to carbomyl phosphate appeared downregulated along with the operon that encodes the machinery necessary to channel it to pyrimidine biosynthesis. This is most likely related to the drop in growth rate that we have characterized when cysteine is omitted from CDM (~15% reduction).

The presence of the B_{12}-biosynthesis gene cluster among the genes that are upregulated in the absence of Cys was quite expected taking into account the phenotype

that we have exposed here. The fact that we cannot find the full length of the cluster back as up-regulated can be easily explained by the large size and fragility of the transcribed mRNA species as previously noted while performing Northern blotting analysis (14). Nonetheless, if we loosen the stringency of our established thresholds, the whole cluster will prove to have a positive M-value, instead of just the genes in its centre. Based on the transcriptome data alone, *de novo* production of riboflavin and folate seem to be negatively affected by the omission of Cys. Since both of these vitamins are actually present in CDM in relative excess and we have recently illustrated metabolic engineering strategies to increase folate production in *L. reuteri* (15), we decided not to pursue this any further.

The omission of Cys has implications on sulfur metabolism that extend beyond its own biosynthesis. The ubiquity of the loci encoding thioredoxin and thioredoxin reductase (EC 1.8.1.9) in both data sets and glutaredoxin in the stationary phase set is illustrative of such. These are known to reduce other proteins by cysteine thiol-disulfide exchange, acting as antioxidants. Here, seems to reside interactions yet to clarify, which could lead to even higher B_{12} production. Partially, related is cobalt bioavailability. We observed the up-regulation of genes for proteins involved in the uptake of cobalt, which is logical since this metal is positioned at the core of the tetrapyrrole macrocycle of B_{12} (10). Adding more cobalt to CDM supplemented with glycerol and without cysteine does not yield significant increase in B_{12} production (data not shown), which was not unexpected since CDM contains an excess of cobalt. This indicates that the rate of cobalt supply is a limiting factor that is compensated by increased expression of one or more cobalt transporters. Bioavailability of cobalt could be the bottleneck that did not allow us to engineer the production past the 20-fold increase.

We have used physiological approaches to modulate the production of B_{12} in *L. reuteri* JCM1112 from nearly undetecTable 6.levels to 20-fold higher than previously reported (15). Furthermore, we have characterized the impact on growth behavior of all cultivation conditions tested, and analyzed by cDNA microarray experiments the transcriptional response of *L. reuteri* to the omission of cysteine. This has lead to new insights that could be used to increase even further the production of B_{12}.

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Content of the supplementary material of Chapter 6

Table 6.S1. Complete list of transcripts from *L. reuteri* that are differentially expressed in the absence of cysteine[a]. Comparisons were established to identify mid-log and early-stationary specific responses.

Locus	Product[b]	Exp		Sta		COG	Acession number
		M[c]	p[d]	M[c]	p[d]		
	Energy production and conversion						
Lreu_0539	Thioredoxin	1.45	0.00	0.96	0.00	C	gi|148543774
Lreu_0631	Pyruvate dehydrogenase E1 component alpha subunit (EC 1.2.4.1)	1.15	0.00			C	gi|148543863
Lreu_0632	Pyruvate dehydrogenase E1 component beta subunit (EC 1.2.4.1)	1.24	0.00			C	gi|148543864
Lreu_0633	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.12)	1.23	0.00			C	gi|148543865
Lreu_0634	Dihydrolipoamide dehydrogenase (EC 1.8.1.4)	1.07	0.01			C	gi|148543866
Lreu_1734	Propanol dehydrogenase (EC 1.1.1.-)	0.93	0.00			C	gi|148544940
Lreu_1735	Propionaldehyde dehydrogenase [CoA-acylating] (EC 1.2.1.-)	0.92	0.00			C	gi|148544941
	Cell cycle control, cell division, chromosome partitioning						
Lreu_1027	Cell filamentation protein fic			-1.92	0.00	D	gi|148544254
Lreu_1939	Glucose inhibited division protein A			1.29	0.02	D	gi|148545138
	Amino acid transport and metabolism						
Lreu_0076	Amino acid permease			0.80	0.02	E	gi|148543318
Lreu_0098	Glutamine-binding protein / Glutamine transport system permease protein glnP			1.22	0.00	E	gi|148543337
Lreu_0099	Glutamine transport ATP-binding protein glnQ			1.59	0.01	E	gi|148543338
Lreu_0185	Glycine betaine transport system permease protein			-0.60	0.02	E	gi|148543422
Lreu_0190	Succinyl-diaminopimelate desuccinylase (EC 3.5.1.18)			-0.70	0.03	E	gi|148543427
Lreu_0293	Cystathionine beta-lyase (EC 4.4.1.8) / Cystathionine gamma-lyase (EC 4.4.1.1)	1.72	0.06			E	gi|148543530
Lreu_0294	Cystine transport system permease protein	1.94	0.00	3.23	0.00	E	gi|148543531
Lreu_0295	Cystine transport ATP-binding protein	2.26	0.00	3.16	0.01	E	gi|148543532
Lreu_0348	Aspartate aminotransferase (EC 2.6.1.1)			-0.87	0.01	E	gi|148543584
Lreu_0357	Amino acid permease			0.70	0.01	E	gi|148543593
Lreu_0377	Succinyl-diaminopimelate desuccinylase (EC 3.5.1.18)			-1.24	0.00	E	gi|148543613
Lreu_0385	Amino acid permease			-0.78	0.00	E	gi|148543621
Lreu_0425	Ornithine carbamoyltransferase (EC 2.1.3.3)	-3.38	0.00	-0.69	0.02	E	gi|148543661
Lreu_0426	Carbamate kinase (EC 2.7.2.2)	-2.27	0.00			E	gi|148543662
Lreu_0445	Arginine deiminase (EC 3.5.3.6)	-2.09	0.02			E	gi|148543680
Lreu_0502	Cysteine desulfurase (EC 2.8.1.7) / Selenocysteine lyase (EC 4.4.1.16)			1.16	0.04	E	gi|148543737
Lreu_0515	Histidine transport system permease protein hisM	-0.99	0.00			E	gi|148543750
Lreu_0570	Dipeptidase A (EC 3.4.13.-)			-1.32	0.00	E	gi|148543805
Lreu_0610	Diaminopimelate epimerase (EC 5.1.1.7)			-1.07	0.00	E	gi|148543844
Lreu_0611	Aspartokinase (EC 2.7.2.4)			-1.51	0.00	E	gi|148543845
Lreu_0612	Diaminopimelate decarboxylase (EC 4.1.1.20)			-0.70	0.02	E	gi|148543846
Lreu_0613	Tetrahydrodipicolinate N-acetyltransferase (EC 2.3.1.89)			-0.65	0.02	E	gi|148543847
Lreu_0726	Arginine transport ATP-binding protein artP			1.95	0.01	E	gi|148543957
Lreu_0727	Arginine-binding protein / Arginine transport			1.73	0.04	E	gi|148543958

Locus	Product[b]	Exp		Sta		COG	Acession number
		M[c]	p[d]	M[c]	p[d]		
	system permease protein artQ						
Lreu_0731	Argininosuccinate synthase (EC 6.3.4.5)	6.71	0.00			E	gi|148543962
Lreu_0732	Argininosuccinate lyase (EC 4.3.2.1)	6.43	0.00			E	gi|148543963
Lreu_1081	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase (EC 2.1.1.14)			-1.36	0.05	E	gi|148544305
Lreu_1313	Hypothetical protein			-0.68	0.00	E	gi|148544537
Lreu_1383	Arginine/ornithine antiporter	0.68	0.00			E	gi|148544599
Lreu_1384	Pyridoxal-dependent aminotransferase, arginine catabolism	1.69	0.00			E	gi|148544600
Lreu_1500	Lysine-specific permease	-0.64	0.05			E	gi|148544712
Lreu_1544	Xaa-His dipeptidase (EC 3.4.13.3)	0.67	0.02			E	gi|148544756
Lreu_1553	Cysteine synthase (EC 2.5.1.47)	1.27	0.00	2.06	0.00	E	gi|148544763
Lreu_1732	Ethanolamine utilization protein eutS	0.87	0.00			E	gi|148544938
Lreu_1748	Propanediol utilization protein pduB	1.04	0.00			E	gi|148544954
Lreu_1791	Homoserine O-succinyltransferase (EC 2.3.1.46)	0.80	0.00	1.27	0.00	E	gi|148544997
Lreu_1792	Cysteine synthase (EC 2.5.1.47)	0.96	0.00	1.16	0.00	E	gi|148544998
Lreu_1866	Amino acid permease			1.45	0.00	E	gi|148545070
Lreu_1938	Asparagine synthetase [glutamine-hydrolyzing] (EC 6.3.5.4)			0.92	0.00	E	gi|148545137
	Nucleotide transport and metabolism						
Lreu_0085	Carbamoyl-phosphate synthase large chain (EC 6.3.5.5)			-2.76	0.03	F	gi|148543327
Lreu_0086	Carbamoyl-phosphate synthase small chain (EC 6.3.5.5)			-2.80	0.03	F	gi|148543328
Lreu_0102	Inosine-uridine preferring nucleoside hydrolase (EC 3.2.2.1)	0.73	0.00	-0.77	0.01	F	gi|148543341
Lreu_0111	Deoxyribose-phosphate aldolase (EC 4.1.2.4)	0.77	0.00			F	gi|148543350
Lreu_0114	Purine nucleoside phosphorylase II (EC 2.4.2.1)	0.94	0.00			F	gi|148543353
Lreu_0123	Aspartate carbamoyltransferase (EC 2.1.3.2)			-5.32	0.00	F	gi|148543362
Lreu_0124	Dihydroorotase (EC 3.5.2.3)			-5.36	0.00	F	gi|148543363
Lreu_0125	Dihydroorotate dehydrogenase, catalytic subunit (EC 1.3.3.1)			-5.09	0.00	F	gi|148543364
Lreu_0126	Orotidine 5'-phosphate decarboxylase (EC 4.1.1.23)			-4.91	0.00	F	gi|148543365
Lreu_0127	Orotate phosphoribosyltransferase (EC 2.4.2.10)			-4.82	0.00	F	gi|148543366
Lreu_0323	Ribonucleoside-diphosphate reductase alpha chain (EC 1.17.4.1)			1.01	0.00	F	gi|148543560
Lreu_0456	Uracil phosphoribosyltransferase (EC 2.4.2.9)			-1.27	0.00	F	gi|148543691
Lreu_0460	Uracil permease			-2.14	0.00	F	gi|148543695
Lreu_0644	ComE operon protein 2			-0.88	0.05	F	gi|148543876
Lreu_1498	Deoxyguanosine kinase (EC 2.7.1.113)			1.78	0.04	F	gi|148544710
Lreu_1617	GMP synthase [glutamine-hydrolyzing] (EC 6.3.5.2)			-1.21	0.00	F	gi|148544824
Lreu_1828	Xanthine permease			0.73	0.01	F	gi|148545034
	Carbohydrate transport and metabolism						
Lreu_0083	Oligo-1,6-glucosidase (EC 3.2.1.10)			-1.79	0.03	G	gi|148543325
Lreu_0112	Phosphopentomutase (EC 5.4.2.7)	0.76	0.00			G	gi|148543351
Lreu_0289	Beta-galactosidase small subunit (EC 3.2.1.23)	0.61	0.01			G	gi|148543526
Lreu_0380	Phosphoglucomutase (EC 5.4.2.2) / Phosphomannomutase (EC 5.4.2.8)			-0.69	0.00	G	gi|148543616
Lreu_0405	D-ribose mutarotase (EC 5.1.3.-)			1.56	0.00	G	gi|148543641

Locus	Product[b]	Exp		Sta		COG	Acession number
		M[c]	p[d]	M[c]	p[d]		
Lreu_0406	Deoxyribose transporter			1.34	0.00	G	gi|148543642
Lreu_0480	L-ribulokinase (EC 2.7.1.16)	1.21	0.00	0.85	0.01	G	gi|148543715
Lreu_0481	L-ribulose-5-phosphate 4-epimerase (EC 5.1.3.4)	0.94	0.00	0.73	0.01	G	gi|148543716
Lreu_0723	Fructosamine kinase family protein			-0.63	0.02	G	gi|148543954
Lreu_0751	Pyruvate kinase (EC 2.7.1.40)			-0.94	0.02	G	gi|148543982
Lreu_0910	Alpha-galactosidase (EC 3.2.1.22)	1.09	0.01			G	gi|148544139
Lreu_1026	Glucosamine-6-phosphate isomerase (EC 3.5.99.6)			-0.60	0.03	G	gi|148544253
Lreu_1319	Acetyltransferase (EC 2.3.1.-)	-0.58	0.00			G	gi|148544543
Lreu_1686	Xylulose-5-phosphate (EC 4.1.2.9) / Fructose-6-phosphate phosphoketolase (EC 4.1.2.22)			-0.71	0.03	G	gi|148544892
Lreu_1765	Glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49)			-1.05	0.00	G	gi|148544971
Lreu_1768	Lactose permease	1.38	0.00	1.03	0.04	G	gi|148544974
Lreu_1777	Galactokinase (EC 2.7.1.6)	0.63	0.02			G	gi|148544983
Lreu_1875	Putative NAD-dependent dehydrogenase	0.96	0.00	1.31	0.00	G	gi|148545079
Lreu_1882	Putative NAD-dependent dehydrogenase	1.41	0.01	1.36	0.00	G	gi|148545086
	Coenzyme transport and metabolism						
Lreu_0155	Nicotinate phosphoribosyltransferase (EC 2.4.2.11)			-0.79	0.00	H	gi|148543394
Lreu_0503	Thiamine biosynthesis protein thiI			1.32	0.00	H	gi|148543738
Lreu_0510	Folylpolyglutamate synthase (EC 6.3.2.17) / Dihydrofolate synthase (EC 6.3.2.12)			-2.33	0.00	H	gi|148543745
Lreu_0641	Phosphopantetheine adenylyltransferase (EC 2.7.7.3)			0.59	0.01	H	gi|148543873
Lreu_0734	Biotin operon repressor / Biotin--[acetyl-CoA-carboxylase] synthetase (EC 6.3.4.15)			-1.45	0.00	H	gi|148543965
Lreu_0878	Diaminohydroxyphosphoribosylaminopyrimidine deaminase (EC 3.5.4.26) / 5-amino-6-(5-phosphoribosylamino)uracil reductase			-0.92	0.00	H	gi|148544108
Lreu_0879	Riboflavin synthase alpha chain (EC 2.5.1.9)			-0.81	0.00	H	gi|148544109
Lreu_0880	GTP cyclohydrolase II (EC 3.5.4.25) / 3,4-dihydroxy-2-butanone-4-phosphate synthase (EC 4.1.2.-)			-0.75	0.01	H	gi|148544110
Lreu_1080	S-adenosylmethionine:2-demethylmenaquinone methyltransferase (EC 2.1.-.-)			1.52	0.00	H	gi|148544304
Lreu_1279	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase (EC 2.7.6.3)			-0.63	0.00	H	gi|148544503
Lreu_1280	Dihydroneopterin aldolase (EC 4.1.2.25)			-0.73	0.00	H	gi|148544504
Lreu_1602	LysM domain protein			1.36	0.02	H	gi|148544809
Lreu_1710	Precorrin-2 C20-methyltransferase (EC 2.1.1.130)			0.71	0.00	H	gi|148544916
Lreu_1711	Sirohydrochlorin cobaltochelatase (EC 4.99.1.3)			0.72	0.00	H	gi|148544917
Lreu_1712	Uroporphyrin-III C-methyltransferase (EC 2.1.1.107) / Uroporphyrinogen-III synthase (EC 4.2.1.75)			0.60	0.01	H	gi|148544918
Lreu_1785	Nicotinamide mononucleotide transporter			0.64	0.03	H	gi|148544991
	Lipid transport and metabolism						
Lreu_0103	3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157)	0.93	0.01	-1.12	0.01	I	gi|148543342
Lreu_0335	Acyl-[acyl-carrier-protein] hydrolase (EC 3.1.2.14)			0.82	0.00	I	gi|148543572
	Translation, ribosomal structure and biogenesis						
Lreu_0007	SSU ribosomal protein S6P			0.60	0.02	J	gi|148543250

