Transcriptome Response of *Lactobacillus plantarum* to Global Regulator Deficiency, Stress and other Environmental Conditions

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Dit onderzoek is uitgevoerd binnen de onderzoeksschool VLAG

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Proefschrift ter verkrijging van de graad van doctor
op gezag van de rector magnificus van Wageningen Universiteit,
Prof. Dr. M. J. Kropff,
In het openbaar te verdedigen op vrijdag
11 januarie 2008 des namiddags om half twee in de aula

Marc J. A. Stevens. Transcriptome Response of *Lactobacillus plantarum* to Global Regulator Deficiency, Stress and other Environmental Conditions.

PhD Thesis. Wageningen University and Research Centre, The Netherlands, 2008. With Summary in Dutch

ISBN: 978-90-8504-869-5

Abstract

Lactobacillus plantarum is a lactic acid bacterium encountered in a variety of food and feed fermentations and as a natural inhabitant of human gastrointestinal tract. To survive in these niches and to maintain its capability, *L. plantarum* has to respond to numerous changing conditions and the cellular processes involved in these responses are frequently regulated at the transcriptional level. For this reason the transcriptome of a cell is of interest, as it contains information about the transcriptional response of individual genes. Transcriptomic approaches allow elucidation of transcriptional behaviour of all genes at once and are therefore a powerful tool to study all the transcriptional response of a bacterium to differential conditions. The complete genome of *L. plantarum* was sequenced and putative genes were identified and annotated, allowing design and production of a full genome amplicon based microarray. Using this array, the transcriptional response of *L. plantarum* to a variety of conditions could be identified.

The regulon of sigma factor 54 (σ^{54}) was elucidated and consisted of a single operon encoding a mannose phosphotransferase system (PTS). This transport system was found to be the only functional mannose transporter in *L. plantarum*. Additionally, a glucose transport function was established for this PTS and deletion of the PTS led to an increase of the intracellular phosphoenolpyruvate (PEP) concentration in resting cells, indicating an important role of this glucose transport system in metabolic control of carbohydrate metabolism.

The mannose PTS was also found to play an important role in hydrogen peroxide caused oxidative stress survival due to its robustness to oxidative stress. The impact of mild oxidative stress caused by aerobic growth was elucidated by comparing an aerobic grown culture with an anaerobic grown culture. The results led to the identification of the underlying mechanism of an observed growth stagnation, and subsequent adjustments of fermentation conditions led to overcome the stagnation.

The role of the global regulator of catabolite control (CcpA) was determined throughout the whole growth curve, showing a global role for this regulator in catabolite control. Additional studies with different carbon sources confirmed this global role of CcpA and suggested further fine-tuning of regulation by local regulators.

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Chapter 1

Introduction and Outline of the Thesis

Lactobacillus plantarum is encountered in a range of environmental niches, including industrial food fermentations. The control, improvement and innovation of such fermentations demands detailed knowledge about the responses of the bacterium during fermentation and processing conditions. Transcriptome research is a powerful tool to investigate transcriptional regulation as it offers a near-complete view on relative mRNA abundance in a cell and therefore this technique was chosen to study the behavior of *L. plantarum* under different conditions.

The main pillars of the research described in this thesis are introduced in the following chapter: the organism *Lactobacillus plantarum*, its characteristics and its place within the group of lactic acid bacteria; the regulation of transcription in bacteria with focus on *L. plantarum*; and the experimental and theoretical background of genome wide transcriptional analyses. At the end of this chapter an outline of the research in this thesis as described in the following chapters is presented.

Lactic acid bacteria

Lactic acid bacteria (LAB) are low G + C Gram-positive bacteria functionally related by their ability to produce lactic acid through homo- and/or hetero-fermentative carbohydrate metabolism. Their natural habitats are commonly anaerobic or micro-aerobic and are related to their generally high demand for nutrients and specialized carbohydrate utilization ({Lambert, 200782). LAB are used in a variety of industrial food and feed fermentations with 6 predominant species of beneficial and non-pathogenic bacteria: *Lactococcus* (milk), *Lactobacillus* (milk, meat, vegetables, cereals), *Leuconostoc* (vegetables, milk), *Pediococcus*, (vegetables, meat, milk), *Oenococcus* (wine), and *Streptococcus* (milk) (49). Some LAB, especially of the genera *Lactobacillus* (e. g. *L. plantarum, L. acidophilus, L. casei, and L. gasseri*) and *Leuconostoc* (*L. argentinum, L. mesenteroides*), are encountered in the human gastrointestinal tract (GI-tract) having a potential beneficial effect on the host (2, 27). The behaviour of LAB in fermentation processes and the GI-tract was extensively studied over the last two decades (85). As this research possibly leads to enhanced performance and functionality of LAB, it is of high relevance for the food industry (49).

The dawning of the LAB genomics era started with the annotation and publication of the complete genome sequence of *Lactococcus lactis* ssp. *lactis* IL1403 in 2001 (16), followed by the genome of *Lactobacillus plantarum* WCFS1 in 2003, the first complete genome within the species of *Lactobacillus*. In the years that followed, genomes of representative species and strains of the genera *Lactobacillus, Streptococcus, Lactococcus, Oenococcus, Leuconostoc,*

and *Pediococcus* were sequenced, representing considerable diversity in ecological habitat (milk, meat, plants, GI-tract) and roles (probiotic, fermentation) (49). The availability of complete genome sequences allowed comparisons between LAB species and revealed major genome decay, lateral gene transfer, sequence conservation, metabolic simplification, and expansion of selected gene families within the group of LAB (reviewed by Makarova (58) and by Claesson (22)). The genome decay, expansions of gene families and metabolic simplification are probably due to adaptation to nutritionally rich environments (57). Moreover, comparison within the genus *Lactobacillus* revealed significant functional differences between Lactobacilli, presenting difficulties or even necessity for revision in terms of their taxonomic classification (57, 86).

The ongoing sequencing projects of LAB genomes will enable advanced comparative genomic analyses, but will also form the basis for extensive functional genomics approaches (ranging from transcriptomic, proteomic, to metabolomic approaches) and the outcome of these efforts will expand our understanding and knowledge of LAB in general and of the genus *Lactobacillus* in particular (22). Eventually, this should enable the design of improved fermentation processes and enhanced probiotic functionality, which will fuel industrial innovation aiming to provide the consumer with tasty and attractive, but also healthy and safe fermented food products.

Lactobacillus plantarum

Lactobacillus plantarum is a facultative heterofermentative lactic acid bacterium found worldwide in the production of fermented food and feed products. Moreover, *L. plantarum* is frequently encountered as a natural inhabitant of the human gastrointestinal tract (2) and as spoilage organism in wine (12), meat (17), and orange juice (3). The complete genome sequence of *Lactobacillus plantarum* strain WCFS1, a single colony isolate of the human saliva isolate NCIMB8826, was determined (50) and was predicted to contain approximately 3,000 protein-encoding genes of which approximately 70% could be assigned with a putative function. The genome encodes all enzymes required for the glycolysis and phosphoketolase pathways, as is expected for a facultative hetero-fermentative LAB. Anaerobically grown with excess of glucose, *L. plantarum* converts glucose to pyruvate which is subsequently converted to approximately equimolar amounts of D- and L-lactate (32). Nevertheless, the chromosome encodes a relatively large number of other pyruvate-dissipating routes that are predicted to catalyze the production of formate, acetate, ethanol, acetoin, and 2,3-butanediol (50), important compounds for flavor and conservation of fermentative food-products (41).

Annotation of the genome sequence revealed the presence of a relatively high number of genes encoding transcriptional regulators (206, approximately 7% of the predicted ORF's (95)), representing 17 regulator families (Table 1). These regulators play a role in controlling the expression of a number of cellular processes such as e.g. metabolism and stress response, and their relative high abundance reflects the capability of *L. plantarum* to adapt to, and survive in a variety of different and possibly rapidly changing environmental conditions.

Table 1: Transcriptional regulator families: characteristics and their abundance (N) in L. plantarum. [1]*Designated Protein-interaction family by Wels (95).*

Family	*N*	*Characteristics/Function*	*reference*
AraC/XylS-family	8	Carbohydrate degradation	(33)
ArsR-family	9	Arsenical/metal resistance	(98)
BglB-family	5	Sugar utilization via anti-termination	(5, 56)
Cell division	3	Cell division	(95)
Crp/FNR-family	3	Global regulation, variety of functions	(51)
DeoR-family	9	Ribonucleotide metabolism	(64)
GntR-family	14	General metabolism	(38)
LacI-family	15	Carbon catabolite control	(94)
LysR-family	22	Carbon and Nitrogen metabolism	(80)
LytR-family	4	Cell lyses	(66)
MarR-family	21	Multiple antibiotic resistance	(97)
MerR-family	10	Metal response and detoxification	(40)
Other	23	Not applicable	
RpiR-family	7	Ribose phosphate isomerization	(88)
Spx-family[1]	5	Stress response	(65)
Sugars	5	Sugars	(95)
TetR/AcrR-family	25	Antibiotic resistance	(75)
Two-component systems	18	Environmental signals transduction	(11)
Total	**206**		

Genes possibly involved in surviving and adaptation are so-called stress proteins and a number of these are identified in the genome, including several proteases such as ATP-dependent proteases (ClpP, HslV, and Lon) which degrade proteins during stress conditions (reviewed in (43)), but also encompassing a range of proteins involved in heat shock response (HrcA-regulon, small heat shock proteins) and three cold-shock proteins (28). In addition, three paralogous alkaline-shock proteins were annotated, which are expected to play a role in pH tolerance (52). Furthermore, genes encoding proteins involved in oxidative stress response such as NADH-oxidases, GSH-reductases, GSH-peroxidase, NADH-peroxidases, and thioredoxins are found in the genome, whereas in agreement with previous observations (60) a superoxide dismutase is absent (50). *L. plantarum* compensates for the absence of the latter

enzyme by high level intracellular accumulation of Mn^{2+} ions (20–30 mM), which at these concentrations can act as a scavenger for oxygen radicals (7, 36).

The preference of *L. plantarum* to reside in nutrient-rich niches is reflected by the high number of transporter it possesses. In total 411 genes in the genome are assigned to transport functions, including genes involved in carbohydrate, purine and pyrimidine, amino acid, peptide and amine transport. The most efficient and thus preferred sugar transport systems in bacteria are the phopshortranferase systems (PTS) (31, 78), in which transport of the sugar is coupled to its phosphorylation (72). In total, 25 complete PTS's and several incomplete PTS's were identified in the genome, which is clearly more than found on average in microbial genomes (50). In addition, 30 alternative transport systems predicted to be involved in sugar uptake were identified and the relatively high number of predicted carbohydrate transporters in the genome supports the experimentally established ability of *L. plantarum* WCFS1 to grow on a wide variety of sugars (68).

The capability of *L. plantarum* to reside in a range of environmental niches as e.g. mammalian GI-tracts is also reflected by the relative high number of putative extracellular proteins (223, approximately 7% of the genome) (50). These proteins provide an important part of the interaction of *L. plantarum* with its environment. *In silico* analyses of these extracellular proteins predicted roles for these proteins in extracellular degradation of polysaccharides and adhesion to host components (15, 84). Interestingly, a large proportion of genes encoding sugar transport and utilization functions appear to be clustered in a 600-kb region near the origin of replication, the so-called sugar island, Furthermore, many of these genes possesses a deviating nucleotide composition, suggesting that they form a lifestyle adaptation region in the chromosome (50). This suggestion was confirmed by genotyping of 20 *L. plantarum* strains that revealed a high variation for the species in this region and the advantage of integrating horizontally transferred genes near the origin was discussed (63).

Annotation of a genome is only the first step toward understanding the function of genes in their biological context. To gain more insight in the biology of *L. plantarum*, a metabolic pathway reconstruction was performed and functionality of the pathways was experimentally verified (92). The experimental analysis revealed that *L. plantarum* is prototrophic for vitamin B_1, B_6, and B_{11}, although the data derived from the genome sequence suggested that these pathways were incomplete. It is therefore assumed that the apparent gaps in the corresponding pathways are filled by other, so far unidentified, enzyme activities. In contrast, the amino acids arginine, glutamate, and tryptophan were shown to be essential for growth, although

their specific pathways appeared to be encoded in the genome. Feedback inhibition of the pathway by other medium compounds (in the case of tryptophan) or mutations in the pathway genes (arginine) possibly explain these observations. The case of glutamate is an example why interpretation of genome sequence data should include additional knowledge of the entire biological system. Although the synthesis pathway for glutamate appears to be present, *L. plantarum*'s incomplete tricarboxylic acid (TCA) cycle leads to an impaired α-ketoglutarate supply for the pathway, resulting in the failure of the bacterium to synthesize this amino acid (93).

Overall, the genome sequence and its interpretation reflect the capability of *L. plantarum* to grow in a variety of anaerobic or micro-aerobic niches with high concentration of nutrients. However, the pathway reconstructions and the subsequent experimental verification of these reconstructions illustrate the importance of post-genomic investigations, as e.g. transcriptome analyses, to understand the biology of this organism.

Bacterial transcriptional initiation and regulation

The central dogma of molecular biology, the flow of genetic information from DNA via RNA to proteins, representing the functional biological units, basically involves two steps: transcription and translation.

Transcription starts at specific sites in the DNA called promoters at which the RNA polymerase (RNAP) binds and transcription starts. Bacterial transcription initiation requires a sigma (σ) factor to direct the RNAP core enzyme (typical composition: α_2ßß'ω) to the promoter, forming a closed promoter complex (Fig. 1). An open complex is formed by DNA-melting of the 16 base pairs downstream of the polymerase complex and, subsequently, nucleotide triphosphates bind to the single stranded DNA forming an initiation complex. Elongation starts as the initiation-complex starts to shift along the DNA and releases the sigma factor, which thus has no further function in RNA-polymerization. Interestingly, bacterial-like transcription involving a sigma factor is also found in chloroplasts of higher plants, making the eubacterial origin of these organelles feasible (42).

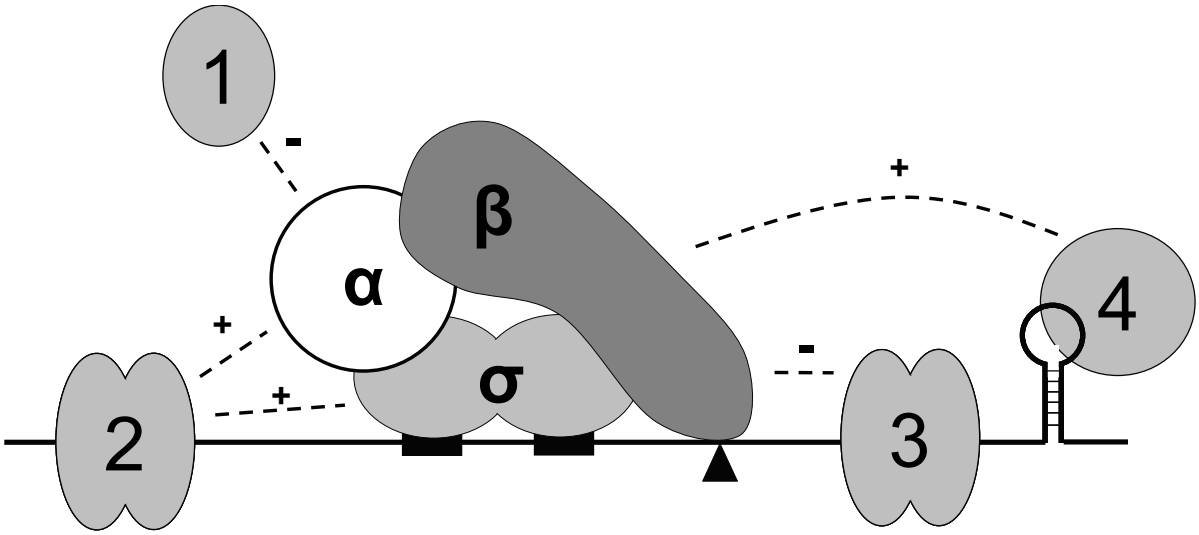

Figure 1: Schematic representation of bacterial transcription initiation and control. The RNAP, consisting of an α-, β-, σ-, and (not visible) ω-subunit, binds to a specific DNA-sequence: the promoter (black boxes). The transcription start site (black triangle) is positioned 10 or 12 nucleotides downstream of the promoter. Transcriptional regulation occurs via proteins (transcriptional regulators) interacting with the RNAP: 1. Negative regulation by regulators which interact with the α-subunit of the RNAP thereby preventing binding of RNAP to the promoter DNA. 2. Positive regulation by upstream bound regulators which direct the RNAP-core enzyme to the promoter. 3. Negative regulation by downstream bound regulators which block transcription elongation (Roadway blocking). 4. Positive regulation by regulators which bind to DNA-loops that block transcription elongation. Negatively regulation by regulators which bind to the promoter thereby preventing RNAP-binding is not shown.

Organisms are continuously exposed to changing external and internal conditions and correct regulation of transcription is an important mechanism for maintaining their viability (83). Bacterial regulation of transcription is mediated via two fundamentally differing mechanism; via altering the binding capacity of RNAP to promoters and via modulating the activity of the RNAP.

An example of the first mechanism is transcriptional regulation by sigma factors. As mentioned above, sigma factors direct the binding of the RNAP core-enzyme to DNA. Most bacteria posses more then one sigma factor, each with its own specific promoter sequence, generating an important mechanism for differential gene expression in bacteria. The nomenclature of sigma factors is complex. In Gram-negative and some Gram-positive bacteria sigma factor encoding genes are designated *rpo* (for RNA polymerase subunit), whereas in most Gram-positive bacteria the genes are designated *sig*. The proteins are mostly designated with a superscript indication of the molecular weight (σ^{70}) or with a single-letter-code (σ^{A}). As molecular weights differ between species, a single-letter-code is useful, although problems occur in those species that posses more than 26 sigma factors (67). However, for historical reasons the letter-codes might differ between species, e.g. the factor

designated σ^N in *Escherichia coli* (regulator of Nitrogen metabolism) is homologous to σ^L in *Bacillus* subtilis (regulator of the Levanase operon). *Lactobacillus plantarum* WCFS1 possesses three putative sigma factors: one highly similar to "house-hold" factor σ^A (73% identity), one, designated σ^{54}, similar to σ^L (35%) and one similar to σ^H (23%) of the Gram-positive model organism *Bacillus subtilis* (Table 2). Comparative analyses of 13 non-pathogenic LAB genomes (58), revealed that σ^{54} is only found in *L. plantarum* and *P. pentosus*, whereas σ^A and σ^H were present in all 12 species.

Table 2: Sigma factors in 13 LAB genomes and their putative binding sequences. The σ^A and σ^H binding sequences are based on the B. subtilis consensus sequence (37). The σ^{54}-consensus was based on 180 promoters reported in literature (10) and on the prediction in chapter 2 of this thesis. Nucleotide sequences are notated as recommended by IUPAC (1).

Species	***Sigma A*** TTGACA-17N-TATAAT	***Sigma H*** RWAGGANNT-14N-HGAAT	***Sigma 54*** TGGCAC-5N-TTGC
L. plantarum	Y	Y	Y
L. johnsonii	Y	Y	N
L. sakei	Y	Y	N
L. delbrueckii subsp. *bulgaricus*	Y	Y	N
L. gasseri	Y	Y	N
L. brevis	Y	Y	N
P.pentosaceus	Y	Y	Y
O. oeni	Y	Y	N
L.mesenteroides	Y	Y	N
L. casei	Y	Y	N
L. lactis subsp. *lactis*	Y	Y	N
L. lactis subsp. *cremoris*	Y	Y	N
S.thermophilus	Y	Y	N

Based on structural and functional criteria sigma factors can be categorized into two main classes (61), a class containing all the *E. coli*- σ^{70} like factors and a class containing only one member, the σ^{54} factor (62). Sigma factors belonging to the σ^{70}-family bind to a sequence lying between 10 and 35 base pairs upstream of the transcription start (-10/-35 region). The σ^{54} transcription factor differs from σ^{70} factors by binding to a promoter with a conserved -12/-24 motif (instead of -10/-35 motif) (19). Furthermore, the promoter bound σ^{54}-RNA-polymerase complex strictly requires a dedicated activator protein that catalyses ATP-dependent transition from a closed to an open complex which enables transcription initiation (79). Sigma factor activity, and as a consequence gene expression, is mediated at many different levels: by enhancing or decreasing the synthesis of sigma factors (at transcriptional and translational level), by post-translational processing, by inactivation of the sigma factors

through binding to anti-sigma factors (post-translational inhibition), or through differential turn-over of the sigma factors (67).
As the binding sequences of sigma factors are highly conserved throughout different species, relatively accurate prediction of sigma factor specific regulons can be achieved using software packages as MEME (8) and MAST (9). The MEME and MAST methodology has been used in this thesis to predict the σ^{54}-regulon in *L. plantarum* (Chapter 2). Another approach to discover sigma factor binding sites is to combine large transcriptomic data sets and sequence information in a statistical model, as for example performed in *B. subtilis* (26). However, this method relies on the availability of large data sets and as these are not available for many organisms, statistical significance is not always obtainable.

The second mode for transcriptional regulation, regulating the activity of the RNAP, is mediated via transcription regulators (TRs) and as mentioned above, 206 TRs are identified in the genome of *L. plantarum* (Table 1). Transcriptional regulators are proteins that frequently contain a DNA-binding domain (helix-turn-helix) and at least one additional domain for the interaction with cellular signals (6). They are classified in 17 transcriptional regulator families based on similarity in function, structure and sequence (75). Most TRs interact with the different domains of a RNA-polymerase complex, preferably at the C-terminal domain of the RNA polymerase α-subunit or at the sigma factor subunit. However, in some cases interaction with N-terminal domain of the α-subunit or with the β-subunit occurs (76).
Previous studies have revealed that some regulators act throughout the entire genome potentially regulating expression of a large amount of genes. For example, in *E. coli* 7 regulators have been identified (CRP, FNR, IHF, FIS, ArcA, NarL and Lrp) that each regulate more than 100 genes and collectively control expression of more than 50% of the genes in the genome (59). In contrast to these global regulators, local regulators regulate only a single or a few genes, which are often genetically linked to the regulator encoding gene.
In the section below a selection of the different mechanisms that mediate transcriptional regulation in bacteria are described. As mentioned above, *L. plantarum* possesses a relative high number of transcription regulators and it is plausible that the described mechanisms occur in this bacterium.
Transcriptional regulators can act by binding to the DNA at a specific binding site in proximity of the promoter, thereby influencing the transcription of downstream genes. Transcription repression occurs by binding of the TR between transcription start point and the downstream gene, thereby blocking the RNA-polymerization-complex and elongation. This

so-called “road-blocking” is one of the predominant mechanisms for bacterial transcriptional repression. The global regulator of catabolite control in low G+C Gram-positive bacteria CcpA functions as a repressor via this mechanism (39). However, binding between transcription start and gene can also lead to activation of expression, as is for example the case for the lactose operon in *Lactobacillus casei*. In this case a transcription regulator binds between the transcription start and the start of the gene thereby resolving a terminator structure that would otherwise block RNA elongation (35).

A different mode of activation occurs via positive interaction of the DNA-bound regulator with the RNA-polymerase. In this case the regulator is generally bound upstream of the promoter and studies of *Bacillus subtilis'* global regulator CcpA revealed that depending on the binding site distance to the putative promoter activation occurs (55). Similar activation occurs by σ^{54} dependent transcription, where the σ^{54}-activator binds upstream of the promoter, although transcription activation requires ATP-hydrolysis catalyzed by the activator (20).

Some regulators do not bind to the DNA, but only interact with RNAP, thereby mediating the mode of gene transcription. Spx in *Bacillus subtilis* binds to the C-terminal domain of the RNAP α-subunit, thereby interfering with the capacity of RNAP to respond to certain activator proteins, leading to abolished transcription activation (65). Putative *spx*-regulator genes have also been identified in the genome of *L. plantarum* (Table 1).

Just as sigma factor binding sites, transcriptional bindings site are often conserved, although the function of the corresponding regulated genes may differ. However, the position of the binding site alone is not sufficient to predict the regulatory mechanism, prediction of DNA loops, genetic organization of the putative regulon genes and knowledge of the regulator’s function, will provide added value to the prediction.

Transcriptional research

In 1961, Brenner and co-workers discovered an unstable RNA-species which transferred genetic information from the DNA to the ribosome. Since that day the RNA-species, designated mRNA, has been studied extensively in biological and medical science. However, due to the unstable nature of mRNA technical difficulties were tremendous in the early years of RNA-research and work was often left to dedicated laboratories (91). Fortunately, the technology evolved and numerous RNA-purification protocols now exist, making RNA-isolation routine. The discovery of reverse transcriptase in the early 1970’s was of great importance as it enabled conversion of RNA to cDNA, which is more stable and can be used

in applications as for example polymerase chain reaction (PCR) and cDNA library production.

Several techniques have been developed in the past 30 years to study gene expression and RNA abundance *in vivo.* Northern blotting was a frequently applied technique to study gene expression in the pre-transcriptomic era (see below). The highly sensitivity technique is based on RNA separation by size followed by transfer of the separated molecules to a membrane. Subsequently, RNA molecules can be detected using labelled probes and detection (4). However, in a northern blot only a few different transcripts can be detected, which has stimulated the development of post-genomic techniques that allowed detection of a high number of transcripts in a single experiment.

Another commonly used technique to study RNA abundance is based on the quantitative polymerase chain reaction (Q-PCR), a technique that allows elucidation of single DNA molecule-abundance compared to an internal standard. Measurement of gene specific RNA levels following RNA to cDNA conversion using reverse transcriptase (qRT-PCR) is frequently used nowadays and is useful as validation technique for microarray data (96).

Microarray technology

Soon after Ed Southern developed his technique to bind nucleic acids to a solid surface and detect them with labelled complementary molecules in the mid 1970s (89), modifications in his technique were made allowing transcriptional profiling (4). Additional innovations had to be made before miniaturization, necessary for full genome transcriptional profiling, could be performed: use of non-porous solid supports, such a glass, and techniques to produce high-density arrays (54). However, after the first paper on the microarray technique was published (81), the tool was still in its infancy, due to high costs, technical difficulties, and difficulties in data handling and storing (54). One reviewer even cynically commented that there were more reviews about arrays than primary research papers applying them (24). The infancy of the techniques in the late 1990s is illustrated by the limited number of microarray papers published in these years (Fig. 2). However, with the start of the new millennium microarrays became popular in a huge number of research fields, including microbiology (29), which led to exponential increase of papers in which microarrays were applied (Fig. 2).

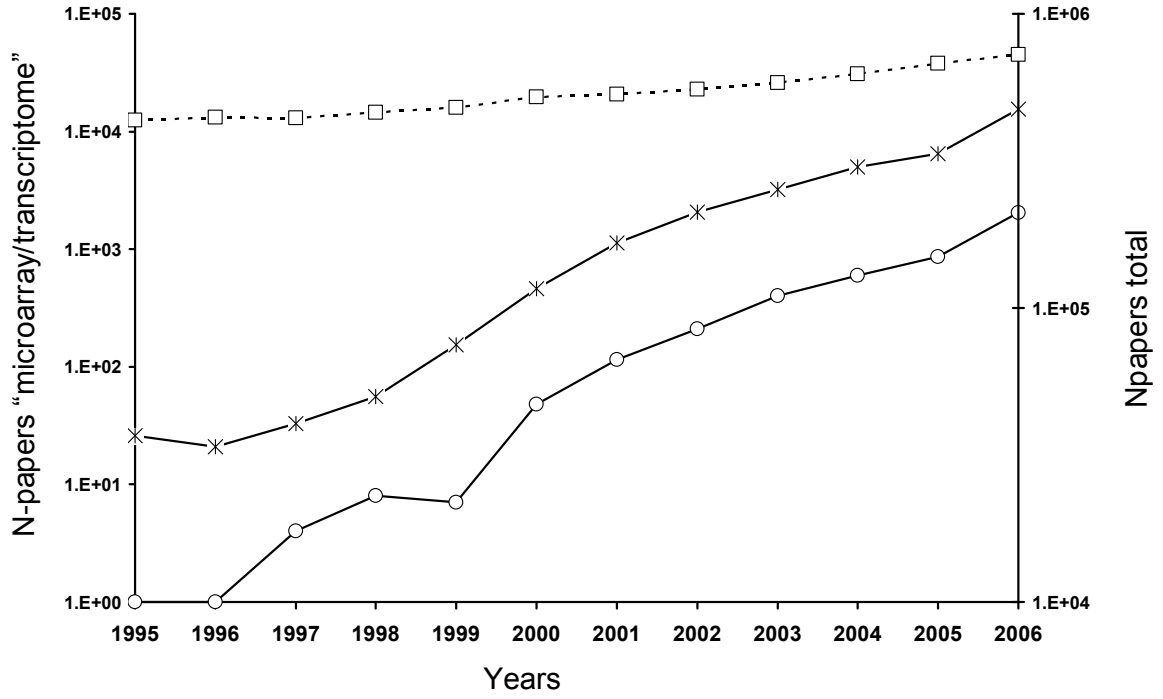

Figure 2: Number of papers found in the Pubmed database using "microarray" (crosses) or "transcriptome" (Circles) as query plotted logarithmic against the total number over papers published that year (squares, values on right axe).

Currently, two formats of DNA micro-arrays are commonly used: microarrays where the single stranded probes (up to ~80 bases) are directly synthesized on the surface of the carrier chip, and microarrays where the synthesized probes are printed or spotted on and bound to the carrier chip. The latter microarray format can be further distinguished in double-stranded DNA microarrays and single-strand oligonucleotide microarrays (30). The transcriptome studies described in this thesis were predominantly performed using double-stranded DNA microarrays with gene specific probes synthesized by a PCR and spotted on glass slides. Advantages of these arrays are that they are relatively inexpensive and relatively easily manufactured (potentially an in-house technique). However, the relative advantages of directly synthesizing single-stranded probes on the chip, which includes a significantly improved signal to noise ratio and a much higher probe density per slide, has led to the standard application of commercial available arrays of this type in the majority of transcriptome analyses performed in our institute.

Experimental design of microarray experiments

Transcriptome experiments aim to identify those genes which respond to the specific condition and therefore probably play a role in cellular adaptation to that condition. However, the genetic response might not solely be due to the condition of interest but also due to biological and experimental variations (secondary effects). In classical, hypothesis-driven, experiments the impact of such variations on data specificity and interpretation is limited since experimental conditions were chosen to confirm or refute a hypothesis. In contrast, in data-driven approaches differentially expressed genes due to secondary effects might end-up in the final selection of results, leading to increased complexity of the data and to time-consuming interpretation studies (69). To be able to separate the biological effects of interest from those caused by experimental setup and variance, a microarray experiment should be carefully designed to yield reliable results.

A microarray experiment can be divided into three organisation levels, so-called layers (21). The top layer consists of the experimental units (also called the "sample"), in microbiology mostly the compared strains or conditions in an experiment. Biological duplicates can only be performed in this upper layer of the experiment. The second layer consist of technical units, in transcriptome experiments the procedure from RNA-isolation to hybridisation on a slide. The third layer is the microarray itself; the design of the array and read-out of the signals. Variance occurs in each phase: biological variance in the upper, technical in the middle, and measurement-errors in the bottom. It is therefore of great importance to select an appropriate experimental design that contains sufficient duplicates and minimizes errors.

The design in the upper layer of the experiment must be chosen in such a way that the variation between the samples is minimal, apart from the condition of interest. In microbiological terms, samples must be taken at identical cell density, growth phase, growth rate etc. Too many variations between the samples makes biological interpretation difficult and additional comparisons might be necessary to obtain a reliable data-set. Differences in growth-rate can be overcome by applying continuous cultures, as was done in a study to unravel the effects of lactate stress in *L. plantarum* (71). Comparisons of mutated strains can be difficult when selection pressures have to be maintained or genetic alteration effects expression of other genes. The development of a gene-replacement vector that enables clean gene deletions in *L. plantarum* (53) may therefore a great advantage in an experimental designs.

Many potentially error-introducing technical steps have to be followed in a transcriptome experiment and it is therefore important that identical and suitable protocols are used

throughout the workflow. Quenching samples in a - 40° C methanol buffer leads to an immediate arrest of cell processes and such a sampling method decrease the errors occurring during sampling and harvesting of the cells, contributing to the reliability and reproducibility of microarray data (70). However, additional steps in the protocol also enlarge the chance of introducing errors and therefore protocols must be chosen carefully and validated extensively.
Apart from using suitable and validated protocols, quality control during each experiment is recommendable to reduce technical error introduction and the constantly advancing technology allows nowadays easy and quick quality checks of RNA, cDNA and labelled cDNA. All these checks contribute to a minimal technical error introduction.
Errors may also be introduced in the third layer of an array experiment, the array design and read-out. Fortunately, due to direct comparison of samples on one array, variations of the array itself (spot-size, spot-intensity) that would otherwise contribute to the error, are accounted for (21, 47). However, due to costs or limited amount of sample material, pairwise comparison of all samples is often impractical and therefore samples are frequently indirect compared in a hybridization-scheme (21). Such a scheme should be designed carefully and a proper hybridisation-scheme beholds direct or indirect comparison of all samples, contains sufficient replications, and should be balanced with respect to the dyes (47). Such a scheme will allow proper statistical analyses to obtain a reliable set of microarray data.

Data handling and storage

In order to draw biological conclusions from transcriptome experiments, data obtained from a microarray analysis have to be processed. Systematic bias, originating from laboratory procedures, such as differences in labelling efficiency, stability, and detection between the two fluorescent dyes, has led to the development of compensating normalization methods (30). The most commonly used normalization method is based on the assumption that the sum of the intensities should be equal in both channels, or in other words: the overall ratio in gene expression of all genes should be one (25). To obtain data according to this assumption, the complete data set is centred on zero by subtracting the average intensity from the specific intensity. However, as the intensity of both channels not always behaves in a linear fashion along the dynamic range of signal intensities, such averaged weight linear regression is only suitable for small data intervals. Therefore a locally weighted least squares regression (LOESS or also known as LOWESS) is commonly applied in microarray data normalization. LOESS is a technique for fitting a smoothing curve to a dataset in which the degree of smoothing is determined by a window width parameter (23). The smoothing curve calculated

by LOESS is used as a locally applied normalization factor. The LOESS based normalization is generally accepted as the most appropriate method of normalisation for microarray data in transcriptome analyses as it can cope with intensity dependent bias (74).

Classical statistical methods are frequently based on the null hypothesis, in microarray experiments the assumption that two compared samples are in principle equal. Only data points which reject this null hypothesis within a certain probability (mostly 95% or $p<0.05$) are assumed to differ between the samples. However, due to the massive amount of data points coming out of a microarray experiment, use of such a methodology would lead to occurrence of many false-positives (73). To control these false-positives, the false discovery rate (FDR) (13) can be calculated, nowadays commonly recognized as a useful measure to control false positive rates in microarray experiments (90). Statistical tests based on this principle are commonly used to calculate differential gene expression in complicated microarray experiments.
Within a hybridisation scheme, a number of comparisons are possible and the interest of the researcher is whether a gene is significantly differentially expressed under certain conditions. A common used tool to identify genes with differential expression in a set of microarray hybridizations is analysis of variance (ANOVA), a method in which the behaviour of a gene is compared to the average behaviour under that condition and to average behaviour under all conditions. This method allows identification of differentially expressed genes corrected for potential interfering effects (48). Additional techniques that aim to combine information across arrays have developed in the past few years and linear models, e.g LIMMA, in which direct comparisons are given more weight as indirect comparisons are more commonly used nowadays (87). The number of different statistical methods seems to be numerous at the moment and is still growing, but as many methodologies are substantially similar understanding these similarities might be sufficient to decide what method to use (46).

The recognition of the fact that the complexity of microarray experiments present a hurdle in the unambiguous interpretation of the data by other researchers has led to the establishment of standards for the exchange of microarray data (18). This MIAME standard (Minimum information about a microarray experiment) has been adopted by almost all scientific journals as a requirement for publication of results derived from microarray experiments. As a consequence, raw microarray data, experimental protocols and analytical procedures have to be made public by deposition in MIAME compliant microarray data repositories like GEO,

ArrayExpress and CIBEX at the time of publication. Such repositories not only present a possibility to for other researchers to verify the published interpretation of microarray data, but are also a rich source for the discovery of new phenomena by meta-analyses.

Data interpretation

After successful culturing and sampling, RNA-isolation, microarray production and optimization, hybridization, scanning, and primary data analyses the researcher ends up with a large set of data containing significant expression and fold-change of expression values for each gene. However, due to the massiveness of microarray data it is still a relatively time consuming and laborious process to uncover the underlying regulatory mechanisms and hierarchical control of gene regulation mechanisms and to formulate an accurate biological conclusion. Therefore, the past years have seen the development of secondary methods to facilitate the comprehensive interpretation of transcriptome datasets.

Principally, there are two approaches for secondary microarray data analyses, a data-based approach and a knowledge-based approach (alternatively termed unsupervised and supervised approach (34)). The data-based approach is an "internal" approach based on mathematical methods and independent of biological knowledge or any other exogenous factors. Biological conclusions are associated to the data, based on the assumption that similar expression patterns indicate related biological function (18). The most frequently used data-driven approaches in microarray analyses are hierarchical clustering, K-means clustering and self-organizing-maps (SOM), and principle component analysis (PCA)(30). The aim of these methods is to group genes with similar expression patterns and visualize these, but the underlying mathematical methodology differs and consequently these methods sometimes generate significantly different results.

In contrast to the data-based approach for microarray data analyses, the knowledge-driven approach compares the data with existing knowledge from "outside" and visualisation tools have been developed to enable straightforward comparison of knowledge. For example, gene-mapping combines gene-context and annotation knowledge with microarray-data by plotting the data on genomic maps using software as e.g. the microbial genome viewer (45). Other methods plot microarray data on metabolic maps using the online database encyclopaedia of genes and genomes KEGG (http://www.genome.ad.jp/kegg/) (44) or the commercially available modelling program Sympheny (Genomatica, San Diego, USA). An example of a combined data- and knowledge-based approach is FIVA (14), a package that calculates and

visualizes significant differential gene expression per functional class or COG category at once (14).

The combination of a variety of methods available for comprehensive analysis of large amounts of transcriptome datasets allows a more effective integration and interpretation of the information captured in the initial data, leading to more rapid disclosure of the biological message enclosed in the datasets. However, as these methods are frequently based on assumptions or incomplete knowledge, their combined use is essential, while in most, if not all, cases additional experiments (often using more classical and biochemical methodology) are required to confirm the biological conclusions drawn from microarray data analyses.

Outline of this Thesis:

The research described in this thesis was initiated to gain more insight in the transcriptional response of *Lactobacillus plantarum* during relevant stress conditions, growth phases, and growth conditions. Bacteria encounter various stress conditions during industrial fermentations, whereas global regulators are involved in the response of numerous conditions, including stress and differential growth conditions.

As the amount of effects and regulator proteins that could be subjected to transcriptome studies is virtually endless, it was decided that most attention would be given to industrial relevant conditions: the role of global regulators, growth control, and stress response. To perform the research an amplicon-based microarray was produced, tested and optimized. Using this microarray, genome-wide transcriptome profiling was performed, data were subsequently analyzed and additional experiments were designed and carried out.

Chapter 2 addresses the impact of inactivation of one of the two alternative sigma factors identified in the *L. plantarum* genome, the σ^{54} encoding gene *rpoN*. Despite its relatively small regulon, encompassing not more than a single mannose PTS encoding gene cluster, the inactivation/deletion of this gene was found to have a significant impact on global physiology of *L. plantarum* through its (in)direct effect on glucose transport and metabolic control of transport of other sugars. The global peroxide-stress response is described in **Chapter 3**. In addition, this chapter exemplifies the global physiological role of σ^{54} in *L. plantarum* by clarifying the role of the σ^{54} controlled mannose PTS system in peroxide resistance in *L. plantarum*. In **Chapter 4**, the anaerobic and aerobic growth of *L.plantarum* was compared by studying both growth-mediated alterations in gene expression patterns as well as those mediated by exposure to oxygen and/or oxidative stress. Comprehensive interpretation of array results allowed us to hypothesize the mechanism underlying an observed aerobic growth

bottleneck, and subsequent adjustments of fermentation confirmed the postulated hypothesis and led to increased aerobic fermentation performance of *L. plantarum*. Global gene regulation was addressed by the multi-level comparative analysis of wild-type cultures with those of a mutant derivative strain that lacks the gene encoding the renowned global regulator of carbon catabolite control, *ccpA* (**Chapter 5**). In this study, transcriptome data were complemented with proteome analysis of the same samples and general physiology and growth studies as well as carbon metabolism end-product analysis. Sugar transport and utilization is addressed in **Chapter 6**, and interpretation of transcriptome data is largely based on knowledge obtained in the other chapters. **Chapter 7** includes the general discussion, the conclusions and future perspectives of the research described in this thesis.

Material and Methods

Data for figure 2 were obtained using the automatic literature search tool Milano (77). Microarray and transcriptome were used as primary keywords and the years 1995-2006 as secondary key words. The total number of papers per year was obtained searching the Pubmed database with limitation “published between” for the specific years, leaving the search query blank.

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Chapter 2

Sigma 54 Mediated Control of the Mannose PTS in *Lactobacillus plantarum* Impacts on Carbohydrate Metabolism

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Abstract

Here we present the elucidation of the σ^{54}-regulon of the lactic acid bacterium *Lactobacillus plantarum*. A prediction made using a sequence-based approach combined with an analysis of the organisation of genetic loci of putative σ^{54}-dependent operons, revealed an operon encoding a mannose phosphotransferase system (PTS) as best candidate for σ^{54}-regulation. A strain lacking a functional *rpoN*-gene (encoding for σ^{54}) was constructed and found to have lost its capability to grow on mannose, thereby confirming the prediction. Further analyses using gene deletion strains revealed the presence of one functional mannose PTS in *L. plantarum* regulated by σ^{54} and a σ^{54}-activator ManR. Additional validation of the prediction was performed by comparing genome-wide transcription of the wild-type and the *rpoN*-deletion strain that revealed only 9 genes to be up regulated in the wild-type, including the genes of the mannose PTS, and 21 in the mutant strain. In addition, it was shown that the σ^{54}-regulated mannose PTS is an important glucose transporter in *L. plantarum* and that presence of the mannose PTS is essential for growth initiation on galactose. Furthermore, the mannose PTS appeared to drain PEP-pools in resting cells as no PEP could be detected in resting wild-type cells, whereas strains lacking the mannose PTS had intracellular concentration of 10-30 μM PEP mg-protein^{-1}. Our data give insight in the role of σ^{54} in *L. plantarum* as regulator of a glucose transporter, which modulates glycolytic intermediate concentrations in *L. plantarum*.