Locus	Product[b]	Exp		Sta		COG	Acession number
		M[c]	p[d]	M[c]	p[d]		
Lreu_0009	SSU ribosomal protein S18P			1.87	0.00	J	gi|148543252
Lreu_0261	Peptidyl-tRNA hydrolase (EC 3.1.1.29)			0.77	0.01	J	gi|148543498
Lreu_0263	Heat shock protein 15			0.89	0.01	J	gi|148543500
Lreu_0313	LSU ribosomal protein L10P			0.66	0.02	J	gi|148543550
Lreu_0314	LSU ribosomal protein L12P (L7/L12)			1.97	0.00	J	gi|148543551
Lreu_0454	Sua5/YciO/YrdC/YwlC family protein			0.66	0.00	J	gi|148543689
Lreu_0529	Queuine tRNA-ribosyltransferase (EC 2.4.2.29)			1.10	0.00	J	gi|148543764
Lreu_0647	SSU ribosomal protein S20P			1.33	0.00	J	gi|148543879
Lreu_0698	LSU ribosomal protein L7AE			0.70	0.01	J	gi|148543930
Lreu_0699	Bacterial Protein Translation Initiation Factor 2 (IF-2)			0.75	0.00	J	gi|148543931
Lreu_0700	Ribosome-binding factor A			0.84	0.00	J	gi|148543932
Lreu_1190	LSU ribosomal protein L27P			1.20	0.00	J	gi|148544414
Lreu_1191	hypothetical ribosome-associated protein			0.73	0.01	J	gi|148544415
Lreu_1224	Peptide deformylase (EC 3.5.1.88)			-1.32	0.00	J	gi|148544448
Lreu_1270	Arginyl-tRNA synthetase (EC 6.1.1.19)	1.66	0.01	0.99	0.00	J	gi|148544494
Lreu_1521	Ribosomal large subunit pseudouridine synthase D (EC 4.2.1.70)			0.73	0.03	J	gi|148544733
Lreu_1605	23S rRNA methyltransferase (EC 2.1.1.-)			1.36	0.05	J	gi|148544812
Lreu_1943	Ribonuclease P protein component (EC 3.1.26.5)			0.82	0.00	J	gi|148545142
	Transcription						
Lreu_0088	Transcriptional regulator, LacI family	0.84	0.03			K	gi|148543330
Lreu_0272	Hypothetical membrane spanning protein			-1.02	0.00	K	gi|148543509
Lreu_0485	Transcriptional regulator, GntR family			-0.62	0.01	K	gi|148543720
Lreu_0550	Catabolite control protein A			0.82	0.00	K	gi|148543785
Lreu_0696	N utilization substance protein A			0.59	0.01	K	gi|148543928
Lreu_0697	Hypothetical cytosolic protein			0.67	0.01	K	gi|148543929
Lreu_0704	Heat-inducible transcription repressor hrcA			-0.71	0.00	K	gi|148543936
Lreu_0831		1.30	0.00			K	gi|148544061
Lreu_0932	Fibronectin-binding protein / Fibrinogen-binding protein			0.93	0.00	K	gi|148544161
Lreu_1109	Phage tail protein	1.81	0.00	0.59	0.01	K	gi|148544333
Lreu_1111	Phage protein	1.76	0.00	0.59	0.02	K	gi|148544335
Lreu_1112		1.77	0.00			K	gi|148544336
Lreu_1505	Cold shock protein			1.44	0.00	K	gi|148544717
Lreu_1528	Transcriptional regulators, LysR family			-0.81	0.00	K	gi|148544740
Lreu_1577	Transcriptional regulators, LysR family			-1.38	0.00	K	gi|148544786
Lreu_1876	Transcriptional regulator, MarR family	0.92	0.01	1.22	0.00	K	gi|148545080
Lreu_1878	Transcriptional regulator, MarR family	1.74	0.00	2.04	0.00	K	gi|148545082
	Replication, recombination and repair						
Lreu_0001	Chromosomal replication initiator protein dnaA			0.76	0.00	L	gi|148543244
Lreu_0002	DNA polymerase III, beta chain (EC 2.7.7.7)			0.97	0.00	L	gi|148543245
Lreu_0008	Phage single-strand DNA binding protein			0.65	0.05	L	gi|148543251
Lreu_0012	Replicative DNA helicase (EC 3.6.1.-)			0.73	0.00	L	gi|148543255
Lreu_0050	ATP-dependent nuclease subunit A			0.60	0.05	L	gi|148543293

Locus	Product[b]	Exp		Sta		COG	Acession number
		M[c]	p[d]	M[c]	p[d]		
Lreu_0151	Transposase			-1.03	0.05	L	gi|148543390
Lreu_0262	Transcription-repair coupling factor			0.67	0.04	L	gi|148543499
Lreu_0383	Excinuclease ABC subunit B			0.81	0.01	L	gi|148543619
Lreu_0384	Excinuclease ABC subunit A			1.03	0.01	L	gi|148543620
Lreu_0511	DNA repair protein radC			-2.12	0.00	L	gi|148543746
Lreu_0526	DNA mismatch repair protein mutL			0.94	0.00	L	gi|148543761
Lreu_0527	Holliday junction DNA helicase ruvA			0.88	0.00	L	gi|148543762
Lreu_0528	Holliday junction DNA helicase ruvB			0.96	0.00	L	gi|148543763
Lreu_0640	Methyltransferase (EC 2.1.1.-)			0.60	0.00	L	gi|148543872
Lreu_0643	COME operon protein 1			-0.96	0.01	L	gi|148543875
Lreu_0661	Single-stranded-DNA-specific exonuclease recJ (EC 3.1.-.-)			0.73	0.03	L	gi|148543893
Lreu_0811	RecT protein	1.26	0.01	0.94	0.04	L	gi|148544041
Lreu_0849	Phage protein	1.49	0.04			L	gi|148544079
Lreu_0918	DNA replication protein dnaD			0.98	0.00	L	gi|148544147
Lreu_1070	Transposase			-0.66	0.02	L	gi|148544294
Lreu_1120	Terminase small subunit	1.54	0.00			L	gi|148544344
Lreu_1139	RecT protein	1.00	0.01	1.04	0.01	L	gi|148544363
Lreu_1369	Putative galactofuranosyltransferase (EC 2.4.1.-)			0.62	0.01	L	gi|148544585
Lreu_1656	Transposase			-0.65	0.03	L	gi|148544862
Lreu_1773	Transposase			-0.88	0.04	L	gi|148544979
	Cell wall/membrane/envelope biogenesis						
Lreu_0317	Large-conductance mechanosensitive channel			0.90	0.02	M	gi|148543554
Lreu_1114	Phage protein	1.75	0.00			M	gi|148544338
Lreu_1370	alpha-D-Glcp alpha-1,6-galactosyltransferase (EC 2.4.1.-)			0.63	0.01	M	gi|148544586
Lreu_1372				0.99	0.00	M	gi|148544588
Lreu_1374	UDP-galactopyranose mutase (EC 5.4.99.9)			0.64	0.00	M	gi|148544590
Lreu_1375	Undecaprenyl-phosphate beta-glucosephosphotransferase (EC 2.7.8.-)			0.69	0.00	M	gi|148544591
Lreu_1562	N-acetylglucosaminyltransferase (EC 2.4.1.-)			0.64	0.03	M	gi|148544772
Lreu_1875	Putative NAD-dependent dehydrogenase	0.96	0.00	1.31	0.00	M	gi|148545079
Lreu_1882	Putative NAD-dependent dehydrogenase	1.41	0.01	1.36	0.00	M	gi|148545086
Lreu_1890	Cellulose synthase catalytic subunit [UDP-forming] (EC 2.4.1.12)			0.68	0.05	M	gi|148545094
	Posttranslational modification, protein turnover, chaperones						
Lreu_0324	Glutaredoxin			0.71	0.01	O	gi|148543561
Lreu_0353	10 kDa chaperonin GROES			-0.83	0.00	O	gi|148543589
Lreu_0354	60 kDa chaperonin GROEL			-0.84	0.00	O	gi|148543590
Lreu_0376	Thioredoxin reductase (EC 1.8.1.9)	1.11	0.00	0.67	0.01	O	gi|148543612
Lreu_0508	Transport ATP-binding protein cydC			0.70	0.04	O	gi|148543743
Lreu_0539	Thioredoxin	1.45	0.00	0.96	0.00	O	gi|148543774
Lreu_0705	GrpE protein			-0.61	0.00	O	gi|148543937
Lreu_0707	Chaperone protein dnaJ			-0.74	0.00	O	gi|148543939
Lreu_1388	Hypothetical protein			-1.08	0.01	O	gi|148544604

Locus	Product[b]	Exp		Sta		COG	Acession number
		M[c]	p[d]	M[c]	p[d]		
	Inorganic ion transport and metabolism						
Lreu_0191	ABC transporter permease protein			-0.86	0.00	P	gi|148543428
Lreu_0192	ABC transporter ATP-binding protein			-0.94	0.00	P	gi|148543429
Lreu_0193	ABC transporter substrate-binding protein			-0.93	0.00	P	gi|148543430
Lreu_0567	Arsenate reductase family protein	1.62	0.00	1.76	0.00	P	gi|148543802
Lreu_1016	Chloride channel protein			0.61	0.01	P	gi|148544243
Lreu_1199	ABC transporter substrate-binding protein			-0.77	0.05	P	gi|148544423
Lreu_1507	Cobalt-zinc-cadmium resistance protein czcD			-0.71	0.03	P	gi|148544719
Lreu_1707	Cobalt transport protein cbiQ			0.85	0.00	P	gi|148544913
Lreu_1708	Cobalt transport protein cbiN			0.63	0.02	P	gi|148544914
Lreu_1709	CbiM protein			0.69	0.00	P	gi|148544915
	Secondary metabolites biosynthesis, transport and catabolism						
Lreu_0133	Alpha-acetolactate decarboxylase (EC 4.1.1.5)			-1.24	0.02	Q	gi|148543372
Lreu_0156	Pyrazinamidase (EC 3.5.1.-) / Nicotinamidase (EC 3.5.1.19)			-0.69	0.00	Q	gi|148543395
Lreu_1738	Ethanolamine utilization protein eutN	0.96	0.00			Q	gi|148544944
Lreu_1740	PduL	1.06	0.00			Q	gi|148544946
Lreu_1741	Propanediol utilization protein pduA	1.02	0.00			Q	gi|148544947
Lreu_1742		1.08	0.00			Q	gi|148544948
Lreu_1743		1.07	0.00			Q	gi|148544949
Lreu_1744	Glycerol dehydratase reactivation factor large subunit	1.07	0.00			Q	gi|148544950
Lreu_1745	Diol dehydratase gamma subunit (EC 4.2.1.28)	1.12	0.00			Q	gi|148544951
Lreu_1747	Diol dehydratase large subunit (EC 4.2.1.28)	1.09	0.00			Q	gi|148544953
Lreu_1749	Propanediol utilization protein pduA	1.04	0.00			Q	gi|148544955
	General function prediction only						
Lreu_0128				-1.73	0.00	R	gi|148543367
Lreu_0334	Tetrapyrrole (Corrin/Porphyrin) methylase family protein			0.79	0.00	R	gi|148543571
Lreu_0347	Carbon-nitrogen hydrolase family protein			-0.91	0.05	R	gi|148543583
Lreu_0381	Hydrolase (HAD superfamily)			-2.02	0.00	R	gi|148543617
Lreu_0386	ATP-binding protein (contains P-loop)			0.59	0.01	R	gi|148543622
Lreu_0479	Arabinose-proton symporter	1.20	0.00	0.70	0.04	R	gi|148543714
Lreu_0519	Peptidase, M16 family			0.97	0.00	R	gi|148543754
Lreu_0522	Colligrin			-1.31	0.00	R	gi|148543757
Lreu_0569	Putative competence protein/transcription factor			-2.84	0.00	R	gi|148543804
Lreu_0614	Putative N-acetyldiaminopimelate deacetylase (EC 3.5.1.47)			-0.61	0.02	R	gi|148543848
Lreu_0645	COME operon protein 3			-0.79	0.01	R	gi|148543877
Lreu_0665	DNA primase			1.64	0.00	R	gi|148543897
Lreu_0833	Terminase large subunit	1.29	0.00			R	gi|148544063
Lreu_0919	Transporter, drug/metabolite exporter family			0.94	0.01	R	gi|148544148
Lreu_0999	Transporter, Drug/Metabolite Exporter family			-1.22	0.00	R	gi|148544227
Lreu_1079	Multidrug resistance efflux pump			1.31	0.00	R	gi|148544303