Introduction

Bacterial transcription initiation requires a sigma (σ) factor to direct the RNA polymerase core enzyme to the promoter. Most bacteria possess more than one sigma factor, each with its own specific promoter sequence, generating an important mechanism for differential gene expression in bacteria. Based on structural and functional criteria, sigma factors can be categorized into two main classes (36), a class containing all the *E. coli*- σ^{70} like factors and a class containing only a single member, the σ^{54} factor (37). Although not immediately recognized as a sigma factor, σ^{54} was originally discovered in the Gram negative bacterium *Salmonella typhimurium* where it was found to regulate expression of the glutamine synthetase (23). The σ^{54} transcription factor (also known as σ^{N} or σ^{L}) differs from all other sigma factors by binding to a promoter with a conserved -12/-24 motif (11),(39) and by the absolute requirement for a dedicated activator protein to initiate transcription by the DNA bound σ^{54}-RNA-polymerase complex (RNAP). This activator protein catalyses ATP-dependent transition from a closed to an open complex of the RNAP, which enables initiation of transcription (49). The activator protein usually binds upstream of the σ^{54}-promoter site, and DNA looping is required for the activator to contact the RNAP, resembling a mechanism similar to transcriptional initiation in eukaryotes, and for this reason the σ^{54}-activators are also called bacterial enhancer binding proteins (EBPs) (54).

Since transcription of σ^{54}-dependent genes absolutely requires the activity of the specific activator protein, transcription of these genes is generally very tightly controlled with low levels of promoter leakage (60). Moreover, another advantage of σ^{54}-dependent regulation is the possibility to modulate gene expression from a promoter over a wide range without requirement for additional transcription regulators, thereby allowing rapid and strict regulation of gene expression under changing physiological conditions (10).

Comparison of 186 σ^{54}-dependent bacterial promoters led to a defined promoter sequence (-12/-24) and spacer-length consensus (5). Deletion of one or more nucleotides in the spacer region of σ^{54}-dependent promoters led to a dramatic decrease or complete loss of promoter activity (9) and although there are no data available about promoter activity after insertion of nucleotides, the spacer-length between the -12 and -24 regions is considered to be essential for promoter function (5). Similarly, a range of σ^{54}-dependent genes has been identified and σ^{54} appears to be involved in a variety of cellular processes, including nitrogen assimilation and fixation, glutamine synthesis and transport, and sugar transport (for a review see Studholme et. al (53)). *In silico* analysis of bacterial genomes has led to the identification of

σ^{54}-encoding genes in various bacteria, representing all bacterial phyla. Bacteria which do have a σ^{54} gene generally also have one or more predicted σ^{54} activator genes, which in many cases are genetically linked to the corresponding regulated genes (45).

In low-GC Gram-positive bacteria σ^{54} dependent genes have been described in several species. In *Bacillus subtilis*, σ^{54} (there designated as σ^{L}) is involved in levanase and acetoin import (2) (18) and in arginine and valine degradation (24) (17). In *Listeria monocytogenes* and *Enterococcus faecalis* the expression of the mannose phosphotransferase system (mannose PTS) encoding operon appears to be strictly σ^{54}-dependent (14, 29).

Lactobacillus plantarum is a gram-positive rod-haped lactic acid bacterium that can be found in vegetable, meat, and dairy fermentations. It is also encountered as a natural inhabitant of mammalian gastrointestinal tracts and specific strains are marketed as probiotics (1, 16). The complete genome sequence of *L. plantarum* strain WCFS1 has been determined and appears to encode three putative sigma factors, i.e., σ^{A} and σ^{H}, both assumable belonging to the σ^{70}-family, and σ^{54} (32).

Here we describe the *in silico* and experimental characterization of the σ^{54} regulon of *L. plantarum* WCFS1. Genome-wide *in silico* predictions as well as transcriptome analyses show that only the mannose PTS operon is directly regulated by σ^{54} in *L. plantarum*. Furthermore, this mannose PTS is found to constitute the main glucose uptake system in *L. plantarum*. As a consequence, σ^{54} does not only control the expression of the mannose PTS but also has an impact on transport of other carbon sources by an indirect regulation mechanism, mediated by the intracellular concentration of glycolytic intermediates.

Material and Methods

Bacterial strains, media, and growth conditions

The bacterial strains used in this study are listed in table 1. *E. coli* DH5α was used as an intermediate cloning host and was grown aerobically at 37° C in TY-medium (48). When appropriate, chloramphenicol was added to a final concentration of 8 µg/ml. *L. plantarum* WCFS1 and its derivatives were anaerobically grown in MRS broth (15) supplemented with 2% w/v of selected carbon source (mannose, glucose, galactose etc.) or in an chemical defined medium designed for *L. plantarum* (55). Cells were grown at 30° C. Carbon sources were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Growth was monitored by

measurement of the optical density at 600 nm (OD_{600}) in a spectrophotometer (Ultrospec 3000, Pharmacia Biotech, Roosendaal, the Netherlands).

Table 1: Strains and plasmids used in this study and their relevant characteristics and references.

Material	***Relevant features***[a]	***Source***
Strains		
E. coli		
DH5α	Cloning host	(61)
L. plantarum		
WCFS1	Wild-type strain, human saliva isolate	(32)
NZ7306	*rpoN* gene replacement (*rpoN::P_{32}cat*) derivative of *L. plantarum* WCFS1	This work
NZ7307	*ManR::Cat* idem	This work
NZ7308	*ManIIC::pNZ7350* idem	This work
NZ7309	*ManIIAB::Cat* idem	This work
NZ7310	*ManIIB::Cat* idem	This work
NZ7311	*ManIIABCD::Cat* idem	This work
NZ7312	*ManIIA+B (86+87)::cat* idem	This work
Plasmids		
pNZ5318	Cm^R, Em^R, gene replacement mutagenesis vector	(35)
pNZ5319	Cm^R, Em^R, gene replacement mutagenesis vector	(35)
pNZ7302	Cm^R, Em^R, pNZ5318 *rpoN::*cat gene replacement mutagenesis vector containing 5'- and 3'-flanking regions of *rpoN*.	This work
pNZ7332	Cm^R, Em^R pNZ5319 *manR::*cat gene replacement mutagenesis vector containing 5'- and 3'-flanking regions of *manR*.	This work
pNZ7350	Cm^R, Em^R pNZ5319 derivative containing a 0.4 kb internal fragment of *manIIC*.	This work
pNZ7364	Cm^R, Em^R pNZ5319 *manIIAB::cat* gene replacement mutagenesis vector containing 5'- and 3'-flanking regions of *manIIAB*.	This work
pNZ7365	Cm^R, Em^R pNZ5319 *manIIB::cat* gene replacement mutagenesis vector containing 5'- and 3'-flanking regions of *manIIB*.	This work
pNZ7366	Cm^R, Em^R pNZ5319 *manIIABCD::cat* gene replacement mutagenesis vector containing 5'- and 3'-flanking regions of *manIIABCD*.	This work
pNZ7372	Cm^R, Em^R pNZ5319 *manIIAB::cat* gene replacement mutagenesis vector containing 5'- and 3'-flanking regions of *manIIA+B*.	This work

[a] Cm^R, chloramphenicol resistant; Em^R, erythromycin resistant.

DNA manipulations and gene disruption

Molecular cloning and DNA manipulations were essentially performed as described by Sambrook et al. (49). Plasmids constructed in this study are listed in table 1. Large scale plasmid DNA isolations from *E. coli* were performed using a Jet Star Maxiprep Kit (Genomed GmbH, Bad Oberhausen, Germany). DNA isolation from *L. plantarum* was performed as described previously (21). Restriction enzymes and *Pwo*-polymerase were obtained from Promega (Leiden, The Netherlands). T4-ligase was obtained from Boehringer (Mannheim, Germany). Primers were purchased from Proligo (Paris, France).

Table 2: Primers used in this study.

Name:	Sequence:	Name:	Sequence:
rpoN-U-5′	*5′-AGTCGAGCTCTGTCCTTCACTGAATCTTGG-3′*	*manIIA+B-U-5′*	*5′-TTATGATGTTCAGGCTTTCG-3′*
rpoN-U-3′	*5′-AACCCTTGTGTTTTTATCCA-3′*	*manIIA+B-U-3′*	*5′-CAAAACTCCCATGACTGACA-3′*
rpoN-D-5′	*5′-AGTCCTGCAGCAACCACCTTATGAACCTGT-3′*	*manIIA+B-D-5′*	*5′-TTTATGGCGCCAGATAAGTA-3′*
rpoN-D-3′	*5′-AGTCAAGCTTAATGTTCTTCATATACGCGG-3′*	*manIIA+B-D-3′*	*5′-GCACACCCCTACTTCTTGAT-3′*
manR-U-5′	*5′-GTTTCAGACACGCTTGGAAC-3′*	*Con-rpoN-5'*	*5′-CTTGCCCTTAGTACCAGATG-3′*
manR-U-3′	*5′-GCACAGGCCGCCCTGTCAGC-3′*	*Con-rpoN-3'*	*5′-GAATGCTAAACGACCGATAC-3′*
manR-D-5′	*5′-CGAAGTGGTACCCACTTGTG-3′*	*Con-manR-5'*	*5′-ACGTTAGCTGTATCATTGGC-3′*
manR-D-3′	*5′-TAGCGGCATCACATCAACCC-3′*	*Con-manR-3'*	*5′-AACATATTGTCCCATGGCGG-3′*
manIIC-I-5′	*5′-CAAACGTTGGTGCCGCTGTT-3′*	*Con-manIIC*	*5′-ATGAATTTGAATGCAATTCA-3′*
manIIC-I-3′	*5′-GCGTAACCAACGGCAACAAC-3′*	*Con-manIIAB-5'*	*5′-GGCGAGGACCAGGCATTGAT-3′*
manIIAB-U5′	*5′-AGTAGATGGTGGTGGCGTAT-3′*	*Con-manIIAB-3'*	*5′-GGTGAGGCAACGTATGGATG-3′*
manIIAB-U3′	*5′-AGCAATGATAATGCTTACCAT-3′*	*Con-manIIPTS-3'*	*5′-ACAAATCTCCCCAATCTGCC-3′*
manIIAB-D5′	*5′-AACATGTTAAATGAACAAAAGTAGTA -3′*	*Con-ManIIB-5'*	*5′-TGCCAGCCACGTGCCGCGCA-3′*
manIIAB-D-3′	*5′-AGAATTCCAAATGACGCTTT-3′*	*Con-ManIIA+B-5'*	*5′-TTCCATGGCACAGGTCGTCG-3′*
manIIB-U-5′	*5′-CGAATCCTAACACTGGCTTT-3′*	*Con-ManIIA+B-3*	*5′-CCCTGTCGCGCGTATTTCTC-3′*
manIIB-U-3′	*5′-GTTATTGTTGGTCGTTGCAG-3′*	*Con-cam-for*	*5′-GATAGGCCTAATGACTGGCT-3′*
manPTS-D-5′	*5′-ATGCATGTTCTTGGTGTAATGT-3′*	*Con-cam-rev*	*5′-CTCTTCCAATTGTCTAAATC-3′*

Construction of plasmids and strains

Gene disruption was performed using a double-cross-over (DCO) gene replacement strategy (except for strain NZ7308, *manIIC::pNZ7350*, see below), which resulted in replacement of the target gene by a chloramphenicol resistance gene-cassette (P_{32}*cat*; (8)). For disruption of the *rpoN*-gene a 1.2 kb fragment of the upstream region of the target locus was amplified using a proof-reading DNA-polymerase (*Pwo*) and the primers *rpoN-U-5'* and *rpoN-U-3'* (Table 2). Similarly, a 1.2 kb fragment of the downstream region of *rpoN* was amplified using primers *rpoN-D-5'* and *rpoN-D-3'* (Table 2). A similar strategy was used for the *manR*-gene, using primers *ManR-U-5'* and *ManR-U-3'* and primers *manR-D-5'* and *manR-D-3'* to amplify the upstream and downstream fragments, respectively (Table 2). The up- and down-stream fragments were sequentially cloned into the SwaI and Ecl136II sites, respectively, of the gene replacement vector pNZ5318 (35), resulting in *rpoN*-replacement vector pNZ7302 and the *manR*-replacement vector pNZ7332 (Table 1). All mutagenesis constructions aimed to replace the target gene by the chloramphenicol resistance cassette with the *cat*-gene in the same orientation as the target gene. Correct plasmid construction was checked by restriction analyses.

A similar DCO strategy was used to disrupt *manIIABCD, manIIAB, manIIB,* and *manIIA+B,* with the modification that the pNZ5318-derivative, pNZ5319 (35) was used as cloning vector, a vector harboring *lox*-sites up- and downstream of the P_{32}-*cat*-cassette, which enables *cat*-gene deletion by a resolvase enzyme resulting in a clean gene deletion as described by

Lambert et. al. (35). Flanking regions of the mannose operon (*manIIABCD*) were amplified using the primer-sets *manIIAB-U-5'* and *manIIAB-U-3'* for the upstream region and *manPTS-D-5'* and *manPTS-D-3'* for the downstream region. Flanking regions of *manIIAB* were amplified using primers *manIIAB-U-5'* and *manIIAB-U-3'* (upstream) and *manIIAB-D-5'* and *manIIAB-D-3'* (downstream), flanking regions of *manIIB* using *manIIB-U-5'* and *manIIB-U-3'* (upstream) and *manIIAB-D-5'* and *manIIAB-D-3'* (downstream), and flanking regions of *manIIA+B* using *manIIA+B-U-5'* and *manIIA+B-U-3'* (upstream) and *manIIA+B-D-5'* and *manIIA+B-D-3'* (downstream). The obtained upstream regions were cloned into the SwaI-site and the downstream regions into the Ecl136-II site of the gene replacement vector pNZ5319, resulting in the *manIIABCD*-replacement vector pNZ7366, the *manIIAB*-replacement vector pNZ7364, the *manIIB*-replacement vector pNZ7365, and the *manIIA+B*-replacement vector pNZ7372 (Table 1).

The resulting plasmids were transformed into *L. plantarum* as described previously (30) and primary plasmid-integrants were selected on MRS-plates with 8 µg/ml chloramphenicol and 1% glycerol at 30° C. To check for erythromycin sensitivity, colonies were picked and transferred to MRS plates with 20, 40, 60, and 80 µg/ml erythromycin and grown over night at 30°C. The anticipated genetic organization after correct gene replacement leads to chloramphenicol resistance and erythromycin sensitivity, integrants with this phenotype were checked by PCR using universal-primers (*C-cat-for* and *C-cat-rev*) annealing in the *cat*-gene and a site-specific primer (*C*-primers in table 2) annealing outside of the chromosomal region used for homologous recombination. The 5'-primers were combined with primer *C-cat-rev* and the 3'-primers were combined with *C-cat-for*.

For the disruption of *manIIC* a single-cross-over strategy (SCO) was used. A 400 bp internal fragment was amplified using primers *manIIC-I-5*' and *manIIC-3'* (Table 2) and cloned into the SwaI-site of pNZ5319 resulting in the *manIIC*-disruption vector pNZ7350. The resulting plasmid was transformed to *L. plantarum* and primary plasmid-integrants were selected on MRS-plates with 8 µg/ml chloramphenicol and 1% glycerol at 30° C. Correct integration of the disruption plasmid was checked by PCR using primer *C-manIIC*, annealing upstream of the integration site, and the *C-cat-rev* primer.

RNA extraction and quality control

RNA was isolated from exponential growing *L. plantarum* WCFS1 cells (OD_{600} = 1.0). The cells were harvested by centrifugation at 3360 x g for 5 minutes at 30°C. Cell pellets were transferred with a spatula to a screw-cap tube containing 500 mg zirconium beads, 500µl of a phenol/chloroform mixture (1:1, v/v), 30 µl 3 M Na-Acetate (pH 5.2), 30 µl 10% SDS, and

400 µl MRS-medium (Merck, Darmstadt, Germany) and immediately frozen in liquid nitrogen. The cells were disrupted by bead-beating four times for 40 s at speed 4.0 using a Fastprep cell disrupter (QBiogene Inc., Cedex, France), interspaced with cooling intervals on ice. The tubes were centrifuged for 1 min at 22800 x g (4°C) and the aqueous-phase was transferred into an Eppendorf-tube. Residual phenol traces were removed by extraction with pre-chilled chloroform. The resulting aqueous phase was mixed with an equal volume of binding buffer (provided in the kit) and applied to a RNA purification column from the High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany). Further purification was performed following the protocol provided by the manufacturer, including on-column incubation with DnaseI for one hour and using a 50 µl elution volume.

The yield and purity of the RNA were determined by measurement of the absorption at 260 nm and 280 nm (Ultrospec 3000, Pharmacia Biotech, Roosendaal, The Netherlands). The RNA quality was assessed using the RNA 6000 Nano Assay in an Agilent 2100 Bioanalyzer (Agilent technologies, Palo Alto, Ca, USA) following the manufacturer's instructions. Only RNA samples displaying 16S/23S-rRNA ratios of 1.6 or higher, were labeled and used for micro array experiments.

cDNA-synthesis, labeling, and hybridization

The Cyscribe Post-labeling kit was used to synthesize cDNA out of 25 µg of total RNA, which was subsequently labeled according to the manufacturer's protocol (Amersham Biosciences, Amersham, UK). Subsequently, labeled cDNA solutions were concentrated in a Hetovac VR-1 (Heto Lab Equipment A/S, Birkerod, Denmark) to a final volume of 10 µl.

Two individual, differentially labeled cDNAs were incubated at 95° C for 3', cooled down to 68° C, and mixed (final-volume 20 µl). To these mixed cDNAs 180 µl of pre-heated (68° C) Slidehyb#1 hybridization buffer (Ambion, Austin, USA) was added and the resulting solution was applied on a pre-heated slide (68° C). Slides were then hybridized at 44° C for 16 hours. Subsequently, slides were washed at 42°C, once in 1 x SSC/0.2% SDS and twice in 1 x SSC and dried by centrifugation (1 x SSC is 0.15 M NaCl and 0.15 M Sodium Citrate).

Hybridizations were performed in duplicate as shown in figure 1.

Open reading frame-based micro array design and spotting

DNA-microarrays were prepared using PCR- amplicons of 2917 genes in the genome of *Lactobacillus plantarum* WCFS1, (EMBL database, accession number AL935263) resulting in a coverage of 97%. Primers were designed to amplify unique regions of these genes using UniFrag and GenomePrimer (58). The optimal amplicon length was set at 750 bp unique regions within the genes. Genes smaller then 750 bp were amplified entirely. Gene-specific

primers were extended with a universal 15 base pair long sequence tag at the 5' end (TGGCGCCCCTAGATG for the 5'-primers and CGCGATGCTGATTGC for the 3'-primers), to enable universal-primer based re-amplification, which was used to generate two-sided terminally aminated gene-specific amplicons. Aminated amplicons were mixed 1:1 with telechem spotting buffer (ArrayIT, Sunnyvale, USA) and spotted in duplicate on SMM superamine slides (ArrayIT, Sunnyvale, USA). After spotting the slides were washed and blocked with Na_2BH_4 as described previously (34). The slide-quality was checked with SpotCheck (Genetix, New Milton, UK) according to the manufacturer's protocol.

Scanning, Data extraction, and analyses

The slides were scanned with a Scan Array Express 4000 scanner (Perkin Elmer, Wellesley, USA) and the images were analyzed with Imagene software 4.2 (BioDiscovery, El Segundo, USA).

Statistical analyses were performed with R (http://www.r-project.org/) using the linear models for microarray data library limma (51). Background corrected spot intensities in both channels (I1 and I2) were converted to M-A coordinates, where M=log2 (I1/I2) and A=log2 (I1/I2)/2 and subsequently normalized using a LOESS fit, assuming that, on average, M is independent of A and centered around 0 (51). Normalized intensities were used for further analysis. Log odds for differential expression (B-value) higher than 1 were taken as a cut-off. As amplicons were spotted in duplicate, two measurements per gene were performed and only genes of which both measurements matched the criteria mentioned above were taken into account.

Physiological characterization

The rate of lactate production was estimated by measuring the acidification rate in acidification buffer (0.5 mM potassium phosphate, 70 mM KCl and 1 mM MgSO4) of pH 6.4 containing 0.5% (W/V) of a specified carbon source, as described previously (43). Cells were grown till OD_{600} = 1.0, washed twice in acidification buffer to remove all residual carbon sources and suspended to a density of OD_{600} = 1.0. Following calibration of the cell suspension, a carbon source was added (t_0) and pH-change was followed in time. Regression linears of $\Delta pH/\Delta t$ were calculated for each suspension within the linear range of acidification using Excel XP (Microsoft, Redmond, USA). The pH-change was converted into H^+-production by calibration of the cell suspension with 2 µl portions of 50 mM HCl.

Transcription analysis of the mannose operon

RNA was isolated from exponentially growing *L. plantarum* WCFS1 cells cultured in chemical defined medium (CDM) (55) supplemented with specific carbon-sources as described above. Total RNA (5 µg) was blotted on Gene Screen filter (New England Nuclear,

Boston, USA) as recommended by the manufacturer. An internal fragment of the *manIIC*-gene was synthesized using the primers *manIIC-I-5'* and *manIIC-I-3'* (Table 2). The fragment was labeled with α-^{32}P-ATP (Amersham Biosciences, Amersham, UK) by nick translation (48) and used for hybridization at 65º C in 6 x SSC/0.2% BSA for 2 hours. Blots were washed 10 minutes with 6 times SSC followed by 30 minutes washing with one time SSC and finally with 0.1 times SSC at 65°C for 30 minutes prior to autoradiography.