Locus	Product[b]	Exp		Sta		COG	Acession number
		M[c]	p[d]	M[c]	p[d]		
Lreu_1082	Polyketide cyclase family protein			-1.64	0.01	R	gi|148544306
Lreu_1117	Terminase large subunit	1.43	0.00			R	gi|148544341
Lreu_1283	CAAX amino terminal protease family			-0.92	0.00	R	gi|148544507
Lreu_1299	Multidrug resistance protein B			-0.60	0.02	R	gi|148544523
Lreu_1449	(R,R)-butanediol dehydrogenase (EC 1.1.1.4) / Acetoin dehydrogenase (EC 1.1.1.5)			-0.61	0.01	R	gi|148544662
Lreu_1516	Zn-dependent hydrolase (EC 3.-.-.-)			0.63	0.00	R	gi|148544728
Lreu_1519	Multidrug resistance protein B			0.81	0.02	R	gi|148544731
Lreu_1552	IRON-SULFUR FLAVOPROTEIN	0.92	0.03	1.04	0.00	R	gi|148544762
Lreu_1582	regulatory protein (pfoS/R)			-0.81	0.05	R	gi|148544791
Lreu_1606	Transporter, MFS superfamily			1.42	0.05	R	gi|148544813
Lreu_1652	Phosphoglycolate phosphatase (EC 3.1.3.18)			-0.79	0.01	R	gi|148544859
Lreu_1659	Short chain dehydrogenase			-2.11	0.00	R	gi|148544865
Lreu_1795	ATPase associated with chromosome architecture/replication	0.79	0.00			R	gi|148545001
Lreu_1877	Nitroreductase family protein	2.69	0.00	3.20	0.00	R	gi|148545081
Lreu_1906	O-acetyltransferase (cell wall biosynthesis)			-0.80	0.02	R	gi|148545110
Lreu_1940	tRNA (5-carboxymethylaminomethyl-2-thiouridylate) synthase			1.66	0.00	R	gi|148545139
	Function unknown						
Lreu_0022	Hypothetical protein			0.59	0.01	S	gi|148543265
Lreu_0177	Hypothetical membrane spanning protein			1.26	0.00	S	gi|148543414
Lreu_0333	Initiation-control protein			0.69	0.00	S	gi|148543570
Lreu_0387	Hypothetical membrane associated protein			0.68	0.01	S	gi|148543623
Lreu_0429	Copper-transporting ATPase (EC 3.6.3.10)	1.74	0.00			S	gi|148543665
Lreu_0430	Copper-transporting ATPase (EC 3.6.3.10)	1.79	0.00			S	gi|148543666
Lreu_0565	DedA family protein			0.62	0.03	S	gi|148543800
Lreu_0665	DNA primase			1.64	0.00	S	gi|148543897
Lreu_0733	BioY protein			-1.26	0.00	S	gi|148543964
Lreu_0752	S1 RNA binding domain			-0.77	0.00	S	gi|148543983
Lreu_0758	Riboflavin transporter			-0.66	0.01	S	gi|148543989
Lreu_0759	Hypothetical protein			-1.20	0.00	S	gi|148543990
Lreu_0803	Phage antirepressor protein	1.11	0.00	0.60	0.05	S	gi|148544033
Lreu_0838	Major capsid protein	1.68	0.00			S	gi|148544068
Lreu_0846	Phage protein	1.57	0.00			S	gi|148544076
Lreu_0946	Hypothetical membrane spanning protein			-1.06	0.01	S	gi|148544175
Lreu_1104	Phage protein	1.67	0.00			S	gi|148544328
Lreu_1106	Phage protein	1.62	0.00			S	gi|148544330
Lreu_1110	Phage tail protein	1.81	0.00			S	gi|148544334
Lreu_1116	Portal protein	1.66	0.00			S	gi|148544340
Lreu_1142	Phage antirepressor protein	1.10	0.00			S	gi|148544366
Lreu_1248	Hypothetical membrane spanning protein			-0.77	0.01	S	gi|148544472
Lreu_1368	Acyltransferase			0.82	0.00	S	gi|148544584
Lreu_1387	Cell wall teichoic acid glycosylation protein gtcA			-0.72	0.01	S	gi|148544603
Lreu_1508	Hypothetical membrane associated protein			-0.98	0.00	S	gi|148544720
Lreu_1509	Hypothetical membrane associated protein			-0.88	0.00	S	gi|148544721

Locus	Product[b]	Exp		Sta		COG	Acession number
		M[c]	p[d]	M[c]	p[d]		
Lreu_1520	Hypothetical membrane spanning protein			0.75	0.02	S	gi|148544732
Lreu_1629	Integral membrane protein			1.07	0.00	S	gi|148544836
Lreu_1736	Protein glcG	0.97	0.00			S	gi|148544942
Lreu_1737	Cobalamin adenosyltransferase family protein	0.98	0.00			S	gi|148544943
Lreu_1802	Hypothetical cytosolic protein			-0.72	0.02	S	gi|148545008
Lreu_1926	Membrane protein, MgtC/SapB family			-1.86	0.00	S	gi|148545125
Lreu_1927	Hypothetical cytosolic protein			-1.32	0.02	S	gi|148545126
	Signal transduction mechanisms						
Lreu_0058	Two-component response regulator			-0.73	0.00	T	gi|148543300
Lreu_0059	Two component system histidine kinase (EC 2.7.3.-)			-0.60	0.00	T	gi|148543301
Lreu_0256	MazF protein			-0.66	0.01	T	gi|148543493
Lreu_0296	Cystine-binding protein	1.61	0.00	2.94	0.00	T	gi|148543533
Lreu_0486	Universal stress protein family			-0.66	0.02	T	gi|148543721
Lreu_0834	Portal protein	1.46	0.00			T	gi|148544064
Lreu_1401	Transcriptional regulator			-0.59	0.02	T	gi|148544617
Lreu_1510	Universal stress protein family			-0.89	0.01	T	gi|148544722
Lreu_1731	Protein tyrosine phosphatase (EC 3.1.3.48)	0.92	0.00			T	gi|148544937
	Intracellular trafficking, secretion, and vesicular transport						
Lreu_0553	ComG operon protein 1			-1.08	0.04	U	gi|148543788
Lreu_0778	Smf protein			-0.91	0.05	U	gi|148544009
Lreu_1115	ATP-dependent endopeptidase clp proteolytic subunit clpP (EC 3.4.21.92)	1.78	0.00	0.59	0.01	U	gi|148544339
Lreu_1289	N-acetylmuramidase (EC 3.2.1.17)			-1.33	0.02	U	gi|148544513
Lreu_1942	60 kDa inner membrane protein YIDC			0.83	0.00	U	gi|148545141
	Defense mechanisms						
Lreu_0812	Phage protein	1.24	0.00	1.12	0.01	V	gi|148544042
Lreu_0869	Type I restriction-modification system methylation subunit			-0.64	0.05	V	gi|148544099
Lreu_1121	Phage endonuclease	1.53	0.00			V	gi|148544345
Lreu_1138	Phage protein	1.24	0.00	1.12	0.01	V	gi|148544362
Lreu_1433	Type I restriction-modification system specificity subunit			0.64	0.02	V	gi|148544646
Lreu_1597				-1.29	0.00	V	gi|148544804
Lreu_1856	ABC transporter ATP-binding protein / ABC transporter permease protein			0.68	0.02	V	gi|148545061
	Unassigned						
Lreu_0120	Branched-chain amino acid transport protein azlD			0.64	0.01		gi|148543359
Lreu_0175				0.62	0.01		gi|148543412
Lreu_0382		0.96	0.05				gi|148543618
Lreu_0551				0.95	0.00		gi|148543786
Lreu_0656				-0.78	0.01		gi|148543888
Lreu_0662				0.81	0.03		gi|148543894
Lreu_0663				1.43	0.03		gi|148543895
Lreu_0664				1.25	0.03		gi|148543896
Lreu_0666				1.66	0.00		gi|148543898
Lreu_0703				-1.09	0.00		gi|148543935

Locus	Product[b]	Exp		Sta		COG	Acession number
		M[c]	p[d]	M[c]	p[d]		
Lreu_0749				1.13	0.00		gi|148543980
Lreu_0802		1.04	0.00				gi|148544032
Lreu_0806		1.33	0.00	0.83	0.03		gi|148544036
Lreu_0813	Phage replication protein	1.29	0.00	1.07	0.00		gi|148544043
Lreu_0814		1.36	0.00	1.25	0.01		gi|148544044
Lreu_0815		1.17	0.00	1.14	0.00		gi|148544045
Lreu_0816		1.30	0.00	1.31	0.00		gi|148544046
Lreu_0821		1.09	0.00	0.92	0.00		gi|148544051
Lreu_0822		1.19	0.00	1.02	0.01		gi|148544052
Lreu_0823		1.11	0.00	0.95	0.00		gi|148544053
Lreu_0827		1.00	0.00	0.87	0.00		gi|148544057
Lreu_0829		1.43	0.00				gi|148544059
Lreu_0830		1.34	0.00				gi|148544060
Lreu_0832		1.29	0.00				gi|148544062
Lreu_0835	Phage protein	1.38	0.00				gi|148544065
Lreu_0836		1.59	0.00				gi|148544066
Lreu_0837		1.68	0.00				gi|148544067
Lreu_0839	Phage protein	1.61	0.00				gi|148544069
Lreu_0840		1.57	0.00				gi|148544070
Lreu_0841	Phage protein	1.62	0.00				gi|148544071
Lreu_0842		1.67	0.00				gi|148544072
Lreu_0843	Phage protein	1.67	0.00				gi|148544073
Lreu_0844	Phage protein	1.76	0.00				gi|148544074
Lreu_0845		1.57	0.00				gi|148544075
Lreu_0847		1.66	0.00				gi|148544077
Lreu_0848	Phage protein	1.47	0.00				gi|148544078
Lreu_0850		1.78	0.00	0.62	0.04		gi|148544080
Lreu_0851		1.89	0.00				gi|148544081
Lreu_0852		1.52	0.03				gi|148544082
Lreu_0854		1.85	0.00	0.73	0.01		gi|148544084
Lreu_0856		1.39	0.00				gi|148544086
Lreu_0857		1.69	0.04				gi|148544087
Lreu_0959				-1.17	0.02		gi|148544188
Lreu_1005		-1.06	0.00	-2.09	0.00		gi|148544232
Lreu_1006		-1.10	0.00	-2.07	0.00		gi|148544233
Lreu_1028				-2.11	0.00		gi|148544255
Lreu_1037				1.12	0.00		gi|148544264
Lreu_1043				0.98	0.03		gi|148544270
Lreu_1069	Transposase			-0.69	0.04		gi|148544293
Lreu_1095		1.84	0.00				gi|148544319
Lreu_1096		1.82	0.00	0.66	0.05		gi|148544320
Lreu_1097		1.84	0.00	0.60	0.04		gi|148544321
Lreu_1098		1.39	0.00				gi|148544322

Locus	Product[b]	Exp		Sta		COG	Acession number
		M[c]	p[d]	M[c]	p[d]		
Lreu_1099		1.85	0.00	0.73	0.01		gi|148544323
Lreu_1100		1.64	0.00	0.61	0.03		gi|148544324
Lreu_1101		1.52	0.03				gi|148544325
Lreu_1102		1.89	0.00				gi|148544326
Lreu_1103		1.81	0.00	0.67	0.04		gi|148544327
Lreu_1108		1.73	0.00				gi|148544332
Lreu_1113	Phage protein	1.78	0.00				gi|148544337
Lreu_1122		0.69	0.00				gi|148544346
Lreu_1127		1.17	0.00	0.88	0.01		gi|148544351
Lreu_1143		0.90	0.00	0.61	0.02		gi|148544367
Lreu_1259				-0.79	0.00		gi|148544483
Lreu_1273				0.64	0.00		gi|148544497
Lreu_1284				-0.91	0.00		gi|148544508
Lreu_1294	Phage protein			-0.86	0.00		gi|148544518
Lreu_1305				0.83	0.01		gi|148544529
Lreu_1363				0.66	0.02		gi|148544579
Lreu_1373				1.08	0.00		gi|148544589
Lreu_1501				-1.49	0.00		gi|148544713
Lreu_1541	Hypothetical secreted protein			0.87	0.00		gi|148544753
Lreu_1573				0.69	0.02		gi|148544782
Lreu_1580				0.59	0.02		gi|148544789
Lreu_1601				1.51	0.02		gi|148544808
Lreu_1630	Hypothetical protein			0.93	0.00		gi|148544837
Lreu_1739		0.85	0.04				gi|148544945
Lreu_1774	Transposase			-1.05	0.00		gi|148544980
Lreu_1798				0.60	0.03		gi|148545004
Lreu_1831		0.86	0.00				gi|148545037
Lreu_1832	Histidine decarboxylase (EC 4.1.1.22)	0.99	0.00				gi|148545038
Lreu_1884	Hypothetical membrane spanning protein	0.92	0.00	0.62	0.00		gi|148545088

[a] Transcripts were considered for analysis if p-value < 0.05 and M > |0.585|.
[b] Annotation retrieved from ERGO (http://ergo.integratedgenomics.com/ERGO) (1).
[c] M, $\log_2$(intensity of signal with glycerol/intensity of signal without glycerol).
[d] p, p-value.

Table 6.S2. Distribution of differentially regulated genes through out the several categories of clusters of orthologuous groups (COG) (2).