Bio-informatics methods

To identify putative σ^{54}-promoters the algorithm for fitting a mixture model by expectation maximization (MEME) (3) was used on 17 experimentally verified σ^{54}-dependent promoters from *E. coli* (45). MEME-parameters were set as follows: one motif per sequence should be found (OOPS) and only the given strand should be searched. The obtained MEME-based motif was used to search the complete genome of *Lactobacillus plantarum* WCFS1, using the motive alignment and search tool (MAST) (4). MAST parameters were a cut-off E-value of $1.00x10^{-6}$, only motifs lying between the start codon and 300 base pairs upstream of the start codon were selected, and overlap with other genes was allowed.

Analyses of glycolytic intermediates

L. plantarum cells grown in chemical defined medium (CDM) containing 1% glucose (55) until OD_{600} of 1.0 were harvested by centrifugation (3360 x g, 30° C, 5 minutes), washed twice with CDM without carbon source and resuspended in CDM without carbon source at an OD_{600} of 5.0. Samples (10 ml) taken after 0, 15, 30, 60, 120, and 180 minutes were quenched in a -40º C 60% methanol-HEPES buffer as described by Pieterse et. al. (41). Quenched cells were harvested by centrifugation at 3500xg at -20º C using a Sorvall RC5B plus centrifuge (Sorvall, Newton, US) and to remove medium components cells were subsequently washed once with quenching buffer (pre-cooled at -40º C) and once with ice-cold water. Subsequently cells were stored overnight at -80º C before lyophilization. The lyophilzate was resuspended in 500 µl water and cell debris was removed by centrifugation (20800xg, 4° C, 5 minutes) to obtain a cell-free extract. The extract was passed through a 0.22 µm filter before HPLC-analysis. HPLC-analyses were performed as described previously (6) (7), using an anion-exchange DX-300 column and using a conductivity detector in combination with an anion self-regenerating suppressor (Dionex) to enhance signal-to-noise ratio. The flow-rate of the elution buffer was adjusted to 1ml/min (half-speed) leading to doubled running time (from 45 to 90 minutes) and allowing better separation of the individual peaks.

Results

In silico regulon prediction of σ^{54} in *L. plantarum* WCFS1

The genome of *L. plantarum* WCFS1 revealed the presence of an *rpoN* -gene encoding a 444-residue protein that displayed high level identity to σ^{54}-proteins in other bacteria, including closely related species, like *Pediococcus pentocaseus* (53% identity), *Enterococcus faecalis* (51%) and *Listeria monocytogenes* (40%). Furthermore, the *rpoN*-gene of *L. plantarum* appeared to be located 220 bp upstream of the *gapA*-operon headed by the central glycolytic genes regulator and encoding the glycolytic enzymes *gapB*, *pgk*, *tpiA*, and *enoA* . This resembles the genetic organization in *P. pentocaseus* and *Listeria* species .

To elucidate the role of σ^{54} in *L. plantarum*, we initially set out to predict the σ^{54}-regulon in this species. Previously, σ^{54}-dependent promoter sequences have been described a range of bacteria and they appear to be conserved throughout the bacterial kingdom (5, 45). To obtain a reliable prediction of the *L. plantarum* σ^{54}-regulon, σ^{54}-dependent promoters from *E. coli*(45) were employed to construct a sequence consensus matrix (conserved -24, and -12 boxes of a fixed length and sequence) using MEME (3). The MEME-derived motif sequence matrix was used to search the *L. plantarum* WCFS1 genome using MAST (4) and a surprisingly limited set of hits were obtained (Table 3). The best hit (score 5.6 10^{-9}) was a sequence encountered 123 base pairs upstream of an operon encoding genes of a mannose phosphotransferase system (mannose PTS) (Table 3). The next-best hit, a sequence in front of a gene encoding an N-acetylglucosamine-6-phosphate deacetylase, had a drastically poorer E-score, but still remains within the significance cut-off employed here (Table 3).

As mentioned before, σ^{54} containing RNA polymerase requires an activator protein to initiate transcription (49). These activators possess a unique σ^{54}-interaction domain (Pfam accession number PF00158 (21, 54)), which can be used to search in bacterial genomes for σ^{54}-activator presence. Such a search revealed a single candidate σ^{54}-activator encoding gene in the *L. plantarum* genome, ORF lp_0585. Detailed analysis of the protein encoded by lp_0585 revealed it to contain 3 additional Pfam domains, i.e., two PTS regulatory domains (i.e., PRD; Pfam PF00874) and a ManIIA domain (Pfam PF02954). PRDs are phosphate accepting domains found in bacterial transcriptional regulators of catabolic operons (59), whereas the ManIIA domain serves in phosphate transfer to mannose in mannose specific PTSs (28). The presence of these domains in the σ^{54}-activator protein suggested a role of this activator in regulation of catabolic genes. Moreover, σ^{54}-activators are often found to be genetically linked to the genes they regulate. Notably, the σ^{54} activator gene in the *L. plantarum* genome

ORF	Gene	Product	Sequence	Score	Position
lp_0575	*pts9AB*	mannose PTS, IIAB	TATTT **TGGC**ACGGAAT**TTGC** ATATA	5.6E-09	-123
lp_0562	*nagA*	N-acetylglucosamine-6-phosphate deacetylase	CCTGG **TGGC**ACGACGG**TTGC** TGGAT	1.2E-07	-169
lp_2712	*lp_2712*	transport protein	CGACT **TGGC**ATAAAAC**TTGC** CCATG	1.8E-07	-154
lp_1048	*rpsN*	ribosomal protein S14	CAATT **TGGC**ATGCCAT**TTGC** TAAAT	3.6E-07	-27
lp_3070	*lp_3070*	hypothetical protein	GCAAG **TGGC**ACAGATT**CTGC** ACCTG	4.0E-07	-271
lp_0220	*gpo*	glutathione peroxidase	CGTGA **TGGC**ACCCCAG**TTGC** TATGA	6.6E-07	-149
lp_0586	*pts10A*	mannose PTS, IIA	TAACT **TGGC**ATGCTTT**TTGC** ATGTA	1.5E-06	-45
lp_3688a	*rpmH*	ribosomal protein L34	ACGAT **TGGC**ATACGAG**TTGC** GGGTC	1.5E-06	-170
lp_1541	*gnd2*	phosphogluconate dehydrogenase (decarboxylating)	TTTTT **TGGT**ACGATAG**TTGC** GTATT	1.7E-06	-28
lp_3331	*ISP2_5-N*	transposase, N-terminal fragment	ATCTA **TGGC**ACAATAG**TTGA** TAAGT	1.9E-06	-273
lp_2613	*lp_2613*	ABC transporter, permease protein	GAACA **TGGC**ATGGAAC**TTGA** AGATT	2.1E-06	-38
lp_1110	*lp_1110*	hypothetical protein	CGCGA **TGGC**ACGATTA**TTGA** TACGA	2.3E-06	-224
lp_1510	*coaE*	dephospho-coa kinase	CGGTG **TGGC**ACAATTA**TTGA** GAAAA	2.6E-06	-58
lp_2271	*mutS*	DNA mismatch repair protein Muts2	GATCA **TTGG**CACGGCA**TCTG** ACCGT	3.2E-06	-237
lp_2954	*lp_2954*	integral membrane protein (putative)	GGTTA **TGGC**ATTGATT**TTGC** GCTAC	5.0E-06	-257
lp_3054	*lp_3054*	aryl-alcohol dehydrogenase	GCGGT **TTAC**ATACTAT**TTGA** AACGA	5.0E-06	-286
lp_1395	*lp_1395*	hypothetical protein	ATTGG **TGGA**ACGCAGG**TTGC** TAGTG	5.6E-06	-274
lp_1596	*efp*	elongation factor P	ACGAC **TGGC**ACCTTAG**TTGC** AAACA	5.6E-06	-206
lp_2925	*lp_2925*	cell surface protein precursor	GTTGC **TGGC**ACGTCTA**TTGA** CAGTC	5.6E-06	-154
lp_1319	*rsuA*	pseudouridylate synthase	ATGAT **TGGA**ACGAAAG**CTGC** TGGGT	6.2E-06	-38
lp_1488	*hpk4*	histidine protein kinase; sensor protein (putative)	CGTAG **TGGC**ATCGGCA**TTGC** CGGGC	6.2E-06	-264

Table 3: Predicted σ^{54} dependent promoters in *Lactobacillus plantarum* WCFS1. The position of the promoter (-12 region) in relation to the start codon is listed in the last column.

is located immediately upstream of a *manIIAB*-gene cluster (lp_0586-0587) that is predicted to encode components of a mannose PTS system, but appears to be incomplete since it lacks a permease-encoding gene. Remarkably, the predicted *L. plantarum* σ^{54}-activator is located downstream of a second mannose PTS operon with the best scoring σ^{54} dependent promoter sequence (Table 3 and Fig. 1). However, these genetic loci appear to be separated in the *L. plantarum* WCFS1 genome by a 15 kb insertion encoding, a non-ribosomal-peptide synthesis machinery (NPS) (Fig.1). It is noteworthy that this NPS encoding cluster appears to be absent in other *L. plantarum* strains as was concluded on basis of genome-wide, array-based comparative genome hybridization (CGH) analysis of this species (38), which could be further substantiated by PCR-based confirmation of the direct linkage between the mannose operon and the *manR* genes in eight other strains of *L. plantarum* (data not shown).

In conclusion, the *in silico* analyses suggested a role for σ^{54} in regulation of mannose PTS encoding operons found up- and downstream of the σ^{54} activator encoding gene. Nevertheless, these findings can not exclude the additional involvement of σ^{54} in modulation of other target genes that appear to be preceded by a promoter resembling the σ^{54} dependent consensus sequence.

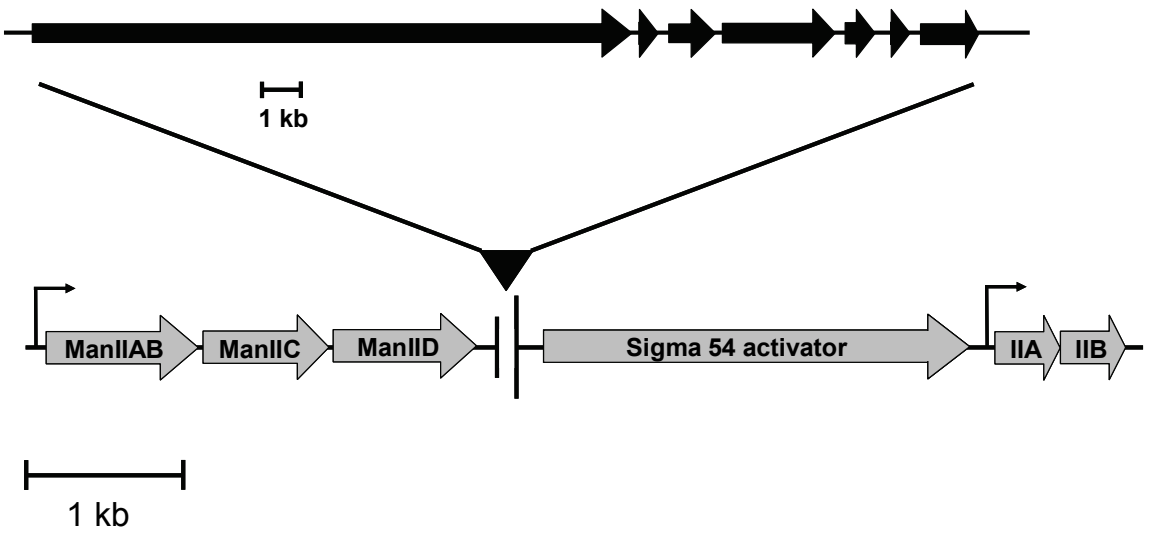

Figure 1: Schematic representation of the mannose gene cluster in L. plantarum. The 15 kb insertion encoding a non-ribosomal-peptide machinery is printed at a smaller scale. Predicted σ^5-dependent promoters are indicated.

Mannose utilization in *L. plantarum* WCFS1

To elucidate the σ^{54}-regulon in *L. plantarum,* a *rpoN* deletion strain was constructed using a double cross-over gene-replacement strategy resulting in strain NZ7306 (*rpoN*::P_{32}*cat*) (Table 1). Since in our *in silico* analysis the best scoring σ^{54}-promoter sequence was predicted upstream of the mannose-PTS operon and a σ^{54} activator gene appeared to be located downstream of that same operon, we evaluated the ability of the *rpoN* deletion strain to grow on mannose. The wild-type strain had almost identical growth rates in MRS medium supplemented with mannose or glucose as carbon source (Table 4). The *rpoN* deletion strain NZ7306 displayed similar growth characteristics as compared to its parental strain when grown on MRS containing glucose. In contrast, when grown on mannose the growth rate was reduced dramatically (Table 4), corroborating a role for σ^{54} in the regulation of genes involved in mannose utilization in *L. plantarum*.

There are two mannose operons in *L. plantarum*, an intact operon (*manIIABCD*) and a truncated operon only encoding ManIIA and IIB proteins (*manIIAB*) (Fig. 4), both containing a predicted σ^{54}-promoter sequence (Table 3) and genetically linked to the σ^{54}-activator gene. To characterize the role of these genes in mannose uptake, gene deletion mutant strains were constructed of the putative regulator (*manR*), both mannose operons, and of the m*anIIAB*-gene and the *manIIB*-gene part of the first, intact operon (Table 1, Fig. 1). Additionally a strain mutated in the IIC-permease was constructed and growth characteristics of all strains were determined (Table 4). The strains deleted in the intact mannose operon or in the σ^{54}-activator gene displayed impaired growth on mannose (Table 4), while the strain mutated in the truncated mannose operon *manIIAB* appeared to be unaffected in terms of growth capacity on mannose containing media. These experiments show that *L. plantarum* contains a single functional mannose PTS system, encoded by the genes lp_0575-0577 (*manABCD*), which is regulated by σ^{54} and by the σ^{54}-activator/mannose operon regulator ManR (lp_0585).

Table 4: Doubling times in minutes of all strains used in this study in MRS or CDM supplemented with 2% (w/v) of different carbohydrates. Doubling times above 4 h are scored as NG (no growth).

Strain	MRS Glucose	MRS Mannose	CDM Glucose	CDM Mannose
WCFS1 *Wild-type*	47.6 ± 2.4	50.4 ± 1.0	55.9 ± 1.1	64.2 ± 0.3
NZ7306 Δ*rpoN*	52.1 ± 1.9	NG	67.9 ± 1.0	NG
NZ7307 Δ*manR*	52.9 ± 0.9	NG	66.4 ± 0.0	NG
NZ7308 Δ*manIIC*	61.4 ± 0.8	NG	68.2 ± 1.2	NG
NZ7309 Δ*manIIAB*	52.4 ± 0.5	NG	65.5 ± 2.3	NG
NZ7310 Δ*manIIB*	54.3 ± 2.6	NG	66.6 ± 0.7	NG
NZ7311 *manIIABCD*	54.3 ± 4.0	NG	66.3 ± 3.4	NG
NZ7312 Δ*manIIA+B*	47.2 ± 0.2	64.7 ± 2.0	65.2 ± 1.6	71.3 ± 3.2

Genome-wide transcription analysis of the *rpoN* deletion strain NZ7306 compared to the wild-type WCFS1

To evaluate the accuracy of the predictions of the σ^{54} target sites, transcriptome profiles of exponentially growing wild-type cells were compared to those of the *rpoN* mutant cells (both grown on MRS medium supplemented with glucose) using genome-wide, amplicon-based DNA-microarrays. The microarray analysis resulted in the identification of 9 genes higher expressed in the wild-type and 23 genes higher expressed in the *rpoN*-mutant (Table 5).

The *rpoN*-gene came out of the analyses as higher expressed in the wild-type, confirming once more the correct deletion of the gene (Table 5A). The most predominant set of genes higher expressed were the *manABCD* genes encoding the mannose PTS, confirming the involvement of σ^{54} in their transcriptional control. Other genes that appeared to be expressed at a higher level had a minor fold-change indicating they were less affected by *rpoN*-deletion. Differential expression of two maltose phosphorylases, an alpha-amylase and an ABC-transporter with high identity to maltose ABC transporters in other organisms (lp_0180) suggests a role for σ^{54} in maltose utilization. However, the predicted regulation of the maltose genes by a LacI-transcription regulator (C. Franken, personal communication) and the virtually identical growth characteristics of the σ^{54}-mutant and the wild-type strain on CDM medium supplemented with maltose (data not shown), contradict such a role for σ^{54}. Furthermore, and a ribonucleoside-diphosphate reductase, alpha chain (lp_0693) was higher expressed in the wild-type, but not the corresponding beta chain lying in the same operon. No putative σ^{54}-dependent promoters could be identified in the upstream regions of higher expressed genes in the wild-type (Table 3), suggesting that the regulation is due to unidentified secondary effect and not to abolished σ^{54}-function.

The gene located immediately downstream of the *rpoN*-gene, the central glycolytic gene regulator (lp_0788; *cggR*) is expressed to a higher level in the mutant, possibly due to read through of the P_{32} promoter upstream of the *cat*-gene that replaces *rpoN* in the mutant. The operon encoding a fatty acid biosynthesis machinery (encoded by the ORF lp_1670-1681) is expressed at a higher level, as are genes involved in membrane modification that are genetically linked to this operon (lp_1682, lp_1684-lp_1686, and lp_1695) (Table 5B). The regulation of this operon might be due to a decreased glycolysis rate of the mutant (see below), which could lead to an altered flux to malonyl-CoA, an essential intermediate in fatty acid biosynthesis which relieves repression of the *fab*-genes in *B. subtilis* (50). The expression of a gene belonging to the DegV family, positively involved in lipid modification (20), indicates changes in lipid composition in the mutant. The higher expression in the mutant of an oxidoreductases encoded by ORF lp_0291 is due to the presence on the plasmid of a 189 bp fragment of this gene directly downstream of the *cat*-gene (35).

The global transcription analysis strongly suggest that the σ^{54}-regulon of *L. plantarum* consists of a single locus, the mannose operon *manABCD*, as was suggested by the outstanding significance of the predicted σ^{54}-promoter sequence upstream of this locus and its genetic linkage to the cognate activator-encoding gene *manR*.

Table 5A: Genes significantly up regulated in the wild-type compared to the mutant NZ7306. Putative operons are indicated bold.

ORF	gene name	Product	Ratio (log^2)	p-value (FDR)	B-value
lp_0179	*amy2*	alpha-amylase	-1.73	1.85E-02	1.02
lp_0180	*msmK1*	multiple sugar ABC transporter, ATP-binding protein	-1.47	7.43E-03	2.63
lp_0181	*map2*	maltose phosphorylase	-2.22	9.11E-03	2.23
lp_0575	*pts9AB*	mannose PTS, EIIAB	-4.45	1.35E-03	5.18
lp_0576	*pts9C*	mannose PTS, EIIC	-3.81	7.55E-04	8.00
lp_0577	*pts9D*	mannose PTS, EIID	-4.68	1.29E-03	5.53
lp_0693	*nrdE*	ribonucleoside-diphosphate reductase, alpha chain	-1.52	1.67E-03	4.81
lp_0787	*rpoN*	DNA-directed RNA polymerase, sigma factor 54	-1.86	1.80E-02	1.06
lp_1730	*map3*	maltose phosphorylase	-1.34	7.43E-03	2.64

Table 5B: Genes significantly down regulated in the wild-type compared to the mutant NZ7306. Putative operons are indicated bold.

ORF	gene name	Product	Ratio (log^2)	p-value (FDR)	B-value
lp_0199		unknown	1.02	9.53E-03	2.11
lp_0291		oxidoreductase	1.47	3.98E-03	3.48
lp_0788	*cggR*	central glycolytic genes regulator	1.20	6.48E-03	2.88
lp_1670	*fabZ1*	(3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase	2.68	1.22E-03	7.12
lp_1671	*fabH2*	3-oxoacyl-[acyl-carrier protein] synthase III	2.36	8.23E-03	2.47
lp_1672	*acpA2*	acyl carrier protein	2.85	1.67E-03	4.70
lp_1673	*fabD*	[acyl-carrier protein] S-malonyltransferase	2.46	8.23E-03	2.42
lp_1674	*fabG1*	3-oxoacyl-[acyl-carrier protein] reductase	2.14	1.48E-02	1.41
lp_1675	*fabF*	3-oxoacyl-[acyl-carrier protein] synthase II	2.56	1.34E-03	5.31
lp_1676	*accB2*	acetyl-CoA carboxylase, biotin carboxyl carrier protein	2.30	1.29E-03	5.45
lp_1677	*fabZ2*	(3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase	2.17	7.18E-03	2.75
lp_1678	*accC2*	acetyl-CoA carboxylase, biotin carboxylase subunit	2.16	2.90E-03	3.95
lp_1679	*accD2*	acetyl-CoA carboxylase, carboxyl transferase subunit beta	1.94	1.49E-02	1.38
lp_1680	*accA2*	acetyl-CoA carboxylase, carboxyl transferase subunit alpha	1.81	9.53E-03	2.14
lp_1681	*fabI*	enoyl-[acyl-carrier protein] reductase (NADH)	2.12	1.49E-02	1.31
lp_1682		phosphopantetheinyltransferase	2.28	1.22E-03	5.78
lp_1684		integral membrane protein	2.05	1.57E-03	4.99
lp_1685		transcription regulator	2.09	1.22E-03	6.44
lp_1686		acyl-CoA thioester hydrolase (putative)	1.81	1.67E-03	4.67
lp_1695		integral membrane protein	1.70	1.40E-02	1.59
lp_1708		unknown	2.49	3.42E-03	3.69
lp_3256		DegV family protein	1.89	1.22E-03	5.83
lp_3487	*galM3*	aldose 1-epimerase	1.05	8.23E-03	2.39

Characterization the mannose PTS in *L. plantarum* WCFS1

The σ^{54}-regulated mannose operon belongs to a separate family of PTS's, the mannose family, characterized by a unique IID enzyme and a fused IIAB enzyme (47, 62). Although it is annotated as a mannose transporter, this PTS is also known to transport glucose in *E. coli* (26) and is presumably the main glucose uptake system in lactic acid bacteria (31). A similar role in *L. plantarum* would require expression of the mannose operon in cells grown on glucose and therefore transcriptional analyses of the mannose PTS genes was performed in cells grown on different carbon sources. The mannose PTS appeared to be only transcribed in cells growing on glucose or mannose and not or only at a very low level in cells growing on lactose, maltose, fructose, cellobiose, or sucrose (Fig. 2), supporting a role for the mannose PTS in mannose and glucose transport.

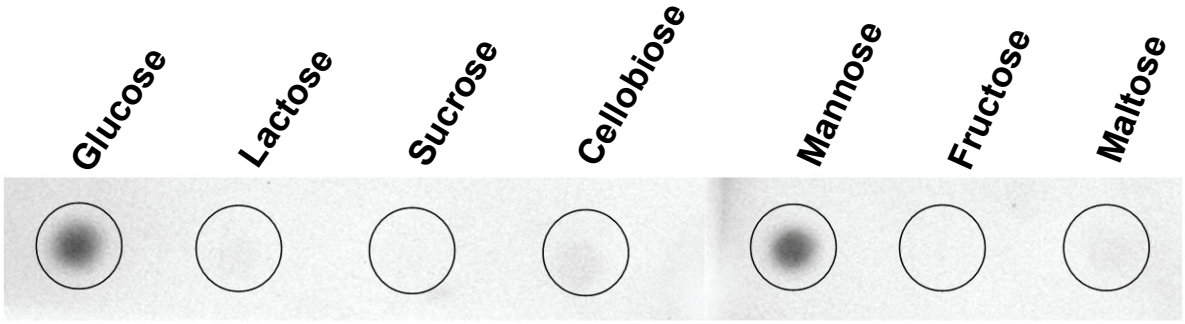

Figure 2: Transcription analysis of the mannose operon on a range of carbohydrates using total RNA from exponential grown cells and an internal 300 bp fragment of the manIIC-gene as probe. The carbohydrates are indicated above the spots.

Involvement of the mannose PTS in glucose transport should lead to decreased glucose uptake in strains lacking this PTS, as already described in *Listeria monocytogenes* and *Lactobacillus pentosus* (12, 57). When grown on MRS a slight difference in growth between the wild-type and mutants affected in the mannose PTS or its regulation was detectable. However, this difference in glucose-growth rates was much more pronounced when cells were grown in chemically defined medium (CDM) (Table 4), supporting a role of the mannose PTS in glucose import.

Reduced glucose uptake rates in *L. plantarum* could result in decreased lactate-formation rates. To evaluate this, the kinetics of lactate production were determined by measurement of the acidification rate in a weakly buffered cell-suspension (43). Wild-type cells were pre-grown on either glucose or mannose and found to produce protons when glucose was used as a substrate in the acidification assay (acidification rate of 238 and 168 nmol protons $mgprot^{-1}$ s^{-1} respectively). This result indicates that that cells grown on mannose apparently contain a glucose transport and utilization machinery, which supports a role of the mannose PTS in glucose import in *L. plantarum* WCFS1. The acidification rate in glucose containing buffer displayed by glucose grown NZ7306 (Δ*rpoN*) cells was lower than that of glucose-grown wild-type cells (acidification rate of 238 and 160 nmol protons mg $prot^{-1}$ s^{-1} respectively indicating reduced glycolysis rate. In contrast to wild-type cells, the glucose-grown NZ7306 cells were not able to convert mannose to lactate, confirming the crucial role of *rpoN* in regulation of mannose utilization capacity in *L. plantarum*.

Overall, these experiments support an important role of the mannose PTS of *L. plantarum* in glucose uptake.

Impact of mannose PTS on overall carbohydrate metabolism

Apart from mannose and glucose, also other carbohydrates have been reported to be transported by homologues of the mannose PTS family (56). The availability of a mutant in this PTS (*manIIABCD::P_{32}cat*) provided the opportunity to evaluate the transport flexibility of the *L. plantarum* mannose PTS system extensively and therefore growth characteristics of the mutant on a range of carbon sources were monitored. The mutant strains were pre-cultured on glucose, washed twice to remove all traces of glucose and inoculated at an OD_{600} of approximately 0.05 in fresh media. To minimize the effects of undefined medium composition cells were grown in chemically defined medium (CDM) (55). No differences in the maximal growth rate of the wild-type compared to NZ7306 were found for a range of carbon sources, including cellobiose, maltose, N-acetyl glucosamine, and sucrose (data not shown).

However, remarkable observations were made when the wild-type and mutant strain were transferred to media containing galactose as the carbon and energy source. The wild-type WCFS1 displayed a growth arrest that lasts for 20-30 h (Fig. 3), stationary phase (24 h of culture) or log-phase (OD_{600} = 1.0) did not affect growth initiation characteristics (data not shown). Remarkably, the delayed growth initiation of the wild-type cells on galactose could be overcome by the addition of a trace amount (0.001% [w/v]) of glucose (Fig. 3). In contrast, mutant strains that lack mannose PTS expression (NZ7306, NZ7307, NZ7308, NZ7309, NZ7310, and NZ7311) were able to initiate growth immediately after inoculation (exemplified by NZ7311 in figure 3), suggesting involvement of the PTS in repression of galactose utilization. The direct involvement of the mannose PTS expression in the delayed growth initiation in the wild-type was further supported by the observation that wild-type cells pre-cultured on CDM with maltose (a carbon source that does not lead to mannose PTS expression, see above) could initiate galactose growth immediately after the medium transfer (Fig. 3).

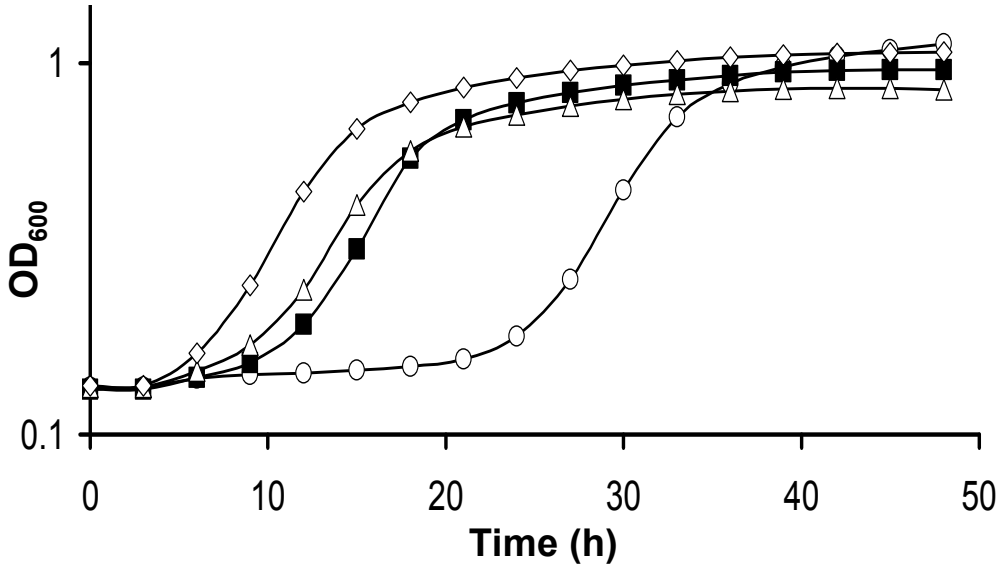

Figure 3: Prolonged growth arrest of L. plantarum WCFS1 wild-type (open circles) compared to the mannose PTS deletion mutant NZ7311 (open diamonds) after transfer from glucose-containing to galactose-containing CDM. Strain WCFS1 pre-grown on maltose (closed squares) or activated with glucose (open triangles). For details see text.

The mannose PTS drains the concentration of PEP

Because no differential expression of genes encoding galactose utilization could be found in the transcriptome analysis, the regulation of galactose catabolism could be due to post-transcriptional regulation, probably by phosphorylation of the galactose transporter by PEP, as had been described in *Streptococcus thermophillus*. In this bacterium galactose is taken up by a lactose/galactose transport protein (LacS) which catalyzes two modes of transport: solute-H^+ symport and lactose/galactose antiport (22). LacS possesses a PTS-IIA-like domain and phosphorylation of this domain leads to an increased antiporter-transport rate, whereas symporters transport rate is not affected (42). *Lactobacillus plantarum* has two putative galactose transporters (*lasS1* and *lacS2*) which are very similar to each other (38% identity) and to the galactose/lactose transporter of *S. thermophillus* (37% and 56% identity respectively). Furthermore, both transporters have a phosphate accepting IIA-domain, suggesting a similar regulatory mechanism in *S. thermophillus* and *L. plantarum* (data not shown).

To evaluate the hypothesis of LacS-phosphorilation, the intracellular concentration of PEP was monitored in *L. plantarum* wild-type and the ManPTS mutant strain grown in glucose-containing CDM and following carbon starvation. To exclude the possibility of Mannose PTS expression due to any leakage from the promoter, the test was performed with the *manABCD* deletion strain NZ7311.In samples taken from wild-type and NZ7311 cultures growing on

CDM containing glucose, no PEP could be detected, indicating that intracellular PEP concentrations during growth are low (data not shown). To follow PEP pool development upon carbon starvation, cultures were grown to mid-exponential growth phase, harvested, washed and resuspended in medium without a carbon source. Samples were taken from these suspensions at different time points after the medium transfer and intracellular PEP concentrations were determined. In the wild-type strain no PEP could be detected in any of the samples, indicating that this strain fails to accumulate an intracellular PEP pool upon carbon starvation (Fig. 4). In contrast, in the mutant strain NZ7311 PEP could be detected immediately after transfer to starvation medium, and appeared to be maintained until several hours after carbon starvation at a concentration of 10-40 μM (Fig. 4). Virtually identical results were obtained for the *manIIC*-mutant strain NZ7308 confirming a role for the mannose PTS in mediating PEP pools in resting cells. These results suggest that the expression of the mannose PTS significantly affects the capacity of the wild-type cells to accumulate PEP during starvation, which could hamper initiation of galactose-growth due to a lack of phosphate-donor molecules required for activation of the galactose transporter by LacS-IIA domain phosphotransfer. In contrast the relatively high PEP pools in resting cells of the mutant strain would support LacS activation, allowing immediate growth initiation upon transfer to galactose containing media. The observation that trace amounts of glucose could facilitate growth initiation of the wild-type strain on galactose are most likely explained by the production of PEP from glucose and corresponding LacS activation under these conditions.

Overall, these results indicate that the mannose PTS system of *L. plantarum* plays a pivotal role in carbon metabolism control in this species. This role clearly exceeds the ‘simple’ role that is predicted on basis of its annotated function, e.g., transport of mannose (and glucose), and includes a role in central carbon metabolism control by affecting the relative levels of important glycolytic intermediates like PEP, which in their turn control the capacity to initiate growth on specific other carbon sources.

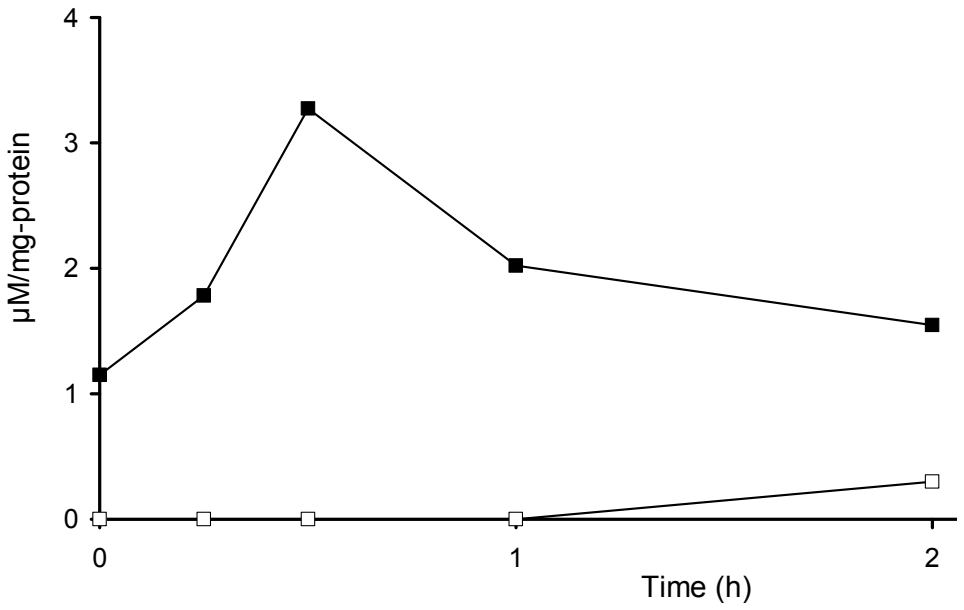

Figure 4: Concentration of intracellular PEP in carbon starved resting cells of the L. plantarum wild-type (open squares) and its mannose PTS-mutant derivative NZ7311 (closed squares).

Discussion

Regulation of gene expression by alternative σ-factors is a well established mechanism for adaptation to environmental conditions in bacteria. Here we describe the prediction and validation of the σ^{54}-regulon in *Lactobacillus plantarum* WCFS1. The conserved binding sequence of this sigma factor enabled a prediction of the specific regulon using the pattern recognition algorithms MEME and MAST (3, 4). However, such a prediction should be verified since complete and accurate predictions are hampered by, variability of the nucleotides in spacer regions, limited numbers of experimentally verified promoter sequences, and the failure of the pattern recognition algorithms used to cope with two (or more) small conserved regions separated by a spacer sequence. Experimental verification was performed by comparative whole genome transcriptome analysis of wild-type and a σ^{54}-mutant derivative. The regulon was concluded to be restricted to the mannose PTS operon, which is in agreement with the genetic linkage between the gene encoding the σ^{54}-activator protein and the target locus of the mannose PTS. Additional genes regulated in the microarray experiment were concluded to be due to secondary effects.

The inability of the σ^{54}-mutant strain NZ7306 to grow on mannose as sole carbon source shows that transcription of the mannose operon is strictly σ^{54}-dependent, which is in

agreement with the observation that σ^{54}-regulated genes are not influenced by other transcription factors (10). Mutants of the mannose PTS showed that ManIIABCD is the only functional mannose transporter in *L. plantarum*.

Homologues of the mannose PTS studied in this paper are found throughout the whole bacterial kingdom. It has been shown that this PTS is also able to transport glucose and it is thought to be in main glucose PTS in lactic acid bacteria (12). The observation that mannose PTS deletion leads to a decreased growth rate on glucose has already been described for *L. monocytogenes* and *Lactobacillus pentosus* (12, 57), a phenotype probably resulting from impaired glucose uptake. Our data provide further evidence for glucose transport by the mannose PTS. Furthermore, analyses of the genes of *L. plantarum* for codon adaptation index (CAI) revealed a high CAI for the mannose genes suggesting high possible expression (32), corroborating the importance of this transport system in uptake of favourable sugars such as glucose.

Regulation of the mannose operon by σ^{54} has already been described in the two other Gram positive bacteria *Enterococcus faecalis* and *Listeria* monocytogenes (29) (14), indicating that this mode of regulation is conserved among some Gram-positive bacteria. In the two bacteria mentioned above abolished σ^{54}-function leads to resistance to mesentericin Y105, a class II bacteriocins, due to impaired *manIIC*-expression (13, 44, 46). Additional studies proposed a common mechanism for bacteriocin sensitivity in which ManIIC acts as a docking protein for anti-microbial peptides (19). However, *L. plantarum* appeared to be resistant to mesentericin Y105 (M. Stevens, unpublished data), suggesting that the *L. plantarum* mannose PTS does not act as a docking protein for at least this bacteriocin. The ManIIC sequence comparison between these three related species revealed a 68-70% overall identity and 67% identity in the putative outer membranes regions of the protein, suggesting that comparative molecular analysis could lead to identification of target residues that play a key role in specificity of bacteriocin docking and the molecular mode of docking interaction.

The major role of the mannose PTS in glucose transport suggest a role of the PTS in canonical systems for control of carbon utilization like carbon catabolite repression (CCR) (12). Indeed, this has been shown in the close relative of *L. plantarum*, *L. casei* in which the mannose PTS regulates lactose operon expression via terminator modulation; only strains lacking CcpA and Mannose PTS activity are able to express the lactose operon (12, 25). However, the expression of both *lacS1* as *lacS2* in a strain lacking a functional CcpA but still harbouring a mannose PTS indicates a different mechanism in *L. plantarum* (52). Since we

did not observe an effect in the transcription analysis, control via the mannose PTS seems to be mediated via another level of control, possibly involving metabolic control of transport.

In *S. thermophillus* galactose transport is depending on the phosphorylation state of the antiporter/symporter LacS. Phosphorylation of a IIA-like domain leads to an increased antiporter-transport rate, whereas symporters transport rate is not affected (42). The antiporter mode (galactose/lactose exchange) is most relevant transport mode as it is much faster as the proton motive force driven (Δp) symporter (Lactose/H^+) mode (33). The wild-type cells in our experiment were washed and depleted of metabolic energy, as no PEP could be detected in these cells, and the prolonged growth arrest could be due to incapability to build up a Δp needed for galactose transport initiation. Adding a trace amount of glucose to resting cells (as in our experiments) leads to a change in concentration of glycolytic intermediates (40) and to generation of Δp (42), which enables to galactose/H^+ transport, explaining the observed growth initiation after adding a trace amount of glucose. In contrast, resting Δ*manIIABCD* cells maintain a high PEP pool build up a Δp and can start growing immediately. Furthermore, phosphorylation of LacS is HPr(His~P)- and PEP-dependent (27), hence it is likely that the high PEP-pool in the mutant strain leads to a permanent LacS-(IIA-P) state, resulting in efficient galactose transport direct after inoculation.

At first sight it may seem that σ^{54} has only a minor role in *L. plantarum* since only the mannose operon, is transcribed from a σ^{54}-dependent promoter. However, the mannose PTS operon encodes the main glucose uptake system in *L. plantarum* and σ^{54}-dependent transcription allows expression of the operon rapidly and at high rate without interference of another transcriptional regulator when glucose is present in the medium. Via the mannose PTS σ^{54} regulates metabolic control due to the effect of the mannose PTS on the concentration of PEP in resting cells, thereby mediating the capability of the cell to grow on galactose and possibly some other carbon sources (Fig. 6).

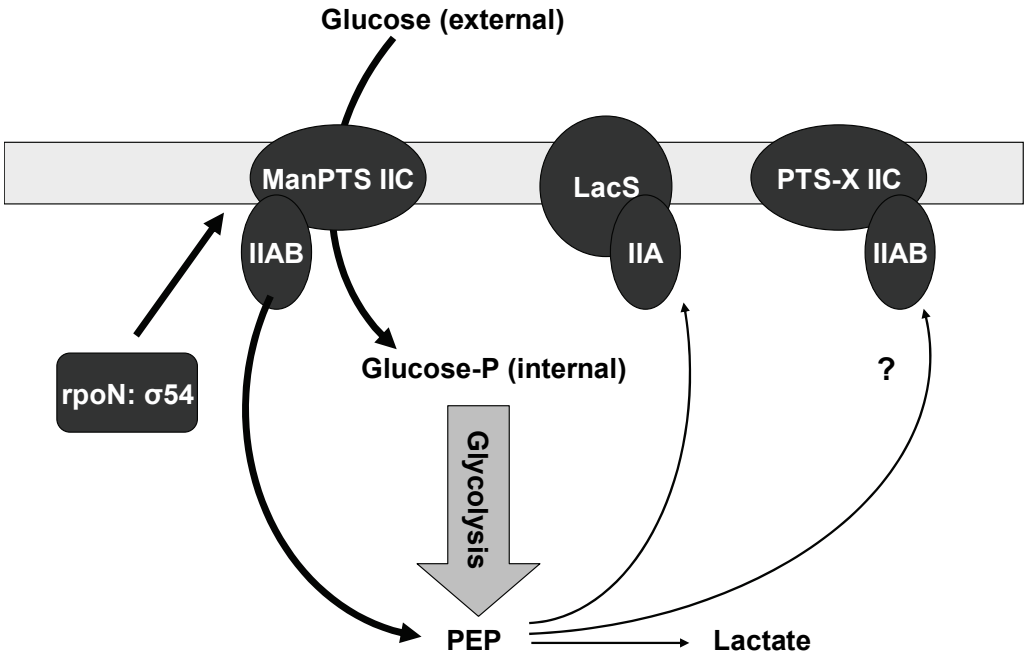

Figure 6: Regulation of sugar transport and catabolite control by σ^{54}. The mannose PTS is regulated by σ^{54} and is a main glucose transporter in the cell. It controls the concentration of PEP, thereby repressing the uptake of galactose and maybe some other sugars.

Acknowledgments

This work was supported by Grant No. IGE1018 from the Dutch IOP-Genomics Program. We thank Guido Starring for performing the HPLC analyses.