COG category		up-regulated when cysteine is omitted	down-regulated when cysteine is omitted	regulated when cysteine is omitted
C - Energy production and conversion (59)	exp	11.86	0.00	11.86
	sta	1.69	0.00	1.69
D - Cell cycle control, cell division, chromosome partitioning (17)	exp	0.00	0.00	0.00
	sta	5.88	5.88	11.76
E - Amino acid transport and metabolism (130)	exp	9.23	3.85	13.08
	sta	10.77	10.00	20.77
F - Nucleotide transport and metabolism (75)	exp	4.00	0.00	4.00
	sta	4.00	16.00	20.00
G - Carbohydrate transport and metabolism (80)	exp	11.25	0.00	11.25
	sta	8.75	8.75	17.50
H - Coenzyme transport and metabolism (71)	exp	0.00	0.00	0.00
	sta	11.27	11.27	22.54
I - Lipid transport and metabolism (34)	exp	2.94	0.00	2.94
	sta	2.94	2.94	5.88
J - Translation, ribosomal structure and biogenesis (139)	exp	0.72	0.00	0.72
	sta	12.95	0.72	13.67
K - Transcription (93)	exp	7.53	0.00	7.53
	sta	9.68	5.38	15.05
L - Replication, recombination and repair (176)	exp	2.27	0.00	2.27
	sta	9.66	3.41	13.07
M - Cell wall/membrane/envelope biogenesis (80)	exp	3.75	0.00	3.75
	sta	11.25	0.00	11.25
N - Cell motility (1)	exp	0.00	0.00	0.00
	sta	0.00	0.00	0.00
O - Posttranslational modification, protein turnover, chaperones (42)	exp	4.76	0.00	4.76
	sta	9.52	11.90	21.43
P - Inorganic ion transport and metabolism (58)	exp	1.72	0.00	1.72
	sta	8.62	8.62	17.24
Q - Secondary metabolites biosynthesis, transport and catabolism (26)	exp	34.62	0.00	34.62
	sta	0.00	7.69	7.69

COG category		up-regulated when cysteine is omitted	down-regulated when cysteine is omitted	regulated when cysteine is omitted
R - General function prediction only (235)	exp	2.55	0.00	2.55
	sta	5.53	6.81	12.34
S - Function unknown (149)	exp	8.05	0.00	8.05
	sta	6.71	8.05	14.77
T - Signal transduction mechanisms (53)	exp	5.66	0.00	5.66
	sta	1.89	11.32	13.21
U - Intracellular trafficking, secretion, and vesicular transpor (23)	exp	4.35	0.00	4.35
	sta	8.70	13.04	21.74
V - Defense mechanisms (34)	exp	8.82	0.00	8.82
	sta	11.76	5.88	17.65

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CHAPTER 7

FUNCTIONAL ENRICHMENT OF FERMENTED FOODS BY INCREASED FOLATE PRODUCTION IN THE B_{12} PRODUCER *LACTOBACILLUS REUTERI* JCM1112

Abstract

We observed that *Lactobacillus reuteri* JCM1112 produces B_{12} and folate. However, the folate/B_{12} mass-to-mass ratio found was far below that desired for human consumption (~170:1). We used metabolic engineering applying genetic and physiological approaches to improve this ratio, and developed a generic and natural process that significantly increases folate production.

In collaboration with Arno Wegkamp (contributed equally), Willem M. de Vos, Eddy J. Smid, Jeroen Hugenholtz

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Humans have an auxotrophic requirement for vitamin B_{12} and folate, with a recommended nutrient intake for healthy adults of 2.4 and 400 μg/day, respectively (6). Sub-optimal intake of either of these compounds has been linked to cardiovascular disease, neuropathy, birth defects, cancer, and different types of anemia, amongst other pathologies (4). Remarkably, the onset of vitamin B_{12} deficiency symptoms is often delayed by the increased intake of folate (22). This masking of B_{12} deficiency has resulted in the restriction of folate intake levels and prevented folate fortification in many countries (6). Strict vegetarian dietary regimes tend to be poor in vitamin B_{12} and rich in folic acid, increasing the risk of vitamin B_{12} deficiency masking. This has boosted the popularity of fortifying vegetarian foodstuffs with B_{12} (1).

Coenzyme B_{12} is synthesized by a few members of bacteria and archaea (15). *In situ* microbial B_{12} production is a convenient strategy to achieve the natural enrichment of fermented foods, notably from vegeTable 7.sources. *Lactobacillus reuteri* is a Gram-positive, heterofermentative lactic acid bacterium with a long history of safe use by the food industry (12). This microorganism ferments several sugars and this flexibility leads to its capacity to thrive on several substrates of vegeTable 7.origin (16). Strain CRL1098 has been reported to produce different forms of B_{12} (20, 27) and the draft genome sequence of strain JCM1112 (accession n.° CP000705) (11) suggests the ability to produce folate, as well as, B_{12}. In this study, we investigated the possibility of using *L. reuteri* for the combined production of both vitamins in a ratio desired for human consumption, ~170:1 (w/w).

In silico analysis of the folate biosynthesis genes of L. reuteri JCM1112. Folate is a tripartite molecule assembled from guanosine-5'triphosphate (GTP), *para*-aminobenzoic acid (*p*ABA) and one or more L-glutamate moieties. Its biosynthesis pathway has been extensively characterized in several lactic acid bacteria, namely *L. plantarum* WCFS1 (Fig. 7.1). The predicted product of each folate biosynthesis gene of this bacterium was used to search the genome of *L. reuteri* JCM1112 using the BLAST algorithm (3). Sequence identity of the bi-directional best hit was calculated on a nucleotide and amino acid level based on separate Needleman-Wunsch global alignments (17) determined using the needle script included in EMBOSS: European Molecular Biology Open Software Suite (19), with default settings. Gene order was analyzed using the ERGO bioinformatics suite (http://ergo.integratedgenomics.com/ERGO/) (18). Both clusters are very similar as expected from the close phylogeny of their hosts (Table 7.1). Sequence identity is high on both the amino acid and nucleotide level, on average 43% and 51%, respectively. Gene order is completely conserved throughout the entire extension of the approximately 4.5 kb cluster composed of six genes.

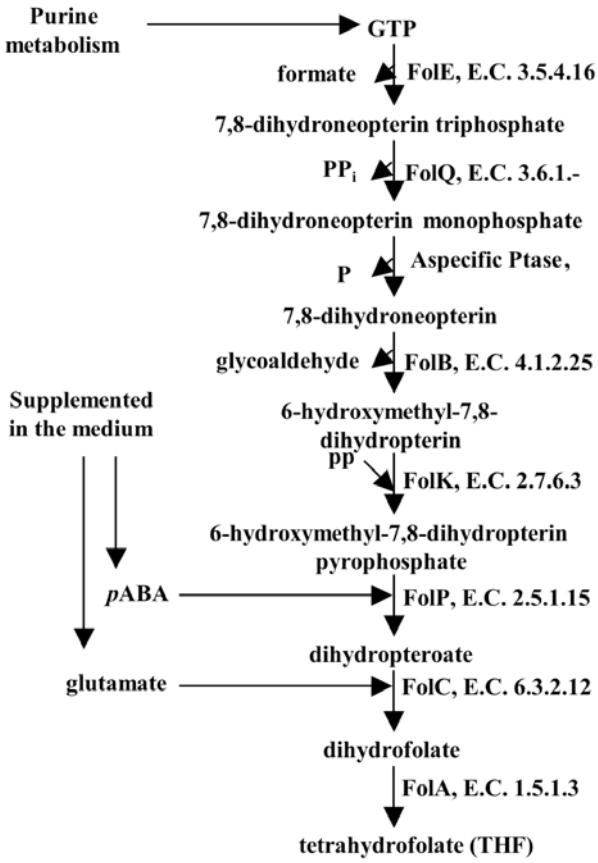

Figure 7.1. The folate biosynthesis pathway in *L. plantarum* WCFS1. GTP, guanosine-5'triphosphate; *p*ABA, *para*-aminobenzoic acid.

Characterization of B_{12} and folate production in CDM by L. reuteri JCM1112 and derivatives. The human isolate *L. reuteri* JCM1112^T was obtained from the Japanese Collection of Microorganisms (Riken, Japan). It was cultured at 37° C in chemically defined medium (CDM) containing 10 mg/L of *p*ABA and lacking vitamin B_{12} and folic acid (28). Folate was quantified from stationary phase cultures as described before (7), with a bioassay using *L. casei* ATCC 7469 as the indicator strain, and including an enzymatic deconjugation of polyglutamate tails (25). Vitamin B_{12} content was determined according to the Official Methods of Analysis of AOAC International, using the *L. delbrueckii* subsp. *lactis* ATCC 7830 vitamin B_{12} assay (8). Cell extracts of stationary phase cultures for B_{12} analysis were prepared as previously described (20). *L. reuteri* JCM1112 produces in CDM around 20 $\mu g.L^{-1}.OD_{600}^{-1}$ of folate, approximately on a 1:1 ratio (w/w) with B_{12} (Fig. 7.2).

Table 7.1. Presence of folate biosynthesis genes in the genome of *L. reuteri* JCM1112 by homology searches with *L. plantarum* WCFS1

***L. plantarum* WCFS1**				***L. reuteri* JCM1112**			
				Orthologue		Identity (%)	
ORF #	Name	Length (aa)*	Assigned function	ORF #	Length (aa)*	Amino acid	Nucleotide
lp3299	folB	122	Dihydroneopterin aldolase (EC 4.1.2.25)	Lreu1280	111	48	58
lp3298	folK	170	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase (EC 2.7.6.3)	Lreu1279	170	43	55
lp3297	folE	189	GTP cyclohydrolase I (EC 3.5.4.16)	Lreu1278	192	57	59
lp3296	folC2	454	Folylpolyglutamate synthase (EC 6.3.2.17) / Dihydrofolate synthase (EC 6.3.2.12)	Lreu1277	419	38	47
lp3295	xtp2	195	Xanthosine triphosphate pyrophosphatase (EC 3.6.1.-)	Lreu1276	195	35	48
lp3294	folP	263	Dihydropteroate synthase (EC 2.5.1.15)	Lreu1275	387	37	39

* Length based on the number of amino acid residues (aa) predicted in the gene product.

We implemented a metabolic engineering strategy as proof of principle for the possibility to influence the ratio of production of these two vitamins. We aimed at increasing folate production through the overexpression of the complete folate biosynthesis gene cluster, as described previously for other lactic acid bacteria (32, 33), ideally leaving unchanged the native B_{12} production. The constructs used in this study cannot be directly used by the food industry, but the application of food-grade alternatives is possible. A wide variety of food-grade systems have been developed for lactic acid bacteria, namely for representatives of the genus *Lactobacillus* (29). *L. reuteri* was transformed by electroporation as described elsewhere (30) with plasmids pNZ7021 (empty vector) and pNZ7026 harboring the folate biosynthesis gene cluster of *L. plantarum* WCFS1 under control of the *pepN* promoter (31). These derivative strains of JCM1112 were cultured and analyzed for folate and B_{12} content in a similar fashion as the parent strain. Chloroamphenicol was used as a selection marker in a final concentration of 10 μg/ml. The constitutive overexpression of the folate biosynthesis genes of *L. plantarum* WCFS1, in

cultures of *L. reuteri* JCM1112 pNZ7026 resulted in an increment of almost 100-fold in folate levels (Fig. 7.2), while the control (*L. reuteri* JCM1112 pNZ7021) did not show any change in folate and B_{12} production. The overproduction of folate was found to have a very small effect on B_{12} production (<10% reduction) resulting in a folate/B_{12} ratio of approximately 100:1 (w/w), sTable 7.over five consecutive transfers (data not shown). The high levels of folate overproduction for the strain transformed with pNZ7026 were as expected, provided that *p*ABA is supplied in the medium. The same construct has been tested with *L. plantarum* resulting in similar folate production levels (31), and similar results were obtained when the same strategy was applied to *Lactococcus lactis* (33) and *L. gasseri* (32).

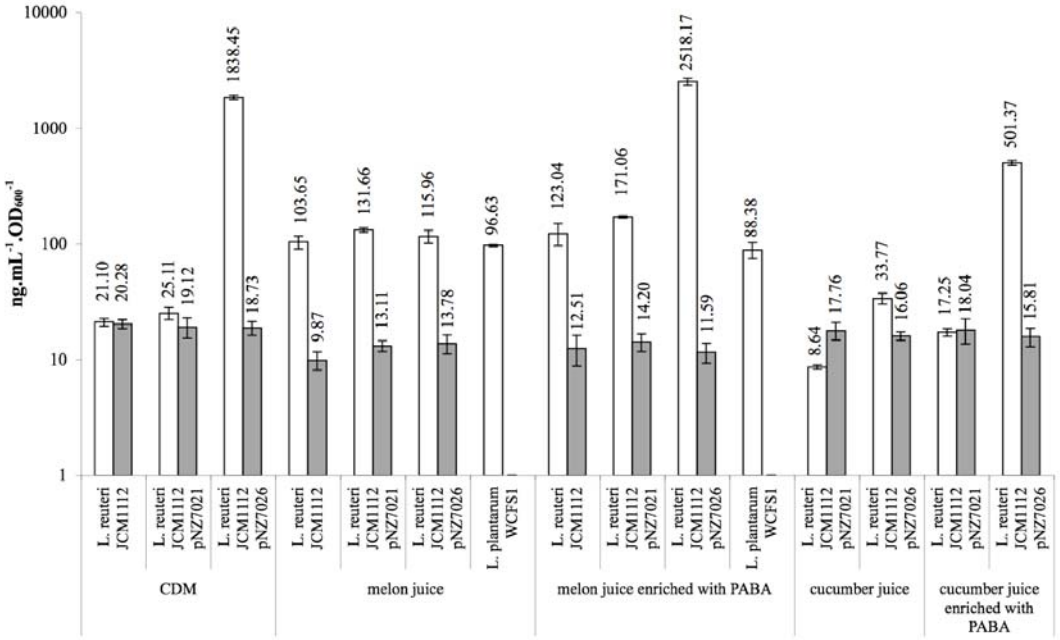

Figure 7.2. Folate (white bars) and B_{12} (grey bars) production by *L. reuteri* wild-type and derivative strains and by *L. plantarum* WCFS1 in different media. Plasmid pNZ7021 is the empty plasmid and plasmid pNZ7026 contains the folate biosynthesis gene cluster of *L. plantarum*. Each bar represents the average of three biological replicates (error bars show standard deviation). All experiments were repeated in at least two different batches of media with similar results.