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Chapter 3

Global Transcriptional Analysis of the Peroxide Response in *Lactobacillus plantarum* and Involvement of the Mannose PTS in Survival

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Abstract

The peroxide stress response of *Lactobacillus plantarum* was addressed as we observed that a strain carrying a deletion of the *rpoN*-gene is more sensitive to hydrogen peroxide treatment as compared with the wild-type strain. Remarkably, genome-wide analyses of the wild-type and its *ΔrpoN* derivative revealed that the transcriptional responses to sub-lethal peroxide treatment were virtually identical in the two strains. The only target was found to be the *manIIABCD* locus, whose expression previously had been found to be abolished in the *ΔrpoN* strain in the absence of oxidative stress. Peroxide stress survival experiments with *L.plantarum* strains lacking expression of a functional mannose PTS established that the sensitivity to peroxide in the *ΔrpoN* strain is due to the impaired expression of this function. It is concluded that increased peroxide sensitivity in strains that fail to express or have a malfunctioning mannose PTS results from a major reduction in energy generating capacity due to a decreased glucose transport capacity.

Introduction

Together with superoxide and hydroxyl radicals, hydrogen peroxide belongs to a group of chemicals known as reactive oxygen species (ROS). ROS can be generated through metabolic activity in presence of molecular oxygen, including pathways like oxidative phosphorylation. Hydrogen peroxide itself is relatively inert towards organic compounds (15), but it reacts readily with metal ions like Fe^{2+} to yield hydroxyl radicals ($^{\cdot}OH$), the so-called Fenton's reaction, that damage DNA, proteins, and membranes (19). All organisms have developed protective mechanism against ROS and various studies have revealed that the protective mechanisms are highly similar in prokaryotic and eukaryotic organisms (38). Protection is generally accomplished by enzymes and cellular compounds that either react directly with the ROS to detoxify it or repair the caused damage. Hydrogen peroxide reacts with α-ketoacids in a non-enzymatic reaction (23) and the cellular α-ketoacids pyruvate and succinate might play a role in protecting the cell against peroxide (2, 37). In addition, cellular sulphur compounds, like thioredoxins and glutathione (GSH), aid in ROS-detoxification, and this has even been proposed to represent the main function of GSH (33). Enzymatic peroxide-detoxification can be performed by catalases and peroxidases. Both enzymes transform peroxide into water and although they catalyze the same reaction, their reaction mechanisms differ. Catalases use only hydrogen peroxide as a substrate and protect against high concentration of peroxide (10), whereas peroxidases use NAD(P)H as a co-factor and protect against lower, physiological, concentrations of hydrogen peroxide (16).

Lactobacillus plantarum is a lactic acid bacterium, found in many dairy, meat, and plant fermentations. Furthermore, it is frequently encountered in the human gastro-intestinal tract (1) and some strains are marketed as probiotics (14). Its natural habitats are usually anaerobic and rich in carbohydrates and nutrients (34). However, analyses of aerobic growth and behaviour of *L. plantarum* is of high interest as it gives insight in the protection against oxygen and survival of the bacterium in (micro)-aerobic niches such as the human GI-tract. Under aerobic condition hydrogen peroxide can be produced by the reaction of oxygen with the enzymes NADH-oxidase and pyruvate oxidase and elimination of hydrogen peroxide is essential for growth (11).

Analyses of the complete genome sequence of *L. plantarum* WCFS1 predicted the presence of genes coding for proteins that might be involved in ROS-detoxification, including NADH-oxidases, GSH-reductases, GSH-peroxidase, NADH-peroxidases, and thioredoxins (25). Previous *in silico* regulatory network predictions suggested that the expression of a

glutathione peroxidase gene (*gpo*) might depend on the alternate sigma factor 54 (36). Investigation of the σ^{54}-regulon by comparative whole genome transcriptome analyses of the wild-type cells and its Δ*rpoN* derivative, revealed only the genes encoding a mannose phosphotransferase system (PTS) as being σ^{54} dependent (36). However, these studies did not include growth under environmental conditions that included the presence of hydrogen peroxide.

Here we describe the higher peroxide sensitivity of the *L. plantarum rpoN*-mutant as compared to the parental strain. Transcriptome analyses of cells grown under peroxide-stress conditions revealed a possible role for a number of genes, including the glutathione peroxidase gene (*gpo*), in the response to peroxide. However, these analyses did not support a role for σ^{54} in the regulation of the GSH-peroxidase. Subsequent experiments revealed that the impaired expression of the mannose PTS operon in an *rpoN* deletion mutant resulted in the observed increased peroxide sensitivity.

Material and Methods

Bacterial strains, media, and growth conditions

Lactobacillus plantarum strains used in this study are listed in Table 1. *L. plantarum* WCFS1 and its derivatives were grown anaerobically in a closed bottle in MRS (13) supplemented with 2% w/v of specific carbohydrates purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Cells were grown at 30° C, except when stated otherwise. Growth was monitored by measurement of the optical density at 600 nm (OD_{600}) in an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Roosendaal, The Netherlands). When appropriate, chloramphenicol was added to the media at a final concentration of 8 μg/ml.

Table 1: Strains used in this study and their relevant characteristics and references.

Strain	Relevant Features	Reference
WCFS1	Wild-type, human isolate	(26)
NZ7306	*rpoN* gene replacement (*rpoN::P_{32}cat*) derivative of *L. plantarum* WCFS1	Chapter 2
NZ7307	*manR* gene replacement (*manR::P_{32}cat*) derivative of *L. plantarum* WCFS1	Chapter 2
NZ7308	*manIIC* gene replacement (*manIIC:pNZ7350*) derivative of *L. plantarum* WCFS1	Chapter 2

RNA extraction and quality control

Exponentially growing *L. plantarum* WCFS1 cells (OD_{600} = 1.0) were harvested by centrifugation at 3360 x g for 5 minutes at 30°C. Subsequently, the cells were transferred with a spatula to a screw-cap tube containing 500 mg zirconium beads, 500µl of a phenol/ chloroform mixture (1:1, v/v), 30 µl 3 M Na-Acetate (pH 5.2), 30 µl 10% SDS, and 400 µl MRS-medium (Merck, Darmstadt, Germany) and immediately frozen in liquid nitrogen.

RNA was isolated as described previously (36), using a phenol-chloroform extraction followed by a purification using the High Pure RNA isolation kit (Roche, Manheim, Germany). The yield and purity of the RNA were determined by measurement of the absorption at 260 nm and 280 nm (Ultrospec 3000, Pharmacia Biotech, Roosendaal, The Netherlands). The RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent technologies, Palo Alto, Ca, USA) following the manufacturer's instructions. Only RNA samples displaying 16S/23S-rRNA ratios of 1.6 or higher, were labelled and used for micro array experiments.

cDNA-synthesis, labeling, and hybridization

The Cyscribe Post-labeling kit (Amersham Biosciences, Amersham, UK) was used to synthesize and to label cDNA out of 25 µg total RNA as described previously (36). Labeled cDNAs were hybridized as described previously on amplicon based microarrays containing fragment of approximately 97% of the genes of *L. plantarum* WCFS1 (36) .

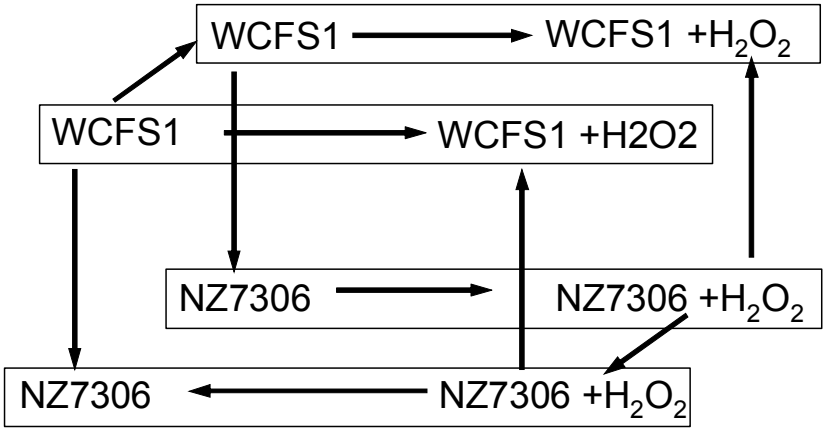

Figure 1: *hybridization scheme. The experiments were performed in duplicate with cross talk between the two sets. Samples in one block were split just before the peroxide treatment. Each arrow represents one hybridization; samples at the base of the arrow were labeled with Cy3 label and samples at the arrowhead with Cy5.*

Scanning, Data extraction, and analyses

The slides were scanned with a Scan Array Express 4000 scanner (Perkin Elmer, Wellesley, USA) and images were analyzed with Imagene 4.2 (BioDiscovery, El Segundo, USA). Statistical analyses were performed with R (http://www.r-project.org/) using the linear models for microarray data library limma (35). Background corrected spot intensities in both channels (I1 and I2) were converted to M-A coordinates, where M=log2 (I1/I2) and A=log2 (I1/I2)/2 and subsequently normalized using a LOESS fit, assuming that, on average, M is independent of A and centered around 0 (35). Normalized intensities were used for further analysis. Log odds for differential expression (B-value) higher than 1 were taken as a cut-off. As amplicons were spotted in duplicate, two measurements per gene were performed and only genes of which both measurements matched the criteria mentioned above were taken into account.

Physiological Characterization

For peroxide-stress adaptation and survival assays cells were grown in MRS at 30° C until an OD_{600} of 1.0. In peroxide-adaptation experiments hydrogen peroxide (Merck, Darmstadt, Germany) was added to the culture at a final concentration of 3.5 mM and incubation at 30° C was continued for 30 min prior to harvesting the cells by centrifugation (3360*g, 5', 30°C). In peroxide-survival determinations, hydrogen peroxide was added to a final concentration of 40 mM, followed by culture sampling after 0, 15, and 30 min. Culture samples were immediately diluted and colony forming units were enumerated by pour-plating appropriate dilutions in MRS-glucose. Plates were incubated for 48 hr at 30° C, after which colonies were counted. Results are presented as relative survival (%), as compared to the initial colony forming unit density observed in the culture (time point '0' = 100 %). The results presented are the average of three independent experiments, including the standard deviation between experiments.

Bio-informatics methods

To identify upstream binding regions the algorithm for fitting a mixture model by expectation maximization (MEME) was used (4). The upstream region sequences used in these analyses were extracted from the genome sequence of *L. plantarum* (Acc. Nr. AL935263), and were limited by the first codon (start or stop) of the preceding gene, and size restricted between 20 and 200 base pairs. MEME searches were performed with the following parameter settings: motif length should be between 5 and 17 base pairs (to obtain biological mean full motifs), zero or one motif per sequence should be found (zoops) and only the given sequence strands should be used. After this an additional search was performed with the same parameters plus the option "force palindromes" to obtain better a defined palindrome motif. To identify

MEME-derived motifs in the *Lactobacillus plantarum* WCFS1 genome, the motive alignment and search tool (MAST) was used (5). Selective restrictions for MAST-based motif identification were set at an E-value below 10^{-6}, and a relative positioning within 200 bp upstream of a predicted protein encoding sequence, while overlap with upstream genes was allowed.

Results

Deletion of the *rpoN*-gene leads to peroxide sensitivity

To complement earlier studies on the σ^{54}-regulon of *L. plantarum* (36), we compared the response of wild-type strain WCFS1 and its isogenic *rpoN* deletion derivative (strain NZ7306) to hydrogen peroxide stress during growth (Fig. 2). After 30 min of peroxide treatment the viable count of the wild-type culture appeared approximately reduced by 4 orders of magnitude while that of the *rpoN* mutant was reduced at least a 100-fold more (>6 orders of magnitude). This could have been caused by σ^{54}-dependent regulation of the oxidative stress response of *L. plantarum*, or alternatively by an impact of σ^{54} mutation on the general stress response in this organism. To exclude the latter possibility, the relative capacities of wild-type and *rpoN* mutant strains to survive lethal levels of UV and heat-shock stress were evaluated and did not appear to differ significantly (data not shown). Hence, the stress tolerance effect observed in the *rpoN* mutant appears to be specific for its response to hydrogen peroxide.

The role of σ^{54} in survival of acute peroxide stress in *L. plantarum* could relate to σ^{54} -dependent adaptation to peroxide stress conditions. To compare peroxide stress adaptation capacity between wild-type and *rpoN* mutant, *L. plantarum* cultures were pre-treated with a sub-lethal peroxide concentration, prior to addition of a lethal peroxide dose. Following peroxide treatment, both the wild-type and NZ7306 were able to re-initiate growth after 2 to 3 h confirming that the peroxide dosage used is sub-lethal for both strains (data not shown). After adaptation, wild-type cells displayed a more than 100-fold improved survival, whereas the *rpoN* mutant displayed similar improvement of survival (Fig. 2). This suggests that at least the relative adaptation capacity is not affected by the *rpoN*-mutation in comparison to the wild-type strain.

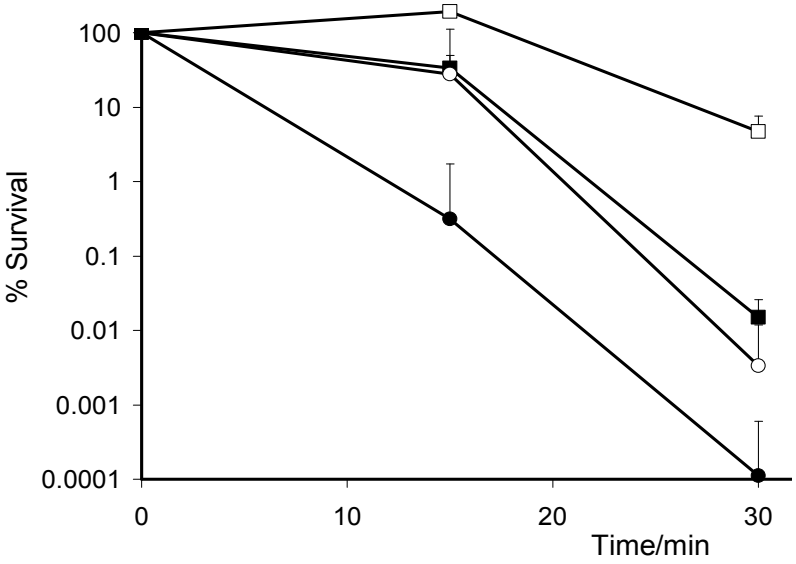

Figure 2: *Relative hydrogen peroxide survival of L. plantarum WCFS1 (wild-type, squares) and NZ7306 (ΔrpoN, circles). Survival was measured without (closed symbols) or with (open symbols) 30 min adaptation with a sublethal level of hydrogen peroxide.*

Genome-wide transcriptional response tot hydrogen peroxide

To evaluate the prossible involvement of σ^{54} in transcription of the glutathione peroxidase (*gpo*) or other genes required for peroxide-stress survival in *L. plantarum*, full genome transcriptome analyses were performed. To this end, strains WCFS1 and its Δ*rpoN* derivative (NZ7306) were grown to mid-exponential phase and treated with hydrogen peroxide for 30 min. Subsequently, cells were harvested and RNA was isolated for transcriptome profiling (see Fig. 1). The transcriptional response was analyzed by focusing on the effects of the mutation, presence of hydrogen peroxide and the combination of these two conditions.

The mutation effect confirmed our previous results that the mannose-PTS operon is most predominant set of genes affected by the mutation (36). Peroxide treatment resulted in 82 up- and 82 down-regulated genes (Table 2). Among the genes up-regulated by sub-lethal peroxide stress, homologues to known stress protein could be identified, including two protein-methionine-S-oxide reductases, two thioredoxins, the universal stress protein UspA, a NADH-peroxidase, a small heat shock protein, and two Clp-proteases. Also two enzymes that react with glutathione (GSH) are induced during oxidative stress, a GSH-peroxidase, with the predicted σ^{54}-dependent promoter (36) and a GSH-reductase. Further, two genes of the NADPH-producing pentose phosphate pathway, a gluconokinase and a phosphogluconate dehydrogenase, are induced. A total of 6 transcriptional regulators are up regulated, one of

them is homologous to *Spx*, an oxidative stress regulator in *Bacillus subtilis* (32), while another (lp_3444) is genetically linked to the higher expressed *pepO* (lp_3445).

Adding a sub-lethal dose of peroxide to growing *L. plantarum* cells leads to a growth arrest that lasts for 2-3 h (data not shown) and the majority of the genes down-regulated under peroxide stress are growthrelated and predicted to encodeproteins involved in cell division and in nucleic acid modification, ribosomal functions, tRNA-ligases, translation initiation factors, cell wall synthesis functions and transporters (Table 2B).

If the difference between the wild-type and the mutant strain is caused by altered expression of the glutathione peroxidase, then this effect should be visible in the mutation-peroxide interaction effect. Genes significantly regulated that belong to this group display differential behaviour when peroxide stress is applied in the wild-type or the σ^{54}-mutant (Fig. 1). The list of genes in this group is relatively small and the ratio's are low (Table 3), and fail to disclose direct clues that could explain the increased peroxide sensitivity observed in the σ^{54}-mutant.

The transcriptome experiments performed led to the identification of a set of genes involved in the peroxide stress response of *L. plantarum*. Furthermore, they confirmed that the mannose-PTS operon is dependent on σ^{54} for expression. These analyses also showed that expression of the glutathione peroxidase encoding *gpo* gene is affected by peroxide stress exposure but that regulation of expression of this gene is not controlled by σ^{54}. Notably, these experiments failed to provide a direct and plausible explanation for the higher sensitivity to peroxide of the *rpoN*-mutant strain NZ7306.

Table 2A: Genes up regulated during peroxide stress compared to standard condition.

ORF	Name	product	Ratio (^{2}log)	P-value (FDR)	B-value
Amino acid biosynthesis					
lp_0775	argG	argininosuccinate synthase	1.40	8.10E-04	4.76
lp_0776	argH	argininosuccinate lyase	1.75	6.99E-04	5.36
lp_2685	dapA2	dihydrodipicolinate synthase	2.16	9.38E-04	4.23
Biosynthesis of cofactors, prosthetic groups, and carriers					
lp_1253	gshR2	glutathione reductase	1.63	2.44E-03	1.44
lp_2270	trxA2	thioredoxin	1.11	1.53E-03	2.57
lp_3437	trxA3	thioredoxin	1.66	6.99E-04	5.31
Cell envelope					
lp_0461	lp_0461	cell surface hydrolase, membrane-bound (putative)	0.96	2.79E-03	1.12
lp_1539	lp_1539	lipoprotein precursor	0.71	2.75E-03	1.15
lp_2101	cps4H	polysaccharide polymerase	0.85	2.09E-03	1.78
Cellular processes					
lp_0220	gpo	glutathione peroxidase	1.38	9.46E-04	4.16
lp_0786	clpP	endopeptidase Clp, proteolytic subunit	1.38	1.08E-03	3.56
lp_1269	clpE	ATP-dependent Clp protease, ATP-binding subunit ClpE	0.88	2.54E-03	1.32
lp_2544	npr2	NADH peroxidase	1.23	2.63E-03	1.23
lp_3342	lp_3342	low temperature requirement C protein (putative)	0.91	1.46E-03	2.75

ORF	Name	product	Ratio (^{2}log)	P-value (FDR)	B-value
lp_3352	hsp3	small heat shock protein	1.38	1.02E-03	3.74
lp_3477	fic	cell filamentation protein Fic	0.95	1.53E-03	2.59
Central intermediary metabolism					
lp_0174	agl1	alpha-glucosidase	0.93	2.36E-03	1.55
lp_0822	glmS1	glutamine-fructose-6-phosphate transaminase (isomerizing)	3.39	7.07E-04	5.02
DNA metabolism					
lp_2860	nth1	DNA-(apurinic or apyrimidinic site) lyase	0.74	2.61E-03	1.26
Energy metabolism					
lp_0826	galM1	aldose 1-epimerase	0.99	2.46E-03	1.37
lp_0931	hpaG	2-hydroxyhepta-2,4-diene-1,7-dioateisomerase / 5-carboxymethyl-2-oxo-hex-3-ene-1,7-dioatedecarboxylase (putative)	0.98	1.72E-03	2.20
lp_1108	citE	citrate lyase, beta chain	0.69	2.69E-03	1.19
lp_1109	citF	citrate lyase, alpha chain	0.69	3.04E-03	0.96
lp_1113	lp_1113	fumarate reductase, flavoprotein subunit precursor,N-term truncated	0.89	2.44E-03	1.46
lp_1250	gntK	gluconokinase	1.25	1.74E-03	2.19
lp_1251	gnd1	phosphogluconate dehydrogenase (decarboxylating)	1.27	1.19E-03	3.32
lp_3092	gabD	succinate-semialdehyde dehydrogenase (NAD(P)+)	1.54	1.24E-03	3.16
lp_3096	lp_3096	short-chain dehydrogenase/oxidoreductase	1.13	1.24E-03	3.16
lp_3469	lacA	beta-galactosidase I	0.94	1.95E-03	1.99
lp_3589	pox5	pyruvate oxidase	0.95	1.53E-03	2.59
lp_3603	lp_3603	sugar-phosphate aldolase	0.74	2.96E-03	1.01
Fatty acid and phospholipid metabolism					
lp_0168	dak1B	glycerone kinase	1.31	1.68E-03	2.34
lp_0169	dak2	dihydroxyacetone phosphotransferase, dihydroxyacetone binding sub-unit	1.22	9.64E-04	4.04
lp_2643	lplA1	lipoate-protein ligase	0.86	1.85E-03	2.06
Hypothetical proteins					
lp_0026	lp_0026	hydrolase, HAD superfamily, Cof family	1.21	1.32E-03	2.98
lp_0089	lp_0089	unknown	1.08	2.44E-03	1.42
lp_0117	lp_0117	unknown	1.16	2.23E-03	1.68
lp_0137	lp_0137	oxidoreductase	1.41	7.07E-04	5.00
lp_0138	lp_0138	unknown	0.83	2.71E-03	1.18
lp_0139	lp_0139	unknown	1.01	1.49E-03	2.67
lp_0146	lp_0146	oxidoreductase	1.30	1.69E-03	2.27
lp_0170	dak3	dihydroxyacetone phosphotransferase, phosphoryl donor protein	1.22	2.09E-03	1.77
lp_0221	lp_0221	oxidoreductase	1.53	6.99E-04	5.12
lp_0244	lp_0244	oxidoreductase	1.97	8.53E-04	4.50
lp_0291	lp_0291	oxidoreductase	1.64	6.99E-04	5.34
lp_0479	lp_0479	unknown	1.09	2.75E-03	1.15
lp_0513	lp_0513	unknown	1.26	1.47E-03	2.71
lp_0554	lp_0554	unknown	1.02	2.00E-03	1.89
lp_0762	lp_0762	unknown	0.93	2.44E-03	1.46
lp_0823	lp_0823	protein containing diguanylate cyclase/phosphodiesterase domain 2 (EAL)	1.88	1.69E-03	2.26
lp_1163	lp_1163	nucleotide-binding protein, universal stress protein UspA family	1.69	1.32E-03	2.97
lp_1257	lp_1257	unknown	0.94	2.33E-03	1.58
lp_1394	lp_1394	unknown	1.23	2.62E-03	1.24
lp_1395	lp_1395	unknown	1.56	1.02E-03	3.81
lp_1703	lp_1703	unknown	1.14	2.23E-03	1.67
lp_1859	lp_1859	conserved hyp. protein	0.93	2.27E-03	1.64
lp_1860	lp_1860	oxidoreductase	0.86	2.20E-03	1.70
lp_1918	lp_1918	oxidoreductase	0.85	2.46E-03	1.38