*Characterization of B_{12} and folate production in fruit fermentation*s. We assessed the applicability of the principle of improving folate/B_{12} ratios through genetic engineering to different media, other than CDM. Most (sub)tropical fruits are perishable and sensitive to chill damage, leading to losses of up to 40% in industrialized countries and far over 50% in

less economically developed nations (5, 14). Fermentation is a secular process of food preservation, which in this case could increase the vitamin content of a raw material. Juice derived from two *Cucumis* spp. (melon and cucumber) was selected for natural enrichment, since this material is low in folate and deficient in B_{12} according to the USDA National Nutrient Database for Standard Reference (9). Melon juice medium was made from *Cucumis melo* var. reticulatus after peeling and removal of seeds. The pulp was liquefied using a kitchen blender (Moulinex, Masterchef 370, France) and the resulting paste was squeezed through a cotton cloth. The flow-through was centrifuged twice at 8000 × *g* for 10 min using a Sorvall centrifuge (Newton, Connecticut, USA). The supernatant was stored at -20ºC until further use. Before inoculation, the melon juice was diluted in a 4:1 ratio (v/v) with potassium-phosphate buffer (0.1 M, final concentration; pH 5.8). Further dilution was found to result in growth impairment (data not shown). The final pH was adjusted to 6.0 and the melon juice medium was forced through a 0.22 µm filter to assure sterility. Cucumber juice medium was prepared from intact cucumber (*Cucumis sativus*) and sterilized using the procedure described for melon with the following modifications: *(i)* an additional filtration step using a cellulose filter (0.15 mm) was implemented before centrifugation; *(ii)* the cucumber juice was diluted in 1 volume of potassium-phosphate buffer (0.1 M, final concentration; pH 5.8). When mentioned, both media were supplemented with 10 mg/L *p*ABA. This concentration does not conflict with exiting food legislation as *p*ABA is listed as a GRAS (generally regarded as safe) compound with an upper intake limit of 30 mg/day (10). If appropriate, 10 µg/ml chloroamphenicol was also added. Biomass formation in the different growth media is indicated in Table 7.2..

Table 7.2. Biomass formation (final OD_{600}) in the different growth media

	CDM	**Melon juice**	**Melon juice enriched with *p*ABA***	**Cucumber juice**	**Cucumber juice enriched with *p*ABA***
L. reuteri JCM1112	2.8	2.9	2.5	n.d.	n.d.
L. reuteri JCM1112 pNZ7021	2.8	2.3	2.0	1.4	1.2
L. reuteri JCM1112 pNZ7026	2.5	1.9	2.9	1.6	1.1
L. plantarum WCFS1	3.5	3.9	3.0	n.d.	n.d.

* Supplemented with 10 mg/L *p*ABA; n.d., not determined.

Folate and B_{12} content were determined from cultures of *L. reuteri* transformed with pNZ7026 and pNZ7021. Background folate levels in melon and cucumber media were found to be 22.5 ± 0.9 and 10.0 ± 0.4 μg/L, respectively. As expected, B_{12} could not be detected in these media. The overexpression of the folate biosynthesis cluster of WCFS1 in *L. reuteri* JCM1112 pNZ7026 leads to high production of folate (2518.2 ± 182.1 $\mu g.L^{-1}.OD_{600}^{-1}$) and a folate/$B_{12}$ ratio of ~250:1 (w/w), but only when *p*ABA is added (Fig. 7.2). *p*ABA availability has been shown to limit folate biosynthesis in several lactic acid bacteria (26, 33). The control experiment using *L. reuteri* JCM1112 with the empty vector (pNZ7021) resulted in the production, in melon media, of 131.7 ± 5.5 $\mu g.L^{-1}.OD_{600}^{-1}$ of folate, which is already more than five times higher than in CDM ($P < 0.001$, pairwise *t* test). In cucumber media, folate production by JCM1112 pNZ7021 was negatively affected in comparison to CDM, regardless of the addition of *p*ABA (Fig. 7.2). The overexpression of the folate biosynthesis genes has a similar affect as described for CDM, but the final folate/B_{12} ratios remain one order of magnitude lower than desired. The 2-fold reduction in B_{12} production observed for the melon juice fermentation can be attributed to the amount of sugars present (~1.5% glucose and ~2% fructose as determined by HPLC analyses performed as described elsewhere (23)). Such concentrations have been shown in previous studies to repress B_{12} biosynthesis at the transcriptional level (2, 21).

The remarkable feature of melon fermentation in comparison to CDM and cucumber is the 5-10 fold higher production of folate for the strain carrying the empty plasmid (pNZ7021). To establish the unique ability of melon juice to induce high folate production, we tested the parent strain, *L. reuteri* JCM1112, and another lactic acid bacterium, *L. plantarum* WCFS1 (13). Both *L. reuteri* and *L. plantarum* showed a 5- to 10-fold increase of folate production in melon juice media compared to CDM (Fig. 7.2). Folate biosynthesis relies on three building blocks (Fig. 7.1) whose availability does not seem to explain this unsuspected observation. We have experimentally ruled out *p*ABA and L-glutamate since both of these compounds are present in excess in CDM. Regarding the other building block, it has been shown that GTP is not the rate limiting substrate in folate biosynthesis (26), which can be explained by the small flux from GTP to folate in comparison to the total GTP pool. This implies that an increase in GTP availability for folate synthesis cannot reasonably explain the increase in folate production observed in melon juice. Folate production is tightly regulated on both transcriptional and translational level (24, 25, 31). We suspect that there might be an interaction between a compound present in melon juice and one of these regulatory factors. However, the nature of the postulated interaction is unclear to us and remains to be elucidated.

In this study, we have demonstrated the possibility of combining the production of folate and B_{12} in *L. reuteri*. We implemented, as proof of principle, a metabolic engineering

strategy to optimize the ratio of production of these two vitamins, and assessed its applicability to fruit fermentations. This resulted in the development of a natural fermentation process to increase folate production by lactobacilli, to levels substantially higher than previously published (26). The findings reported here may lead to the development of (fermented) foods based on perishable fruits, such as melons, with an extended durability and higher nutritional value. A well-tasting fermented melon juice or melon squash, containing high folate and vitamin B_{12} could be the start of a longer shelf-life product line specially targeting vitamin deficient populations.

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CHAPTER 8

SUMMARY, DISCUSSION AND CONCLUDING REMARKS

Introduction

The observation that a compound isolated from *Lactobacillus reuteri* CRL1098 cultured in B_{12}-free media was found to satisfy the auxotrophic B_{12} requirement of three different indicator strains, led to the first report in 2003 of a B_{12}-producing lactic acid bacterium (38). *L. reuteri* has a long history of safe use in the food and feed industries and it was suggested that *L. reuteri* could be used to increase the vitamin B_{12} content of fermented foods.

The aim of this thesis was to characterize the biochemical, genetic and physiological aspects related to vitamin B_{12} production by *L. reuteri* using a multidisciplinary approach, and to explore its potential applications. **Chapter 1** provides an overview of the state of the art of vitamin B_{12} research and attempts to place this into an historical perspective. It also describes the industrial and scientific importance of lactic acid bacteria, focusing on *L. reuteri*. Moreover, it briefly addresses the opportunities created by the availability of genome sequences and the scientific tools that can derive thereof.

Pseudovitamin B_{12} – an ubiquitous vitamin B_{12} co-factor

In **Chapter 2**, using powerful analytical chemistry techniques, including HPLC, MS and NMR, evidence is provided that the native corrinoid extracted from *L. reuteri* grown under anaerobic conditions is Coα-[α-(7-adenyl)]-Coβ-cyanocobamide (27). Commonly known as pseudovitamin B_{12}, this compound differs in the α-ligand, where adenine instead of 5,6-dimethylbenzimidazole is the aglycon bound by a glycosyl link from its N-1 to the C-1 of the ribose.

It has been proposed that *L. reuteri* is a suiTable 8.source of vitamin B_{12}. (38). However, *in vivo* studies validating the ability of pseudovitamin B_{12} to correct vitamin B_{12} deficiency remain to be carried out. In absence of these, some theoretical considerations can be deduced based on presently available *in vitro* data and knowledge on the processing of vitamin B_{12}. Human absorption of vitamin B_{12} is a complex process involving at least three enzymes, namely haptocorrin (HC), intrinsic factor (IF) and transcobalamin II (TCII) (Fig. 8.1). One complicating factor in the utilization of pseudovitamin B_{12} by humans may be the 500-fold lower binding affinity of human-IF for pseudovitamin B_{12} than for vitamin B_{12} as measured *in vitro* (33). The other enzymes that participate in the absorption of vitamin B_{12} in humans do not seem to pose a bottleneck in the uptake of pseudovitamin B_{12}. HC binds all corrinoids with similar efficiency independently of the strength of their Co-N coordination, or the structure of their Coα-ligands. In addition, TCII is able to bind to pseudovitamin B_{12} at a significant rate, although, *in vitro*, it displays a slightly decreased affinity (80%) in comparison to cyanocobalamin (33).

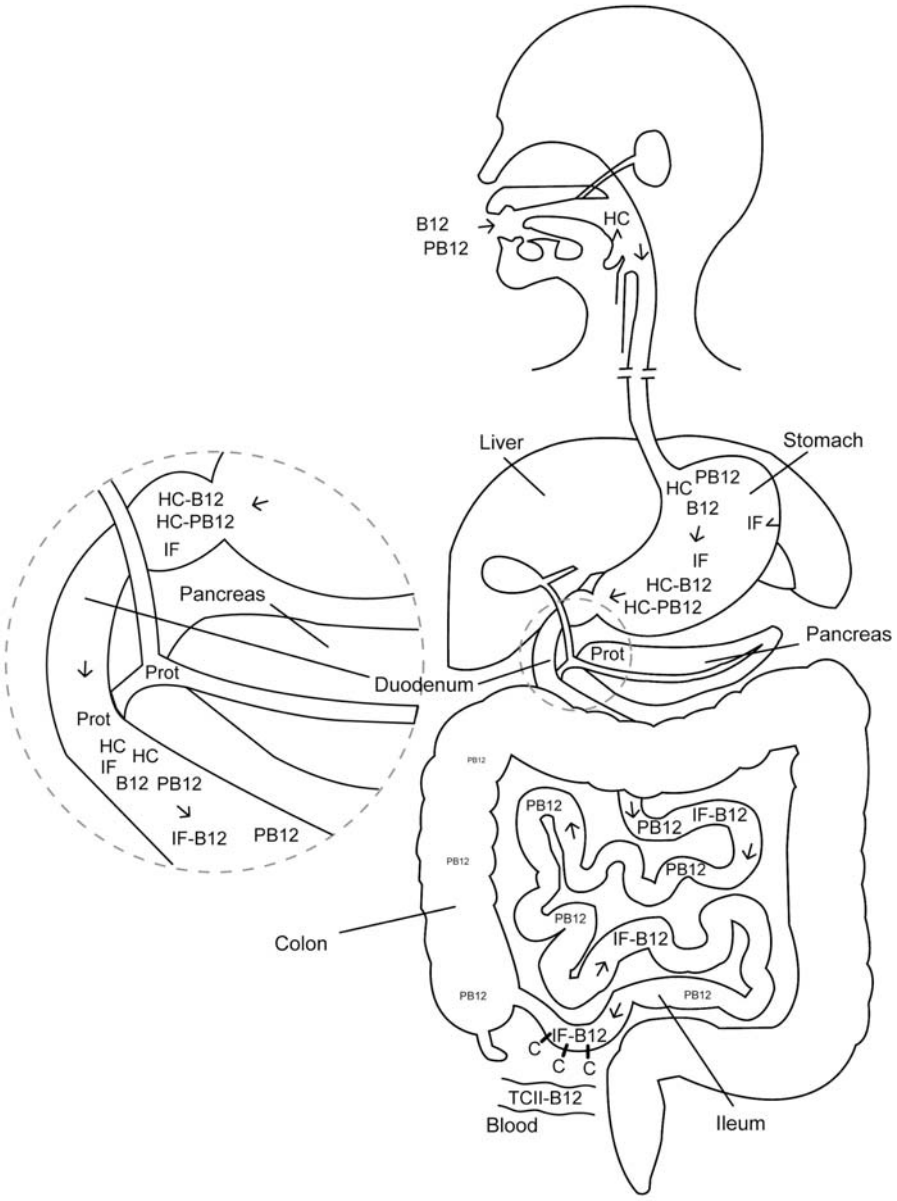

Figure 8.1. Proposed fate of dietary vitamin B_{12} and pseudovitamin B_{12} in healthy adults based on *in vitro* studies (33). In the stomach, haptocorrin (HC) produced in the salivary glands binds both dietary vitamin B_{12} (B12) and pseudovitamin B_{12} (PB12). In the duodenum, intrinsic factor (IF) produced in the gastric parietal cells binds vitamin B_{12}, but not pseudovitamin B_{12}, once they are released from haptocorrin following its degradation by pancreatic proteases. At the distal third of the ileum, vitamin B_{12} is internalized, mediated by specific receptors, cubilin (C), that recognize the intrinsic factor-vitamin B_{12} complex. Vitamin B_{12} is distributed to other tissues by serum transport aided by transcobalamin II (TCII).

It has been proposed that B_{12}-analogues, such as pseudovitamin B_{12}, could play a role in assessing the capability of B_{12}-dependent enzymes to utilize alternative cofactors (21), and in understanding the impact of B_{12} analogues in vitamin B_{12} metabolism (13, 16). Hitherto, the provision of pseudovitamin B_{12} relied on guided biosynthesis or chemical synthesis (9, 36, 42) since it is not commercially available. The fact that *L. reuteri* is capable of producing pseudovitamin B_{12} as its sole corrinoid under anaerobic conditions, turns it into an attractive source for this compound (27). The cultivation of *L. reuteri* as described in **Chapter 2** results in the production of pseudovitamin B_{12} in an accessible manner, with no need for further separation from different corrinoid species. This might instigate research on the role of the lower ligand and Co-N coordination for enzymatic activity, helping to unravel yet unknown enzyme-cofactor interactions.

Another important aspect of the identification of pseudovitamin B_{12} as the sole corrinoid produced by *L. reuteri* under anaerobic conditions, concerns the physiological role of B_{12} co-factors. Cobalamin, the most intensively studied corrinoid, has generally been regarded as the most important, because it was the only form that was thought to be physiologically active in all organism, including microbes (37). However, the finding that 5,6-dimethylbenzimidazole is not in the Coα-ligand of the corrinoid produced by *L. reuteri* (27), and also not in those of some genera of cyanobacteria (41, 42), *Salmonella enterica* (2) and *Clostridium cochlearium* (12), amongst a few others (37), clearly illustrates that cobalamin is not the only biologically active form of vitamin B_{12}. Furthermore, amongst Firmicutes and γ-Proteobacteria it seems that adenine or adenine derived moieties are particularly abundant in the lower ligand of B_{12} co-factors.

During the course of the studies described here, an important discovery was made when an enzyme capable of synthesizing 5,6-dimethylbenzimidazole was identified (8). In subsequent studies, it was shown that the product of *bluB* from *Sinorhizobium meliloti* catalyses the oxygen-dependent fragmentation and contraction of flavin mononucleotide originating D-erythrose 4-phosphate and 5,6-dimethylbenzimidazole (36).

We determined the phylogeny of the deduced amino acid sequences found to be homologous to BluB using the same methodology as described previously (28). We found that BluB is only present in B_{12}-producing α- and β-Proteobacteria, in some Crenarchaeota and in Actinobacteria (Fig. 8.2). This could explain the role of different bases in other clades of Eubacteria, since it is unlikely that 5,6-dimethylbenzimidazole will be abundantly available in naturally occurring environments. The phylogenetic tree deduced for BluB is mostly in accordance to canonical phylogeny. One exception is the BluB homologue of the Bacteroid *Chlorobium tepidum*, which could be indicative of a possible horizontal gene transfer event, but further evidence to challenge this hypothesis, has to be gathered.

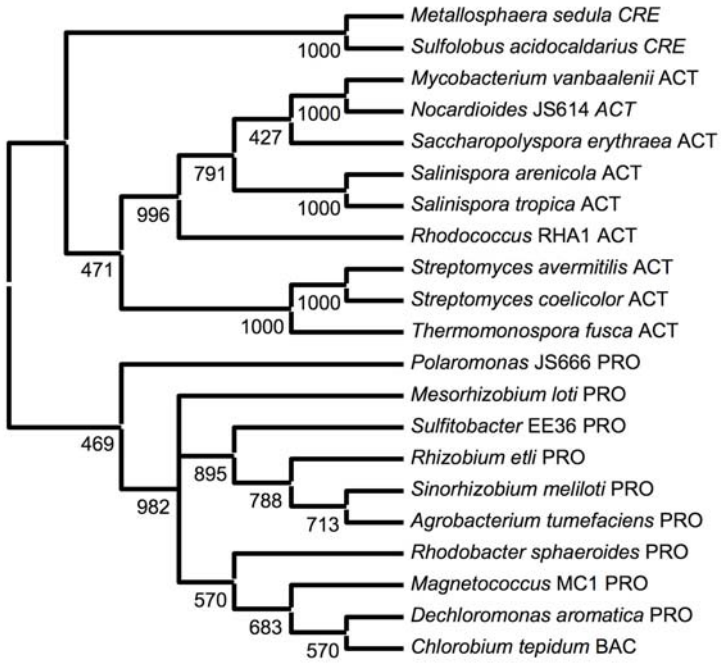

Figure 8.2. Bootstrapped neighbor-joining phylogenetic tree of BluB protein. CRE, Crenarchaeota; ACT, Actinobacteria; PRO, α- or β- Proteobacteria; BAC, Bacteroides.

Vitamin B_{12} biosynthesis in *L. reuteri*

In **Chapter 3**, we report the complete sequence analysis of the flanking regions of the original fragment found to contain genes associated to the anaerobic vitamin B_{12}-biosynthesis pathway. Quite remarkably, and unlike any other vitamin B_{12}-biosynthesis gene cluster found before (or since), *L. reuteri* encodes the complete enzymatic machinery necessary for vitamin B_{12} production in a single continuous stretch of the chromosome. In this same study, we also looked at the transcriptional organization of the cluster and found that it was expressed in two multicistronic operons of 22 and 4 kb, and that vitamin B_{12} influenced its expression by a negative feedback loop mechanism (28). We found that the genes encoding the synthesis of uroporphyrinogen III, the synthesis of the corrin ring, and the active transport of cobalt are transcribed together in the large transcript. The smaller transcript encodes the attachment of the amino-propanol arm and assembly of the nucleotide loop connecting the α-ligand to the Co at the centre of the corrin ring.

Cobalt is likely to be transported into the cell using the ATP-dependent transporter complex CbiMNQO. In Chapter 5, while studying the regulation of vitamin B_{12}-

biosynthesis in *L. reuteri*, we have identified another operon containing hypothetical ATP-dependent transporters, which could also be putatively assigned to the transport of cobalt. It is not surprising that *L. reuteri* possesses multiple systems to scavenge cobalt from the environment, since the availability of metal ions is often the limiting factor of microbial growth in natural environments (30). The synthesis of uroporphyrinogen III in *L. reuteri* derives from the five-carbon backbone of glutamate as evidenced by the presence of *hemA* (Fig. 8.3). This is expected since only α-Proteobacteria are known to use the Shemin-way, which starts with glycine and succinyl-CoA (20). The synthesis of the tetrapyrrole-derived corrin ring is achieved in *L. reuteri* via the early cobalt insertion route (non-oxygen dependent) (Fig. 8.3). This is suggested by the presence of several genes which are unique to this route, including *cbiDGK* (25).

There is great interest in analyzing the genes encoding the assembly of the lower ligand, however we could not find genetic evidence that could help predict whether an organism incorporates 5,6-dimethylbenzimidazole or adenine. It has been shown *in vitro* for *Salmonella*, that the phosphorybosyltransferase encoded by *cobT* can use substrates other than 5,6-dimethylbenzimidazol (19). We presumed that this was also the case in *L. reuteri* (28). Very recently, evidence gathered *in vivo* for *Salmonella* indicates that not only can adenine be incorporated in the lower ligand, but that the resulting corrinoid, pseudovitamin B_{12}, is also physiologically active (2).

The major step, which truly remains enigmatic, is the reduction of cobalt cob(II)yrinic acid a,c-diamide to cob(I)yrinic acid a,c-diamide before adenosylation. The nature of this reaction has remained elusive until very recently, when a cob(II)yrinic acid a,c-diamide reductase (*cobR*) was identified in *Brucella melitensis* (17). However, in *L. reuteri* we have not found any suitable candidate to encode this activity.

In early 2006 the draft genome sequence of *L. reuteri* JCM1112 was made available by JGI. The comparisons established in **Chapter 3** between the B_{12} biosynthesis clusters of the strains JCM1112 and our strain CRL1098 were indicative that much of the work carried out until this stage would be applicable, and so we decided to focus on the newly sequenced strain. In the meantime, the genome of yet another *L. reuteri*, strain100-23, was also released. This resident of the GI-tract of rodents does not seem to have the vitamin B_{12} biosynthesis cluster, although this cannot be confirmed at this point since the genome has not yet been circularized. In retrospect, the fact that the genome of JCM1112 was released and closed before the one of 100-23 had a huge impact on the way we directed our research – we entered the -omics phase.

In order to integrate and visualize the large data sets that are typical of -omics experiments (11), we developed a genome-scale network model of *L. reuteri*. This was done in a semi-automated way (22), benefiting from the manually-curate models that were

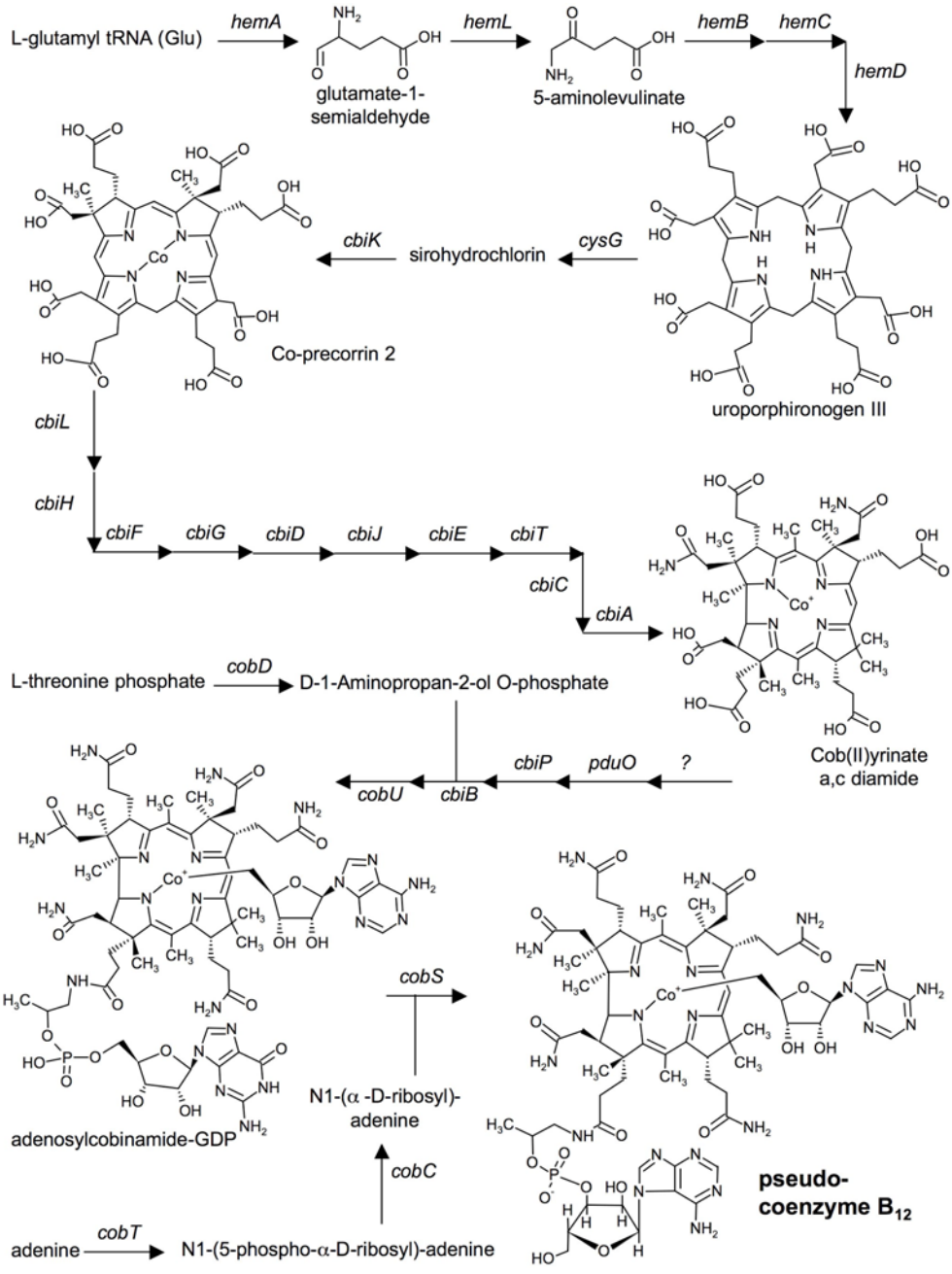

Figure 8.3. Overview of the proposed pseudovitamin B_{12} biosynthesis pathway of *L. reuteri* based on current biochemical knowledge and on the gene content of the B_{12} biosynthesis cluster.

available, including those of *Lb. plantarum* (39), *L. lactis* (24), *B. subtilis* (23) and *E. coli* (26). Meanwhile, we designed a custom-made cDNA microarray platform for *L. reuteri* and used the highly studied glucose/glycerol co-fermentation to benchmark our newly developed toolbox (**Chapter 4**).

The model enabled us to identify stoichiometrically equivalent metabolic routes, attuned with the maximal flux towards an objective function. Additionally, it aided us in comparing measured with predicted fluxes and allowed us to visualize the transcriptomic data sets. Modeling *L. reuteri* turned out to be a lot more predictive than it had previously been for other lactic acid bacteria (39). Paradoxically, the model was crucial in addressing this issue as well. It turned out that it is the absence of pyruvate formate lyase that makes Flux Balance Analysis (FBA) more predictive since the production of acetate, in detriment of lactate, yielding extra free energy (39), is no longer stoichiometrically possible.

One of the remaining questions that are tentatively addressed in **Chapter 4** is why some lactic acid bacteria, including *L. reuteri*, display a heterofermentative behavior. Recently, the phosphoketolase pathway has been shown to be dominant over the Embden-Meyerhof glycolytic route in *L. reuteri* (3). FBA made it clear that without this constraint heterofermentation could not be predicted. This does not fully explain why natural selection would favor such a behavior in central carbon distribution. Nonetheless, it certainly sheds some light on the issue, by pinpointing what is crucial for it to take place. A consequence of the heterofermentative pathway is a chronic dependency on external electron acceptors, which is quite revealing of the sort of niche where such a trait may come to evolve.

Another important outcome of this study were leads for follow-up experimentation. The global transcriptome response revealed targets for metabolic engineering of industrially relevant compounds, such as vitamin B_{12} and 1,3-propanediol, which we pursued in **Chapter 5**. Additionally, it revealed some previously unsuspected liaisons between the central carbon metabolism, and amino acid and secondary metabolite metabolism, which were pursued in **Chapter 6**.

Regulation of vitamin B_{12} synthesis in *L. reuteri*

The evidence that considering vitamin B_{12} production, *L. reuteri* is related to enteric bacteria, more specifically to representatives of γ-Proteobacteria, started building up when we identified pseudovitamin B_{12} as its native corrinoid [**Chapter 2**, (27)]. However, this was not recognized immediately, since it had not yet been reported that *Salmonella* also produces pseudovitamin B_{12} (2). Nevertheless, once the sequence of the cluster was deduced (**Chapter 3**) comparative genomics made this association obvious. The gene order of the vitamin B_{12} clusters is strikingly similar, and the phylogeny of the predicted gene products clearly contrasts that of 16S RNA sequences. The fact that the G+C skew is not

drastic between the vitamin B_{12} clusters and their hosts implies that the postulated horizontal gene transfer event was not recent.