ORF	Name	product	Ratio (^{2}log)	P-value (FDR)	B-value
lp_2690	lp_2690	unknown	1.09	1.68E-03	2.40
lp_3134	lp_3134	unknown	0.94	2.08E-03	1.84
lp_3305	lp_3305	unknown	1.66	8.53E-04	4.47
lp_3323	lp_3323	unknown	1.10	1.78E-03	2.16
lp_3351	lp_3351	unknown	1.24	1.87E-03	2.05
lp_3353	lp_3353	unknown	1.07	1.19E-03	3.33
lp_3356	lp_3356	acetyltransferase (putative)	1.31	9.64E-04	4.03
lp_3438	lp_3438	unknown	1.01	2.44E-03	1.47
Protein fate					
lp_1339	mrsA1	protein-methionine-S-oxide reductase	1.29	1.02E-03	3.82
lp_1979	msrA4	protein-methionine-S-oxide reductase	1.70	9.64E-04	4.00
lp_3445	pepO	endopeptidase PepO	0.85	2.44E-03	1.43
Regulatory functions					
lp_0836	spx1	regulatory protein Spx	1.29	1.38E-03	2.83
lp_0889	lp_0889	transcription regulator	1.76	9.46E-04	4.18
lp_2800	lp_2800	transcription regulator (putative)	1.70	1.09E-03	3.53
lp_2804	lp_2804	transcription regulator	0.91	2.64E-03	1.22
lp_3444	lp_3444	transcription regulator	2.23	6.99E-04	5.13
lp_3655	srlM2	sorbitol operon activator	0.83	2.27E-03	1.62
Transport and binding proteins					
lp_0171	dhaP	dihydroxyacetone transport protein (putative)	1.23	2.44E-03	1.47
lp_1327	lp_1327	glycerol-3-phosphate ABC transporter, substrate binding protein (putative)	0.79	2.46E-03	1.38
lp_2312	glnH2	glutamine ABC transporter, substrate binding protein	0.75	7.01E-03	-0.63
lp_3171	lp_3171	transport protein	1.01	1.53E-03	2.58
lp_3466	brnQ3	branched-chain amino acid transport protein	0.84	1.97E-03	1.93
lp_3468	lacS1	lactose transport protein	1.36	1.32E-03	2.93
lp_3658	rbsU	ribose transport protein	0.83	3.33E-03	0.80

Table 2B: Genes down regulated during peroxide stress compared to standard conditions.

ORF	name	product	Ratio (^{2}log)	p-value (FDR)	B-value
Amino acid biosynthesis					
lp_0255	metC1	cystathionine beta-lyase	-2.33	2.40E-04	1.03
lp_0254	cysE	serine O-acetyltransferase	-1.85	2.03E-05	3.68
Biosynthesis of cofactors, prosthetic groups, and carriers					
lp_1437	ribA	GTP cyclohydrolase II	-1.31	2.61E-05	3.42
lp_1438	ribH	riboflavin synthase, beta chain	-1.03	7.62E-05	2.28
Cell envelope					
lp_0618	lp_0618	cell surface hydrolase, membrane-bound (putative)	-1.11	1.31E-04	1.7
lp_1462	murC	UDP-N-acetylmuramate--alanine ligase	-1.14	9.22E-05	2.08
lp_2021	pbpX2	serine-type D-Ala-D-Ala carboxypeptidase	-1.17	1.97E-04	1.25
lp_2200	pbp2B2	penicillin binding protein 2B	-1.12	1.37E-04	1.64
lp_3421	lp_3421	extracellular protein, gamma-D-glutamate-meso-diaminopimelate muropeptidase (putative)	-2.15	3.29E-05	3.18
lp_0856	lp_0856	acyltransferase (putative)	-1.01	2.45E-04	1.01
lp_1178	cps1B	polysaccharide biosynthesis protein	-1.65	4.79E-05	2.78
lp_1180	cps1D	glycosyltransferase	-1.44	2.02E-04	1.22
lp_1182	cps1F	exopolysaccharide biosynthesis protein	-1.27	1.11E-04	1.88
lp_1185	cps1I	polysaccharide polymerase	-1.39	1.07E-04	1.92

ORF	name	product	Ratio (^{2}log)	p-value (FDR)	B-value
lp_1220	cps3D	polysaccharide biosynthesis protein (putative)	-1.79	4.89E-05	2.76
lp_1221	cps3E	polysaccharide biosynthesis protein (putative)	-1.03	1.14E-04	1.85
lp_1222	cps3F	polysaccharide polymerase	-1.37	1.49E-04	1.55
lp_1226	cps3I	O-acetyltransferase	-1.14	9.42E-05	2.05
lp_0267	tagD1	glycerol-3-phosphate cytidylyltransferase	-0.97	1.25E-04	1.75
lp_0268	tagF1	teichoic acid biosynthesis protein	-1.49	2.37E-04	1.05
lp_2018	dltB	D-alanyl transfer protein DltB	-1.53	7.81E-05	2.26
lp_2019	dltA	D-alanine activating enzyme DltA	-1.41	2.20E-05	3.6
Cellular processes					
lp_1632	smc	cell division protein Smc	-1.29	3.33E-05	3.16
lp_2319	mreB1	cell shape determining protein MreB	-1.4	6.25E-05	2.5
DNA metabolism					
lp_0938	hsdR	type I site-specific deoxyribonuclease, HsdR subunit	-1.83	5.39E-06	5
lp_0939	hsdM	site-specific DNA-methyltransferase (adenine-specific), HsdM subunit	-1.65	1.48E-05	4
Energy metabolism					
lp_1573	glk	glucokinase	-1.21	2.33E-05	3.54
Hypothetical proteins					
lp_0259	lp_0259	integral membrane protein	-2.85	4.28E-05	2.9
lp_0524	lp_0524	unknown	-1.37	1.51E-04	1.54
lp_1561	lp_1561	unknown	-1.22	1.35E-04	1.66
lp_2715	lp_2715	unknown	-1.26	1.99E-04	1.24
lp_3425	lp_3425	unknown	-2.28	1.04E-05	4.36
lp_2580	lp_2580	alkaline phosphatase superfamily protein	-1.05	1.27E-04	1.73
lp_2714	lp_2714	protein containing diguanylate cyclase/phosphodiesterase domain 2 (EAL)	-1.93	4.64E-05	2.81
lp_1357	lp_1357	extracellular protein, membrane-anchored (putative)	-1.34	7.12E-05	2.36
lp_2809	lp_2809	unknown	-1.36	1.85E-04	1.32
Other categories					
lp_2451	lp_2451	prophage P2a protein 6	-1.42	3.35E-05	3.16
lp_3390	lp_3390	prophage P3 protein 1, integrase	-1.31	2.52E-05	3.45
Protein fate					
lp_3687	yidC2	preprotein translocase, YidC subunit (putative)	-1.17	4.71E-05	2.8
lp_1619	pkn1	serine/threonine protein kinase (putative)	-0.83	1.80E-04	1.35
Protein synthesis					
lp_2044	lp_2044	unknown	-1.02	8.56E-05	2.16
lp_0512	rpmE	ribosomal protein L31	-1.12	1.57E-04	1.5
lp_1032	rpsJ	ribosomal protein S10	-1.82	4.40E-06	5.2
lp_1033	rplC	ribosomal protein L3	-1.53	2.30E-05	3.55
lp_1034	rplD	ribosomal protein L4	-1.59	4.63E-05	2.82
lp_1077	rplM	ribosomal protein L13	-0.83	1.63E-04	1.46
lp_1636	rpsP	ribosomal protein S16	-0.98	1.38E-04	1.64
lp_1515	infC	translation initiation factor IF-3	-1.35	1.71E-04	1.41
lp_2146	typA	GTP-binding protein TypA	-1.47	3.74E-05	3.04
lp_0454	metS	methionine--tRNA ligase	-1.4	3.08E-05	3.24
lp_1558	pheS	phenylalanine--tRNA ligase, alpha chain	-1.32	3.43E-05	3.13
lp_1559	pheT	phenylalanine--tRNA ligase, beta chain	-1.32	1.61E-04	1.47
lp_1965	glyQ	glycine--tRNA ligase, alpha chain	-1.1	2.06E-04	1.2
lp_2277	alaS	alanine--tRNA ligase	-1.45	6.69E-05	2.42
lp_2807	tyrS	tyrosine--tRNA ligase	-0.99	1.52E-04	1.53
lp_1555	lp_1555	rRNA methylase	-0.9	1.77E-04	1.37
lp_3688	rnpA	ribonuclease P	-1.26	6.85E-05	2.4
Purines, pyrimidines, nucleosides and nucleotides					
lp_1011	dgk1	deoxyguanosine kinase	-1.42	5.51E-05	2.63
lp_0693	nrdE	ribonucleoside-diphosphate reductase, alpha chain	-1.21	2.28E-05	3.56
lp_1562	udk	uridine kinase	-1.13	1.38E-04	1.64
lp_1883	cmk	cytidylate kinase	-1.06	9.05E-05	2.1

ORF	name	product	Ratio (^{2}log)	p-value (FDR)	B-value
lp_2698	pyrF	orotidine-5'-phosphate decarboxylase	-1.95	1.21E-04	1.79
lp_2701	pyrAA	carbamoyl-phosphate synthase, pyrimidine-specific, small chain	-3.19	6.86E-07	6.84
lp_2702	pyrC	dihydroorotase	-2.7	9.31E-06	4.47
lp_2703	pyrB	aspartate carbamoyltransferase	-3.26	1.07E-05	4.33
lp_2931	nrdG	anaerobic ribonucleotide reductase activator protein	-1.61	7.36E-05	2.32
lp_2932	nrdD	anaerobic ribonucleoside-triphosphate reductase	-1.81	1.52E-05	3.98
lp_1176	glf1	UDP-galactopyranose mutase	-1.58	7.78E-05	2.26

Table 3: Genes that are affected by peroxide treatment and rpoN-deletion (Interaction effect) of the wild-type (Ratio's > 0) and the mutant (Ratio's < 0).

ORF	name	product	Ratio (log^2)	p-value (FDR)	B-value
Cell envelope					
lp_0618	*lp_0618*	cell surface hydrolase, membrane-bound (putative)	0.68	1.43E-02	2.14
lp_1185	*cps1I*	polysaccharide polymerase	0.75	1.70E-02	1.57
Central intermediary metabolism					
lp_1686	*lp_1686*	acyl-CoA thioester hydrolase (putative)	-0.74	1.40E-02	3.16
Fatty acid and phospholipid metabolism					
lp_1670	*fabZ1*	(3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase	-1.01	1.40E-02	3.45
lp_1675	*fabF*	3-oxoacyl-[acyl-carrier protein] synthase II	-0.89	1.43E-02	2.49
lp_1682	*lp_1682*	phosphopantetheinyltransferase	-0.78	1.43E-02	2.16
Hypothetical proteins					
lp_0199	*lp_0199*	unknown	-0.57	1.43E-02	2.07
lp_0291	*lp_0291*	oxidoreductase	-0.61	1.84E-02	1.27
lp_1098	*lp_1098*	unknown	-0.69	1.43E-02	2.49
lp_1684	*lp_1684*	integral membrane protein	-0.84	1.40E-02	3.79
lp_3256	*lp_3256*	DegV family protein	-0.73	1.92E-02	1.09
lp_3353	*lp_3353*	unknown	-0.58	1.59E-02	1.81
Other categories					
lp_1687	*lp_1687*	GTPase	-0.59	1.88E-02	1.15
Purines, pyrimidines, nucleosides and nucleotides					
lp_0693	*nrdE*	ribonucleoside-diphosphate reductase, alpha chain	1.01	8.88E-03	5.04
Regulatory functions					
lp_1685	*lp_1685*	transcription regulator	-0.78	1.40E-02	3.72
Transcription					
lp_0520	*rhe1*	ATP-dependent RNA helicase	0.78	1.43E-02	2.01
Transport and binding proteins					
lp_0180	*msmK1*	multiple sugar ABC transporter, ATP-binding protein	0.89	1.43E-02	2.4
lp_0265	*pts5ABC*	PTS system, trehalose-specific IIBC component	0.7	1.43E-02	2.15
lp_0315	*potD*	spermidine/putrescine ABC transporter, substrate binding protein	0.46	1.90E-02	1.13
lp_0344	*tagH*	teichoic acid ABC transporter, ATP-binding protein	0.51	1.86E-02	1.2

Peroxide sensitivity of strains lacking a mannose PTS

The transcriptome analysis of the wild-type strain compared to the mutant NZ7306 in the presence and absence of hydrogen peroxide showed that the responses of both strains are similar and that expression of the GSH peroxidase gene is not altered in the mutant. This raises the question whether the peroxide sensitivity of NZ7306 is caused by the lack of expression of the mannose PTS operon, the only operon shown to be σ^{54}-dependent in *L. plantarum* (36). To test whether this is the case, the relative peroxide tolerance levels of *L. plantarum* grown on alternative carbon sources that would not require a functional mannose PTS was evaluated. The mannose PTS is the main glucose transporter and the only mannose transporter in *L. plantarum* and it is not expressed in cells growing on maltose (36). Therefore, we performed survival tests with maltose as carbon source similar to the tests performed with glucose. When pre-grown on maltose and following treatment with lethal dosages of peroxide, the viable count of the wild-type decreased about 5 orders of magnitude in 30 min, slightly more than the decrease seen in glucose-grown cells. Analogously, the viable count of maltose-grown NZ7306 (Δ*rpoN*) also reduced by 5 orders of magnitude (Figure 3), paralleling that observed for wild-type cells but markedly less than observed for glucose grown NZ7306. Hence, the peroxide sensitivity of the σ^{54}-mutant strain was not observed when cells were grown on a carbon source on which no mannose PTS expression occurs (maltose), confirming the role of the glucose/mannose PTS.

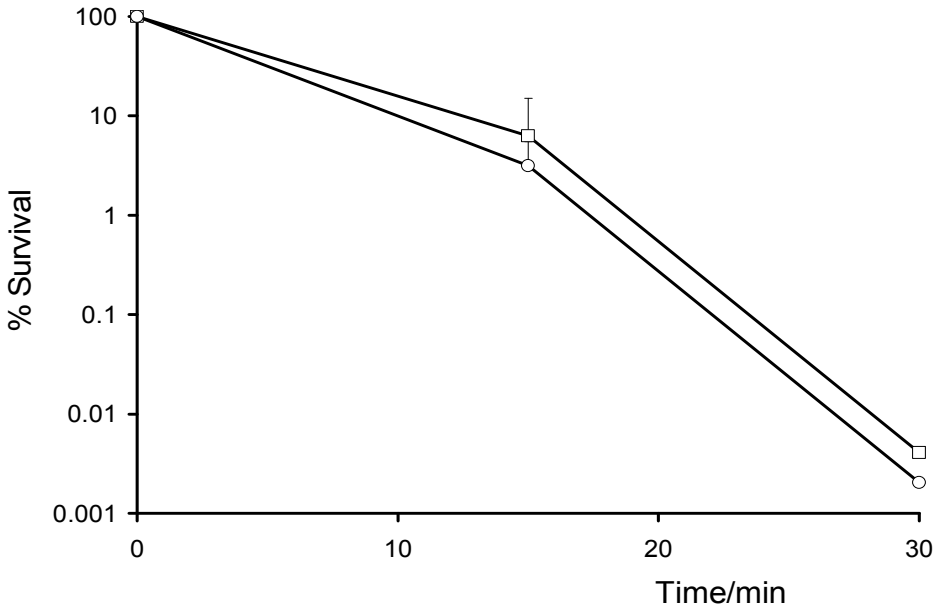

Figure 3: relative peroxide survival of L. plantarum (wild-type, squares) and NZ7306 (ΔrpoN, circles) grown in culture containing maltose as sole carbon source.

To further investigate the putative role of the mannose PTS in *L. plantarum* peroxide tolerance the hydrogen peroxide sensitivity of two additional strains was tested, i.e., NZ7307, which lacks a functional *manR* gene, and NZ7308, which lacks *manIIC* (Table 1). ManR is a putative σ^{54}-activator which co-regulates the expression of the mannose operon and deletion of its gene leads to a growth-defect on mannose, indicating that the expression of the mannose operon is impaired (36). ManIIC is the permease unit of the mannose PTS, and deletion of the *manIIC*-gene also leads to impaired growth on mannose, due the loss of mannose uptake capacity (36).

When treated with a lethal dose of hydrogen peroxide the viable cell count of NZ7307 and NZ7308 reduced by 6 orders of magnitude in 30 min. This reduction is clearly higher then that observed for the wild-type and parallels that seen for the *rpoN* mutant, NZ7306 (Figure 4). Since NZ7306, NZ7307, and NZ7308 share the lack of expression of a functional mannose PTS, these results seem to point again at a direct relation between the presence of a functional mannose PTS and peroxide tolerance in *L. plantarum*. This feature might be due to the high robustness of the transporter to oxidative compounds compared to other sugar transporters (21).

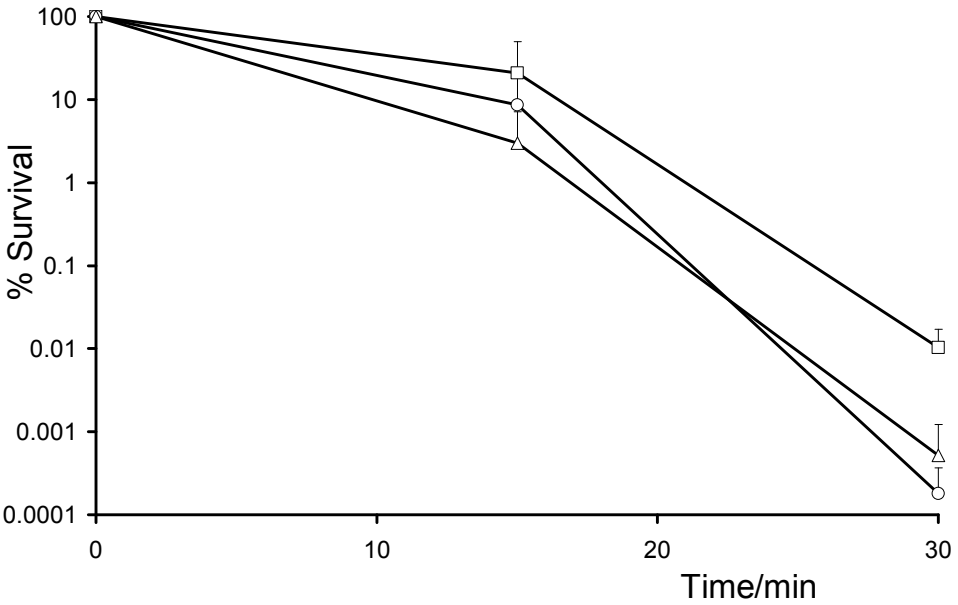

Figure 4: Relative peroxide survival of L. plantarum (wild-type, squares) and the mutant strain, NZ7307 (ΔmanR, triangles), and NZ7308 (ΔmanIIC, circles).

Discussion

Hydrogen peroxide is toxic for organisms as it generates highly reactive hydroxide radicals (˙OH) that lead to damage of DNA, proteins, and membranes (19). This study presents evidence that deletion of the *rpoN*-gene, encoding an alternative sigma factor (σ^{54}), leads to increased sensitivity to peroxide in *L. plantarum*, which is shown to be caused by impaired expression of the mannose operon in this strain.