The regulation of vitamin B_{12} biosynthesis in *Salmonella* is well-characterized (1, 4), and due to the arguments just stated, we hypothesized that vitamin B_{12}-biosynthesis in *L. reuteri* would be controlled in a similar fashion. In *Salmonella*, a single regulatory protein, PocR, regulates both vitamin B_{12} biosynthesis and diol dehydratase activity. In the genome of *L. reuteri*, *lreu_1750* is the most likely ortholog of the gene encoding PocR but some uncertainty surrounds this functional annotation, mostly related to lack of homology. In **Chapter 5** we overexpressed this putative transcription factor of the AraC family and quantified its biotechnologically relevant impact on the production of vitamin B_{12} and 1,3-propanediol. Furthermore, the characterization of the transcriptome in response to the overexpression of *lreu_1750* led to the identification of the PocR regulon in *L. reuteri*. Again the similarity between *L. reuteri* and *Salmonella* is striking, adding one more argument to the already long list that supports the occurrence of a lateral gene transfer event between Firmicutes and γ-Proteobacteria (18, 28, 40).

Included in the same regulon as vitamin B_{12} biosynthesis, we found the genes encoding the several subunits of the diol dehydratase, and the genes encoding the assembly of metabolosomes (Fig. 8.4). The presence of such structures in *L. reuteri* has recently been shown (32), indicating some kind of primordial compartmentalization of a cell. The diol dehydratase is localized inside these polyhedral organelles and *L. reuteri* appears to possess 5-15 of these compartments per cell, when the genes encoding their assembly are induced (32).

The metabolosomes have been suggested to be important in the encapsulation of toxic intermediates such as 3-hydroxypropanaldehyde (reuterin) or propionyl-CoA (32). Additionally, it has been proposed that they might be important in isolating the diol dehydratase from molecular oxygen (5). It has been reported for *Salmonella* that, with as little as 100 molecules of vitamin B_{12} per cell, growth dependent on the activity of the B_{12}-dependent diol dehydratase can occur (2). Another role that the metabolosomes might play is in increasing the local concentration of vitamin B_{12} around the enzyme. How vitamin B_{12} enters the metabolosomes, or even whether (part of) it is synthesized there, remains unclear at this stage. It is pertinent to emphasize that the entire genetic sequences necessary to encode vitamin B_{12}-biosynthesis, cobalt transport, metabolosome assembly and diol dehydratase activity, are located in one single stretch of the chromosome (Fig. 8.4). They account for as much as 2% of the whole length of the genome.

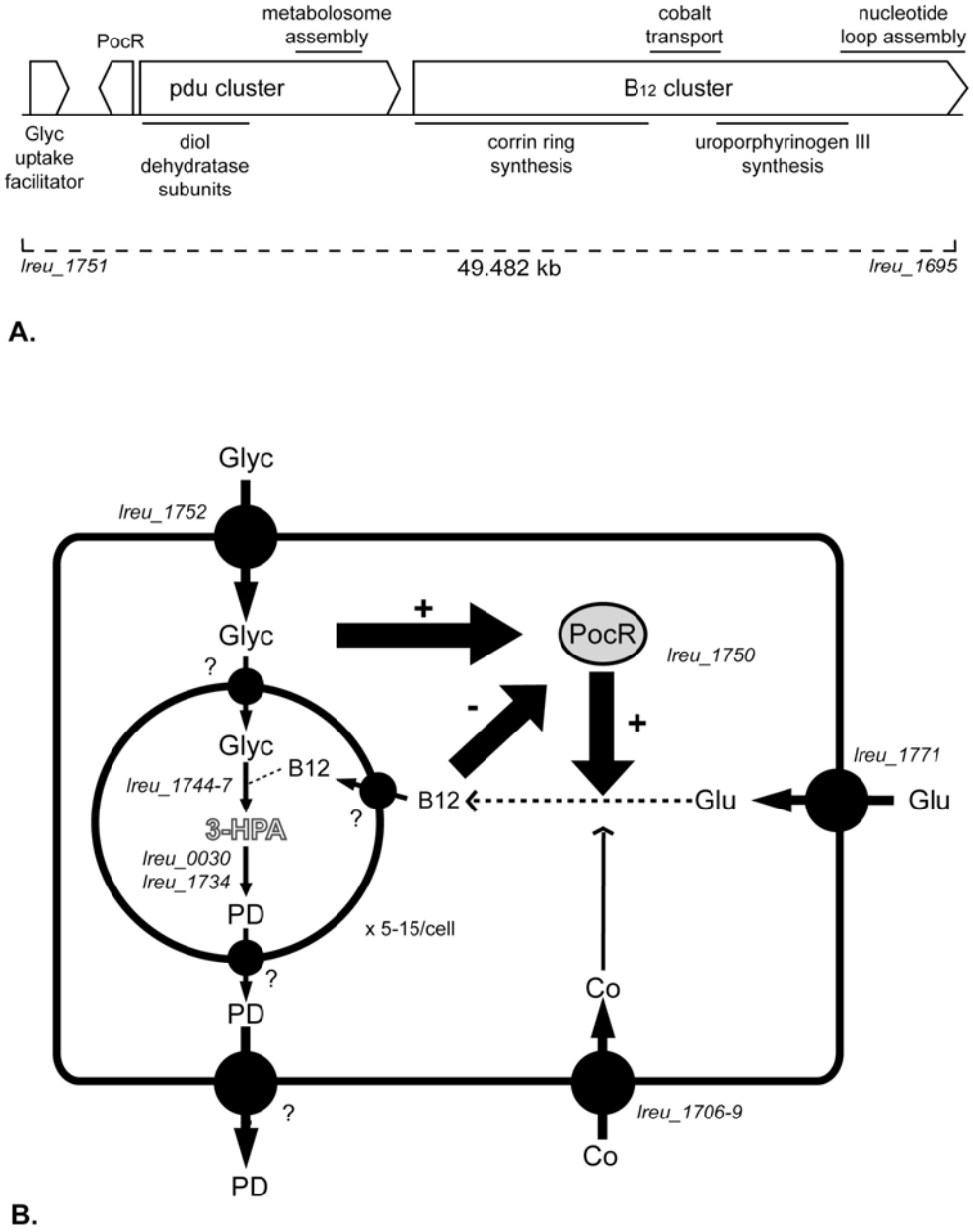

Figure 8.4. (A) Schematic representation of genomic region comprising *pdu* and B_{12} clusters (not to scale). (B) Proposed pathway for the B_{12}-dependent glycerol reduction in a metabolosome in a *L. reuteri* growing cell.

Strategies to increase vitamin B_{12} production in *L. reuteri*.

Following the leads obtained in **Chapter 4**, we pursued two different strategies to increase vitamin B_{12} production in *L. reuteri*. One was the already mentioned overexpression of PocR, which yielded little over 40% increase, and the second was the systematic omission of amino acids from the growth media, described in **Chapter 6**. The latter proved to be much more effective in increasing production levels. In fact, by omitting either isoleucine or cysteine, we were able to modulate the production from barely detecTable 8.to nearly 20-fold higher, respectively. cDNA microarray experiments revealed that it is most likely previously unreported effects of sulfur metabolism on vitamin B_{12} biosynthesis that are responsible for this phenotype, but conclusive evidence remains to be gathered.

The transport of cobalt was highlighted in both **Chapter 5** and **Chapter 6** as a potential target that could lead to further improvements of vitamin B_{12} production. We added extra cobalt to the growth broth in an initial attempt to follow this lead, but no significant changes in vitamin B_{12} production were observed. This is not surprising since cobalt is already available in great excess in the media used. However, the omission of other metals has to the best of our knowledge never been tried. Some ion transporters have a very small specificity and in the absence of certain metals, might permit the scavenging of more cobalt from the environment, increasing its bioavailibity.

Vitamin B_{12}-research in lactic acid bacteria – open questions.

The work presented in this thesis, was built up from an isolated report of B_{12} production in lactic acid bacteria (38). This implied, that vitamin B_{12} research in relation to lactic acid bacteria was an open field. By studying it, we exposed several research questions/opportunities that remain to be addressed. Illustrative, are the several leads for increased vitamin B_{12} production generated in this study. Like in most, if not all, research efforts carried out, not all the lines pursued turned out to be successful. Amongst these, there are two that we consider to be worth highlighting here.

(i) Attempt to transfer vitamin B_{12} biosynthesis cluster to other lactic acid bacteria. As previously mentioned, the vitamin B_{12} biosynthesis cluster of *L. reuteri* has the idiosyncrasy of encoding in one continuous fragment, the full machinery to make this industrially relevant compound. Furthermore, we have provided evidence supporting that this cluster has a precedent of shifting between genomes. We attempted to transfer the cluster to *L. plantarum* WCFS1 (14), because of its close proximity with *L. reuteri*, and because a considerable functional genomics platform had already been developed for this organism.

We started by trying to turn *L. plantarum* into a vitamin B_{12}-dependent organism hoping that this would provide the basis for a high throughput screening method for conjugants. *L. plantarum* WCFS1 is known to contain two genes annotated to convert L-homocysteine in L-methionine, namely *metE* (*lp_1375*) and *metH* (*lp_1374*). The latter is known to encode the well characterized vitamin B_{12} dependent methionine synthase (10). We hypothesized that a *metE* deletion mutant would not be able to sustain growth in CDM lacking methionine unless vitamin B_{12} was exogenously supplied. A derivative of WCFS1 (NZ7740) containing a *lox66*-P_{32}-*cat*-*lox71* replacement of *metE* (*lp_1375*) was constructed using a system described elsewhere (15). We compared the growth behavior of *L. plantarum* WCFS1 and NZ7740 in CDM variations including or not methionine and vitamin B_{12} in its composition (Fig. 8.5). The result did not quite match up to our expectations as it turned out that *lp_1374* was wrongly annotated as *metH*. Strain NZ7740 cannot grow in CDM unless methionine is supplied regardless of the presence of vitamin B_{12}. The WCFS1 derivative with the *metE*::*lox66*-P_{32}-*cat*-*lox71* genotype is available upon request for further studies.

At this stage, we tried to transfer the cluster to *L. plantarum* by cloning it in a low copy vector, pIL252 (31). We were able to amplify by PCR the full extension of the cluster, and purify it by gel extraction with low melting agarose followed by an agarase treatment and dialyses (details available upon request). However, the cloning proved to be the bottleneck and none of the many attempts turned out to be successful.

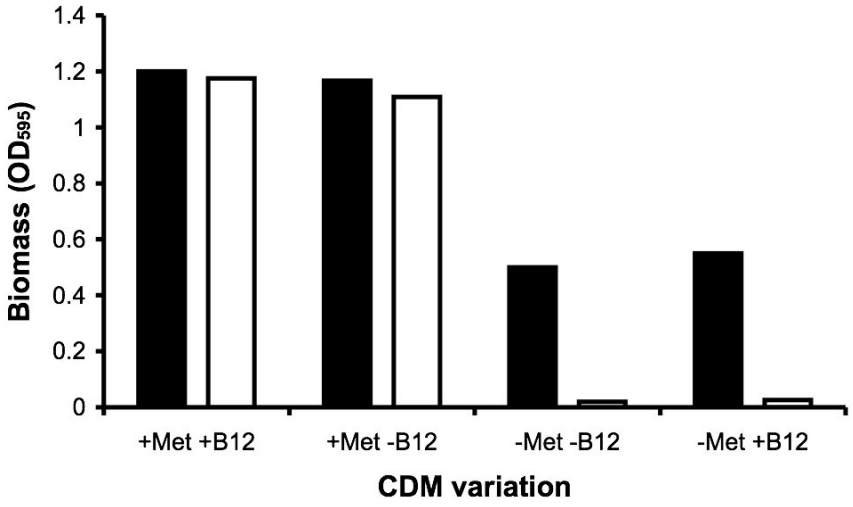

Figure 8.5. Growth of *L. plantarum* strains WCFS1 [(wild type) black bars] and NZ7740 [(*metE*::*lox66*-P_{32}-*cat*-*lox71*) white bars] on CDM variations including or not methionine and vitamin B_{12} in its composition.

(ii) Attempts to engineer the production of different corrinoids in L. reuteri. Since the identification of pseudovitamin B_{12} as the sole corrinoid produced by *L. reuteri* under anaerobic conditions, that we have been trying to devise strategies to engineer the production of different corrinoid species in *L. reuteri*. We first tried the physiological approaches described in Chapter 2 (27). Since these did not yield any major breakthrough, when BluB was implicated in the synthesis of DMB (8, 36), we decided to switch to a metabolic engineering strategy. This was done in collaboration with Dr. Amanda A. Brindley and Prof. Martin J. Warren of the Department of Biosciences, University of Kent, United Kingdom. We constitutively overexpressed *bluB* from *Rhodobacter capsulans* under control of the *pepN* promoter in a similar fashion as described in **Chapter 5**. Unfortunately, to date this strategy has also not been successful. Western blotting (data not shown) indicated that there might be a problem in the expression of *bluB*, and currently efforts are underway to try to overcome this bottlekneck.

Mining the genomes of lactic acid bacteria for the production of vitamins.

We consider vitamin B_{12} research in lactic acid bacteria to be far from an exhausted field. However, we present here the highlights of a genome mining exercise for the production of vitamins and co-factors in non-pathogenic lactic acid bacteria (Table 8.1), which might constitute the basis for future research. Folate is the vitamin that has been more extensively studied. Yet, in **Chapter 7** we describe how folate can still have unexplored features, which can be interesting both from a scientific and industrial perspective. It provides an example of how the production of multiple vitamins can be combined in one single host, like it has been done for folate and riboflavin in *Lactococcus lactis* (34). In this study, we combined the production of folate and vitamin B_{12} in *L. reuteri* (29) and stumbled across an unexpected interaction between melon fermentation and increased folate production which is extendable to other lactobacilli (43).

Concerning potential targets for research, riboflavin has been extensively studied in *Lactococcus lactis* (7), but very little in other lactic acid bacteria, which could potentially be better cell factories or have certain appealing phenotypic traits, such as fermenting cheap substrates. The presence of biotin genes in *Leuconostoc mesenteroides* ATCC8293 is a very odd observation, since this is the only lactic acid bacterium to putatively encode such pathway. It was a peculiarity like this that led to the study of vitamin B_{12} in *L. reuteri* in the first place. Finally, the production of vitamin K and molybdenum cofactor can also in principle be possible, based on the genome content alone.

Table 8.1. Mining the genome of non-pathogenic lactic acid bacteria for the production of vitamins and co-factors. Black square means that over 90% of the pathway is predicted to be encoded. Grey squares indicate that between 50 to 89% of the pathway is present. Numbers in brackets refer to studies were experimental data has been gathered that supports our assetions.

	Molybdenum cofactor	Vitamin B_2 (riboflavin)	Vitamin B_7 (biotin)	Vitamin B_9 (folate)	Vitamin B_{12}	Vitamin K_2 (menaquinone)	n.° of Contigs
Lactobacillaceae family							
Lactobacillus acidophilus NCFM				(35)			1
Lactobacillus brevis ATCC367		■				■	3
Lactobacillus casei ATCC334				(35)			2
Lactobacillus delbrueckii bulgaricus ATCC11842				(35)			1
Lactobacillus delbrueckii bulgaricus ATCCBAA-365				(35)			1
Lactobacillus fermentum IFO 3956	■	■		■		■	1
Lactobacillus gasseri ATCC-33323				(44)			1
Lactobacillus helveticus DPC 4571				■			1
Lactobacillus johnsonii NCC 533							1
Lactobacillus plantarum WCFS1	■			(35)			1
Lactobacillus reuteri F275		■		(29)	(28)	■	1
Lactobacillus sakei sakei 23K				■			1
Lactobacillus salivarius salivarius UCC118						▒	4
Pediococcus pentosaceus ATCC25745		■					1
***Leuconostocacaea* family**							
Leuconostoc mesenteroides ATCC8293		■	▒			■	2
Oenococcus oeni PSU-1						■	1
***Streptococcaceae* family**							
Lactococcus lactis lactis IL1403		■		(35)		■	1
Lactococcus lactis cremoris MG1363		(7)		(35)		(6)	1
Lactococcus lactis cremoris SK11		■		(35)		■	6
Streptococcus thermophilus CNRZ1066				(35)			1
Streptococcus thermophilus LMD-9				(35)			3
Streptococcus thermophilus LMG18311				(35)			1

Final remarks

We have used a wide array of techniques ranging amongst the several disciplines of science to characterize and understand the role of vitamin B_{12} in *L. reuteri*. This resulted in findings that might be considered of interest to the scientific community and at the same time be applicable in the improvement/development of industrial processes. Ultimately, the work developed here might also be used to ensure an adequate vitamin B_{12} intake in humans.

Vitamin B_{12} is definitely a challenging and ancient molecule. While studying it, one will always be surprised to find how much it reveals about the world and its workings.

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SUPPLEMENTS

Samenvatting*

Vitamine B_{12} is een fascinerend molekuul dat in veel organismen als cofactor dient bij cruciale processen. Het is een essentiele component van ons dieet en tevens een belangrijke cofactor voor industriele biokatalyse processen, zoals de productie van 1,3-propaandiol. *De novo* synthese van vitamine B_{12} vindt in slechts een beperkt aantal vertegenwoordigers van de bacteria en de archaea plaats. Eerder was beschreven dat *Lactobacillus reuteri* vitamin B_{12} produceert. De kennis over dit proces was echter, voor aanvang van het hier beschreven onderzoek zeer beperkt. Het hier beschreven werk had als doel om alle aspecten van de vitamin B_{12} productie in *L. reuteri* door middel van een interdisciplinaire benadering te onderzoeken, en om potentiele toepassingen te ontwikkelen.

Het natieve, door *L. reuteri* onder anaerobe omstandigheden geproduceerde corrinoide werd door middel van HPLC, MS and NMR geanalyseerd, en bleek adenine in plaats van 5,6-dimethlbenzimidazool in het Coα-ligand te bevatten. Deze verbinding, bekend onder de naam pseudovitamine B_{12}, kan gebruikt worden om vast te stellen of vitamin B_{12} afhankelijke enzymen alternatieve cofactoren kunnen gebruiken, en om kennis op te doen over de effecten van analoga van vitamin B_{12} in het metabolisme.

We vonden dat de anaerobe biosynthese route, welke de omzetting van glutamaat in vitamine B_{12} katalyseert, op een aaneengesloten gebied op het chromosoom gecodeerd ligt, naast genclusters welke betrokken zijn bij glycerol reductie, metabolosoom assemblage en transport van kobalt. De expressie van deze clusters blijkt onder controle te staan van een regulon waarin één enkel regulatieeiwit een doorslaggevende rol speelt. Genoom brede analyses door middel van cDNA microarrays en metabole netwerkmodellen openbaarden onverwachte verbanden tussen het gebruik van glycerol, vitamine B_{12} synthese en aminozuur metabolisme. De hieruit opgedane inzichten leidden tot een proces waarin, zonder gebruik van genetische manipulatie, een 20-voudige verhoging van de vitamine B_{12} produktie gehaald kon worden. Tevens werd aangetoond dat het mogelijkis om de produktie van folaat en vitamine B_{12} in *L. reuteri* te combineren. Dit proces werd toegepast in een fermentatie op basis van plantaardige substraten.

Afgezien van hun wetenschappelijke betekenis kunnen de hier beschreven vindingen toegepast worden in de ontwikkeling en verbetering van vitamine B_{12} productieprocessen.En uiteindelijk kunnen ze dienen om een toereikende inname van vitamine B_{12} door de mens zeker te stellen.

* Kindly translated from English by Douwe Molenaar

Sumário

A vitamina B_{12} é uma molécula fascinante que actua como co-factor em diversos processos essências a vários organismos. É ainda, um constituinte essencial da nossa dieta e um co-factor importante em processos industriais de biocatalise, tal como a produção de 1,3-propanodiol. A síntese de vitamina B_{12} está limitada a alguns microorganismos. A produção de vitamina B_{12} pelo *Lactobacillus reuteri* já tinha sido relatada, mas antes deste estudo, muito pouco era conhecido sobre este processo. O trabalho aqui apresentado ambicionava esclarecer os vários aspectos da síntese de vitamina B_{12} em *L. reuteri* usando uma abordagem multi-disciplinares e explorando as suas potenciais aplicações.

O corrinoide produzido por *L. reuteri* em condições de anaerobiose foi analisado por HPLC, MS e NMR (siglas internacionais), tendo sido descoberto que continha adenina em vez de 5,6-dimethylbenzimidazol no ligando Co-. Conhecido por pseudovitamina B_{12}, este composto pode ser importante para determinar a capacidade de enzimas dependentes de vitamina B_{12} actuarem utilizando moléculas análogas, e ainda compreender o impacto das últimas no metabolismo da vitamina B_{12}.

A via biosintética que converte ácido glutâmico em vitamina B_{12} foi encontrada codificada num único pedaço do cromossoma, rodeado por genes envolvidos na redução do glicerol, formação de metabolossomas, e transporte de cobalto. O controle da transcrição destes genes aparenta fazer parte de uma mesma unidade de regulação, controlada por uma única proteína reguladora. Instrumentos de análise à escala do genoma como cADN microarrays e modelos da rede metabólica, expuseram ligações inesperadas entre a utilização do glicerol, a síntese de vitamina B_{12} e o metabolismo de amino ácidos. Isto serviu de base a novas hipóteses que resultaram num incremento de 20 vezes na produção de vitamina B_{12} sem recorrer à manipulação genética de organismos. A possibilidade de combinar a produção de ácido fólico juntamente com vitamina B_{12} nas mesmas células de *L. reuteri* também foi exemplificado e aplicado à fermentação de substractos de origem vegetal.

As descobertas aqui relatadas, para além da sua relevância cientifica, podem ser aplicadas ao melhoramento e desenvolvimento de fermentações com um maior conteúdo de vitamina B_{12}. Em última instancia, o trabalho aqui apresentado poderá contribuir para assegurar um aporte de vitamina B_{12} adequado.

Acknowledgments

Scientific research is ever so often multidisciplinary. It requires the contribution of different fields that can rarely be mastered by a single individual.

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Science has to be exciting and fun to be worthwhile. It is easier to achieve this when one is surround by enthusiastic colleagues who willingly dedicate their free time to tackling some of the most intriguing questions. I am convinced that Herwig, Douwe and Bas would be scientists whether, or not, they would so desire. I thank you for your enthusiasm and readiness to share your knowledge. Zé Nuno, you were restless in helping me out with scripting and strongly encouraged me to get into it. – Thanks.

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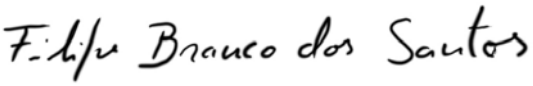
Finally, my deepest love to my newly formed *familee*… who I have burdened the most with my PhD studies.

Sincerely,



About the Author

Filipe Santos was born on the 24th of January 1979 in Lisbon, Portugal. He studied Nutrition and Food Engineering at the Instituto Superior de Ciências da Saúde from Monte da Caparica graduating in January 2003. During his final internship project he had his first contact with scientific research, working in a collaboration between WCFS and the NMR Group of Instituto de Tecnologia Química e Biológica of Universidade Nova de Lisboa. He focused on the *in situ* production of folate by lactic acid bacteria to enhance the nutritional value of fermented foods, searching for metabolic bottlenecks using nuclear magnetic resonance. He then enrolled shortly at the NMR group of ITQB headed by Prof. Helena Santos on a research fellowship working on the purification of a novel enzyme with α-PGM activity from *Lactococcus lactis*. In late 2003, he began his PhD studies working in a WCFS and Kluyver Centre for Genomics of Industrial Fermentation project, which resulted in this thesis. Currently, he is working as a PostDoc stationed at the Vrije Universiteit Amsterdam, in a project titled adaptive evolution of *Lactococcus lactis* studied by functional genomics.

List of Publications

Neves, A. R., W. A. Pool, R. Castro, A. Mingote, **F. Santos**, J. Kok, O. P. Kuipers, and H. Santos. 2006. The alpha-phosphoglucomutase of *Lactococcus lactis* is unrelated to the alpha-D-phosphohexomutase superfamily and is encoded by the essential gene *pgmH*. J Biol Chem 281:36864-73.

Bachmann, H., **F. Santos**, M. Kleerebezem, and J. E. van Hylckama Vlieg. 2007. Luciferase Detection during Stationary Phase in *Lactococcus lactis*. Appl Environ Microbiol 73:4704-6.

Santos, F., J. L. Vera, P. Lamosa, G. F. de Valdez, W. M. de Vos, H. Santos, F. Sesma, and J. Hugenholtz. 2007. Pseudovitamin B(12) is the corrinoid produced by *Lactobacillus reuteri* CRL1098 under anaerobic conditions. FEBS Lett 581:4865-70.

Wegkamp, A., **F. Santos**, E. J. Smid, and J. Hugenholtz. 2007. Increased folate production levels by fermenting melon juice. Patent EP 07122444.8-1521.

Santos, F., J. L. Vera, R. van der Heijden, G. Valdez, W. M. de Vos, F. Sesma, and J. Hugenholtz. 2008. The complete coenzyme B_{12} biosynthesis gene cluster of *Lactobacillus reuteri* CRL1098. Microbiology 154:81-93.

Santos, F., A. Wegkamp, W. M. de Vos, E. J. Smid, and J. Hugenholtz. 2008. High-Level folate production in fermented foods by the B_{12} producer *Lactobacillus reuteri* JCM1112. Appl Environ Microbiol 74:3291-4.

Santos, F., D. Molenaar, M. van Heck, M. Wels, R. A. Notebaart, W. M. de Vos, J. Hugenholtz, B. Teusink. 2008. The impact of glycerol on the metabolism of *Lactobacillus reuteri* studied by functional genomics and genome-scale modeling. *Submitted for publication*.

Santos, F., D. Molenaar, B. Teusink, W. M. de Vos, J. Hugenholtz. 2008. PocR regulates glycerol reduction, assembly of metabolosomes and vitamin B_{12} biosynthesis in *Lactobacillus reuteri* JCM1112 as in otherwise unrelated enteric bacteria. *Manuscript in preparation*.

Santos, F., B. Teusink, D. Molenaar, M. van Heck, S. Sieuwerts, W. M. de Vos, J. Hugenholtz. 2008. Effect of amino acid availability on vitamin B_{12} production in *Lactobacillus reuteri. Manuscript in preparation*.

Brooijmans, R.J.W., **F. Santos**, W. M. de Vos, J. Hugenholtz. 2008. Screening for respiration in lactic acid bacteria. *Manuscript in preparation*.

Training and Supervision Plan

Discipline specific activities

- Food Fermentation, VLAG, Wageningen, 2004
- Bioinformation Technology-1, VLAG, Wageningen, 2004
- Genetics and Physiology of Food-associated Micro-organisms, VLAG, Wageningen, 2004
- Microbial Physiology & Fermentation Technology, BODL, Delft, 2005
- Cell Physiology & NMR Laboratory of ITQB, Oeiras, Portugal, 2005
- Kluyver Center for Genomics of Industrial Fermentation Symposium (oral and poster presentations), 2004-8
- 8th International Symposium on Lactic Acid Bacteria, Egmond aan Zee (poster presentation), 2005
- Netherlands Bioinformatics Conference, Ede, 2006
- 232nd American Chemical Society National Meeting, San Francisco, USA (oral and poster presentations), 2006
- Gordon Research Conference on Vitamin B12 & Corphins, University of New England, USA (poster presentation), 2007
- 9th International Symposium on Lactic Acid Bacteria, Egmond aan Zee (oral and poster presentations), 2008

General courses

- Safe Handling with Radioactive Materials and Sources, Larenstein, Velp, 2003
- Teaching and Supervising Thesis Students, OWU, Wageningen, 2004

Optional courses and activities

- Attendance of PhD Study Trip to Japan, 2004
- Organization and Attendance of Programme 3 WCFS Fall WE-days, Cork - Ireland, 2005
- PhD/Postdoc meetings, Laboratory of Microbiology Wageningen University, 2005-7
- Organization and attendance of PhD Study Trip to California, 2006

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