Full genome transcriptome analyses resulted in the identification of 82 up- and 82 down-regulated genes during adaptation to sub-lethal levels of hydrogen peroxide (Table 2). Various unspecified oxidoreductases genes are found to be up- regulated, suggesting a role for these genes in maintaining the redox-balance in the cell during oxidative stress. Additionally, several known stress-response genes are encountered in the up-regulated genes, including genes involved in protein-damage repair by oxidation (protein-methionine-S-oxide reductases (6), and enzymes that catalyze ROS-detoxification reactions (thioredoxins and NADH-peroxidase (3). Other typical stress proteins induced are the universal stress protein UspA, which is highly conserved and appears to respond to any stress condition applied but still has not been associated with a molecular function (26), a small heat shock protein, which has been proposed to act as a chaperone preventing protein aggregation during stress conditions (27), and ClpP and ClpE, belonging to the damaged protein degrading Clp-protease system (30). Two enzymes involved in glutathione (GSH) metabolism are also induced during oxidative stress, namely GSH-peroxidase and GSH-reductase. The latter enzyme can detoxify hydrogen peroxide accompanied by the production of water and oxidation of GSH (31), which subsequently can be reduced by GSH reductase. In view of the high degree of conservation seen in the oxidative stress response in bacteria (38) the induction of these genes in *L. plantarum* under hydrogen peroxide stress conditions was expected.

A number of the stress-induced enzymes mentioned consume NADPH. NADPH can be generated in the pentose phosphate pathway and the up regulation of genes of this pathway is probably due to the increased need for NADPH during stress conditions. Another way to produce NADPH might be using glutamate as a substrate, which can be converted to succinate-semialdehyde and subsequently into succinate. In the latter step, an NADPH is gained and the corresponding enzyme, the succinate-semialdehyde dehydrogenase, is induced during peroxide stress. An additional advantage for the cell is the proposed protection of succinate against ROS (2, 37).

Peroxide can disturb membrane integrity by oxidizing lipids (19). The up-regulation of the genes *dak1B,2,3,P*, encoding the genes of a dihydroxyacetone phospho-transferase system might be due to this effect. This system produces dihydroxy-acetone phosphate (DAP) (18), a compound that serves as precursor for membrane lipids (22). The concomitantly up-regulated acetyltranferase might play a role in the acetylation of DAP. Analogously, up-regulation of the low temperature requirement C protein, with a predicted phosphatidylglycerophosphatase A-domain (NCBI conserved domain search(29)) that catalyzes the formation of the multifunctional phospholipid phosphatidylglycerol (9), indicates membrane modification.

The unusual guanosine nucleotide (p)ppGpp is thought to have a range of functions in bacteria that are all related to unfavourable, stressful conditions and this alarmone is mainly involved in the repression of genes (24). The regulation of the GTP pyrophosphokinase suggests a function for the (p)ppGpp-signaling system in regulating the temporal growth arrest observed in *L. plantarum* during sub-lethal stress conditions.

Altogether, the transcriptome analyses led to identification of genes involved in the oxidative stress response. However, the regulatory mechanism underlying these responses mostly remains unknown. Nevertheless, a conserved candidate regulatory binding site consisting of a palindrome sequence interspaced with a three base pair wide AT-rich region was identified upstream of 3 genes encoding oxidoreductases as well upstream of a thioredoxin gene (Fig. 6, Table 4). Employing this motif to search the complete *L. plantarum* WCFS1 genome identified ten additional sequences upstream of genes, of which a remarkably high number is likely to be involved in stress response (Table 4). Regulators that play a specific role in oxidative stress response, as well as their specific binding sites, have been identified in *B. subtilis* (7, 20). However, a search with the identified motif in the *B. subtilis* database for transcriptional regulators (DBTBS) (28) did not yield any matches. Nevertheless, the fact that this conserved motif is found in the upstream region of genes that appear to be functionally related and the fact that it forms a nearly perfect palindrome makes it likely that this motif represents a functional binding site.

Figure 5: Visualization of the putative upstream binding sequence found in front of stress related genes using weblogo (12). The palindrome sequences are CTTAC from position 6 to 10 and GTAAG from position 16 to 20. The adenines in the spacer at position 12-13 appear to be conserved as well. The score for each base pair is reflected by the size of the character.

Table 4: Genes in the genome of L. plantarum WCFS1 that are preceded by a sequence element with significant sequence homology to the putative regulatory element identified in this study using MEME. The genes indicated bold are higher expressed during peroxide treatment. Distances are calculated from the last G (or A) of the candidate cis-sequence to the start codon of the downstream gene.

ORF	Product	Dir.	Dist	E_Score	Sequence
lp_0146	**oxidoreductase**	+	24	2.1E-08	GAAACTAGAT**CTTACA**AAA**AGTAAG**CAAAACTAGC
lp_0244	**oxidoreductase**	-	52	7.4E-09	CTTCGTTTCA**CTTACT**AAT**AGTAAG**TATATCAAGT
lp_0752	stress-responsive transcription regulator	-	50	7.1E-07	CTAACCACAT**CTTACT**AAT**TGTAAA**AGCCGGTTGC
lp_0852	pyruvate oxidase	+	112	9.5E-09	TGTAATTTCA**CTTACG**AAA**AGTAAG**CATATTAATT
lp_1320	unknown	+	73	2.7E-07	CTGTCGTCAA**CTTAAT**AAT**CGTAAG**TAACCGCTTT
lp_1321	dipeptidase	-	67	2.9E-07	CTGTCGTCAA**CTTAAT**AAT**CGTAAG**TAACCGCTTT
lp_1556	unknown	-	100	8.1E-08	TTAGCATTTT**CTTACT**AAT**TGTAAG**TAGCGATATA
lp_1860	**oxidoreductase**	+	41	3.7E-09	ACATCAACCA**CTTACT**AAA**AGTAAG**TAAAATAAAA
lp_2270	**thioredoxin**	-	43	2.3E-07	AGCTTTATCT**GTTACT**AAT**AGTAAG**TATACCAGTA
lp_2633	**thioredoxin H-type**	-	57	1.5E-07	TAGTTTACCA**CATACT**AAA**AGTAAG**TAAAGTCTTT
lp_3279	Potassium uptake protein	+	123	5.1E-07	TAAAGTTCAC**CTAACG**AAA**CGTAAG**TTGAAACTTG
lp_3433	unknown	+	115	9.3E-08	CGCTCACCGG**CTGACG**AAC**AGTAAG**CCAACTAAAT
lp_3448	unknown	+	85	9.3E-08	CGCACATCGG**CTGACG**AAC**AGTAAG**CCAACTAAAT
lp_3575	integral membrane protein	-	55	5.9E-07	TACCCTCATT**CTTAAT**AAA**AGTAAA**TATTACTTTT

The peroxide-sensitive *rpoN*-deletion strain NZ7306 was still able to adapt to peroxide stress (Figure 2), which would contradict a direct role of σ^{54} in the peroxide-stress adaptation in *L. plantarum*. Whole genome transcription analyses of the wild-type compared to the *rpoN*-mutant with and without peroxide confirmed previous findings that indicated that the mannose PTS operon is the sole locus displaying σ^{54}-dependent expression. The mannose PTS is the main glucose-transporting PTS in various lactic acid bacteria (8) and functional

disruption of the genes encoding the mannose PTS in *L. plantarum* resulted in decreased growth and glycolysis rate, indicating reduced glucose uptake capacity and energetic limitations for the cell (36). Furthermore, mannose PTS expression is only observed in cells growing on mannose or glucose, while it is repressed on other sugars like maltose (36). Indeed, the increased peroxide-sensitivity of the *rpoN* mutant was not observed in maltose grown cells, confirming the role of the mannose PTS in this discriminative phenotype. Moreover, experiments with mutants affected in genes involved in mannose uptake (*manR* and *manIIC*) clearly established that the absence of a functional mannose PTS leads to higher sensitivity to hydrogen peroxide.

There are several possible explanations for the increased sensitivity of NZ7306 for peroxide stress. The reduced glucose uptake capacity in cells lacking a functional mannose PTS could lead to reduction of the energy generation rate (36), which in its turn could lead to increased hydrogen peroxide sensitivity. However, this "lack-of-energy" explanation would also predict an increased sensitivity to other forms of stress (e.g. UV, heat, etc.), which could not be experimentally confirmed, suggesting that this explanation is not valid.

The mannose PTS homolog in *E. coli* is highly resistant to oxidizing agents (21) and the described close relationship between the systems (39) suggests a similar robustness for the *L. plantarum* mannose PTS. In mutants lacking a functional mannose PTS, glucose uptake must be accomplished by other transporters, likely a glucose PTS, since these transport systems represent the most efficient mode of carbohydrate transport (17). As other glucose PTS's are sensitive to oxidizing agents (21), mutants that fail to express the mannose PTS (Δ*rpoN*, or Δ*manR*), or express a non functional variant of this system (Δ*manIIC*), will loose their capacity to transport glucose. This leads to major problems in energy generation processes and thus to hydrogenperoxide sensitivity. This possibility appears to explain all the observations reported here, and in contrast to the "lack-of-energy" explanation can also explain the lack of effect of σ^{54}-deletion during UV-, or heat- stress survival.

Acknowledgments

This work was supported by Grant No. IGE1018 from the Dutch IOP-Genomics Program.

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Chapter 4

Improvement of *Lactobacillus plantarum* WCFS1 Aerobic Growth by Comprehensive Transcriptome Analysis

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Abstract

Lactobacillus plantarum is used worldwide to initiate food fermentations, but little is known about the overall transcriptional response of this bacterium in the presence of oxygen. Here we present the physiological and transcriptional comparison between *Lactobacillus plantarum* cultures grown in presence or absence of oxygen. Both conditions led to typical bi-phasic growth and appeared to support comparable growth rates. However, the aerobic culture displayed growth stagnation in the early growth phase of approximately 30 min. Whole genome transcriptome profiles were obtained for cells harvested at early, mid, late logarithmic phases of growth as well as the stationary phase of growth of both *L. plantarum* cultures. The analyses revealed the differential expression of a total of 67 genes due to aerobic growth during the complete growth curve. Furthermore, the transcriptome analysis revealed a remarkable activation of CO_2-producing pathways in aerobically grown mid-logarithmic cells as compared to early logarithmic cells, suggesting low CO_2-concentration in the medium led to a temporary reduction of the growth rate and to increased expression of CO_2-producing enzymes. This hypothesis could be confirmed by supplementation of the aerobic growth conditions by providing higher CO_2 gas pressure during these growth conditions, which was shown to eliminate the observed growth delay.

Introduction

Lactobacillus plantarum is a facultative heterofermentative lactic acid bacterium involved worldwide in production of fermented food and feed products. Moreover, *L. plantarum* is frequently encountered as a natural inhabitant of the human gastrointestinal tract (2). Its natural habitats are anaerobic or micro-aerobic and rich in carbohydrates (32). Nevertheless, the responses of lactic acid bacteria such as *L. plantarum* to aerobic growth conditions and corresponding oxidative stresses are relevant for a variety of industrial processing conditions (20). Moreover, the potential to respire and increase biomass yield during aerobic growth has been shown for several LAB including *Lactococcus lactis* (5, 13).

Under anaerobic conditions and in the presence of excess of glucose, *L. plantarum* converts glucose exclusively to pyruvate which is subsequently converted to approximately equimolar amounts of D- and L-lactate (11). Research performed in the 1960's concluded that no oxygen consumption occurred during exponential growth under excess of glucose, leading to a similar fermentation pattern as under anaerobic conditions (26). Aerobic growth was even thought to be impossible for the bacterium as it possesses no superoxide dismutase to protect against superoxide produced during reduction of oxygene (16, 23). However, even earlier studies in the late 1950's showed that cell-free extracts of lactobacilli, including *L. plantarum*, can oxidize a number of substrates like glucose, pyruvate, lactate, and glycerol, suggesting a oxidative system with potential metabolic significance (40). Nowadays, it is known that *L. plantarum* is able to consume oxygen under certain circumstances, including glucose-limitation (15). The absence of the ubiquitous defensive reaction catalyzed by superoxide dismutase in *L. plantarum* has found to be compensated by the capacity of this bacterium to accumulate very high concentrations of intracellular Mn(II)-ions (up to 35 mM), which acts as a scavenger system for superoxide (3, 17). Manganese is also shown to bind to transcriptional regulators involved in oxidative stress response like PerR in *Bacillus subtilis* and OxyR *salmonella typhimurium* in thereby mediating regulation of stress genes (27).

Metabolic activity in presence of molecular oxygen leads to the production of reactive oxygen species (ROS), which damage DNA, membranes and proteins (12). The ROS-scavenging enzyme activity of NADH-peroxidase has been detected in aerobically grown *L. plantarum* cells (15), although the activity is apparently too low to detoxify the formed hydrogen peroxide (29). The disclosure of the complete genome sequence of *Lactobacillus plantarum* strain WCFS1 revealed that a number of genes encode functions involved in the detoxification of ROS, like GSH-reductases, a GSH-peroxidase, NADH-peroxidases, and thioredoxins,

suggesting that *L. plantarum* possesses a sophisticated defence mechanism that allows the bacterium to grow under aerobic conditions (19).

A well-documented activity of *L. plantarum* under aerobic conditions is the conversion of lactate, which was produced during earlier growth phases, to acetate. This conversion generates additional ATP and thereby supports an increase of biomass (25,35). The pathway involved in the lactate to acetate conversion has recently been studied in some detail, revealing the involvement of lactate dehydrogenase, pyruvate oxidase, and acetate kinase. This pathway is thought to play a role in pH homeostasis and has been shown to significantly influence stationary phase survival of *L. plantarum* under aerobic conditions (25,14). A key enzyme in this pathway is the pyruvate oxidase that catalyzes the conversion of pyruvate and phosphate to acetylphosphate and produces carbon dioxide and hydrogen peroxide as side-products. Expression of especially the Pox enzyme production has been shown to be repressed by excess glucose, involving the catabolite control protein A (CcpA), while it is also strongly regulated by the presence of oxygen via an unknown mechanism (14, 21). The Pox side-product hydrogen peroxide is a key source of hydroxyl radicals ($^{\cdot}OH$) that can be formed via the reaction of hydrogen peroxide with metal ions like Fe^{2+} (Fenton's reaction). The hydroxyl radicals cause damage to DNA, proteins, and membranes (12) and the accumulation of hydrogen peroxide in aerobically grown lactobacilli has been reported to lead to growth inhibition (7).

Here we present the comparative analysis of global transcriptome profiles of *L. plantarum* WCFS1 grown under aerobic and anaerobic conditions at different growth phases during batch culture. In the aerobically grown cultures a consistent temporary stagnation of growth was observed during the early logarithmic phase, while fermentation patterns shifted from homofermentative under anaerobic conditions, to a more heterofermenative pattern under aerobic conditions. Transcriptome analyses revealed genes that were differentially expressed due to aeration in early, mid, and late logarithmic as well as stationary phases of growth. Comprehensive interpretation of these differentially expressed genes revealed that the observed growth stagnation in aerobically grown cultures corresponded with the activation of a range of CO_2 producing metabolic pathways, leading to the hypothesis that the delay was due to CO_2-limitation. Analogously, it could be shown that increasing the gas-supplementation regime, including elevated CO_2 levels, relieved growth stagnation, confirming the CO_2 limitation hypothesis. This study exemplifies the power of post-genomic approaches to discover bacterial fermentation-limiting factors, which provides a first step to the rational adjustment of fermentation conditions in order to improve bacterial performance.

Material and Methods

Bacterial strains and growth conditions:

Lactobacillus plantarum strain WCFS1 (19) was routinely grown in MRS broth prepared without Tween 80 (8) in a Biocontroller ADI 1030 fermenter (Applikon, Schiedam, the Netherlands) at 37º C. Glucose was added to the medium (final concentration 2% (w/v)) and the initial pH of the medium was set at 6.5 with 0.1 N HCl. Anaerobic cultures were flushed with nitrogen (0.1 volumes per minute), and aerobic cultures were flushed with air (1 volume per minute), while both cultures were mixed vigourously (250 rpm). Growth was monitored by measuring the optical density at 600 nm (OD_{600}) in a spectrophotometer (Ultrospec 3000, Pharmacia Biotech, Roosendaal, the Netherlands). Pre-pre-cultures were grown overnight, at 37º C without agitation (anaerobic) in MRS medium, which were used to inoculate two 100 ml cultures (MRS without tween; inoculum 1:100; initial OD_{600} of approximately 0.02) and cultured at 37 º C either anaerobically or aerobically (500 ml Erlenmeyer flasks shaken at 200 rpm in an Innova 4340 incubator (New Brunswick Sciences, New Brunswick, Canada)). These pre-cultures were incubated for 14 hours, resulting in an exponentially growing culture with an OD_{600} of approximately 1.8. These growing cultures were used for inoculation of the anaerobic and aerobic cultures using the corresponding pre-culture, starting the fermentations at an OD_{600} of 0.2. The average OD_{600} of 2 biological independent cultures was plotted on a logarithmic scale as a function of time (Figure 1). The specific growth rate was calculated as the change of the logarithm of the cell density (log(N)) in time (t), $\Delta\log(N)/\Delta t$ (Figure 2).

Sampling

To avoid undesired alterations in the transcriptome due to a cellular responses after sampling, 1 volume of culture was quenched in 4 volumes of -40º C 60% methanol-HEPES buffer as described by Pieterse (28). After quenching, cells were harvested by centrifugation (13182 * g, 10 min, -20º C) using a Sorvall RC5B plus centrifuge (Sorvall, Newton, US). Immediately after harvesting, cell pellets were rapidly transferred with a pre-chilled spatula to a screw-crap tube containing 500 mg zirconium beads, 500µl phenol-chloroform-mix 1:1, 30 µl 3 M Na-Acetate (pH 5.2), 30 µl 10% SDS, and 400 µl MRS-medium (Merck, Darmstadt, Germany), carefully avoiding thawing of the cell pellet material. The tubes containing the cells were shaken, frozen in liquid nitrogen and stored at -80º C.

Culture supernatant samples were taken isolated by removal of the cells by centrifugation (20800 * g, 1 min), in an Eppendorf table centrifuge 5417C (Eppendorf, Hamburg, Germany). The supernatant was transferred to a new tube and stored at -20ºC until further use.

RNA extraction and quality control

RNA was isolated as described previously (39), using a phenol-chloroform extraction followed by a purification using the High Pure RNA isolation kit (Roche, Manheim, Germany). The yield and purity of the RNA were determined by measurement of the absorption at 260 nm and 280 nm (Ultrospec 3000, Pharmacia Biotech, Roosendaal, The Netherlands). The RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent technologies, Palo Alto, Ca, USA) following the manufacturer's instructions. Only RNA samples displaying 16S/23S-rRNA ratios of 1.6 or higher, were labeled and used for micro array experiments.

cDNA-synthesis, labeling, and hybridization

The Cyscribe Post-labeling kit (Amersham Biosciences, Amersham, UK) was used to synthesize and to label cDNA. Labeled cDNAs were hybridized as described previously on amplicon based microarrays containing fragment of approximately 97% of the genes of *L. plantarum* WCFS1 (39).

Scanning, data extraction, and analyses

The slides were scanned with a Scan Array Express 4000 scanner (Perkin Elmer, Wellesley, USA) and the images were analyzed with Imagene software 4.2 (BioDiscovery, El Segundo, USA). Statistical analyses were performed with R (http://www.r-project.org/) using the linear models for microarray data library (limma), which includes a lowess normalization of the data (6) and allows comparisons between many RNA targets simultaneously in complex experimental designs (36). Log odds for differential expression (B-value) higher than 1 were taken as a cut off. After this selection only genes with duplicate matching of these criteria were taken into account.

Cluster analysis was performed using the "cluster" software (10), taking the ^{2}log value of the ratio's determined by the limma software as shown in table 2. Distances between genes were calculated using the complete linkage method. Graphical output of the clustered data was obtained using Tree View (http://rana.lbl.gov/EisenSoftware.htm).

Determination of organic compounds in the supernatant

Fermentations end-products (Lactate and Acetate) were determined using a high-performance liquid chromatography (HPLC) as described previously, with an HPX-87P anion exchange column (Bio-Rad, Inc.) using 0.01 M H_2SO_4 as the elution fluid (37).

Glucose concentration was determined using Diabur Test 5000 stripes (Roche Diagnostics, Mannheim, Germany) following the manufacturer's protocol.

Results

Aerobic growth of *L. plantarum* in MRS medium

Aerobically and anaerobically growing cultures of *Lactobacillus plantarum* were monitored until stationary phase and samples were taken at early-log phase (P1), mid-log phase (P2), late-log phase (P3), and stationary-phase (P4) (Figure 1, Table 1). Aerobically and anaerobically growing cultures of *Lactobacillus plantarum* were monitored until stationary phase and samples were taken at early-log phase (P1), mid-log phase (P2), late-log phase (P3), and stationary-phase (P4) (Figure 1, Table 1). *L. plantarum* displayed bi-phasic growth (Fig. 1 and 2), which is in agreement with previous observations (9) and the final cell density was higher in the aerobic culture (OD_{600} of 5.5) compared to the anaerobic culture (OD_{600} of 4.5). Interestingly, a temporary stagnation of growth occurred during aerobic fermentation after approximately 2 h, a feature not observed in the anaerobic culture (Fig.1). Following stagnation, growth resumed, reaching a maximum growth rate comparable to that observed in anaerobic cultures (Fig.2).

Table 1: Characteristics of the aerobic and anaerobic culture at the 4 sample points. The growth rate was estimated from the graphs in figure 2B.

Feature:	***Aerobic:***	***Anaerobic:***
Phase 1:		
OD_{600}	0.43± 0.01	0.41 ± 0.02
pH	6.03 ± 0.01	6.01 ± 0.01
Specific growth rate	0.6 h^{-1}	0.7 h^{-1}
Phase 2:		
OD_{600}	1.164 ± 0.01	0.98 ± 0.02
pH	5.37 ± 0.03	5.47 ± 0.01
Specific growth rate	0.8 h^{-1}	0.8 h^{-1}
Phase 3:		
OD_{600}	3.0 ± 0.04	3.07 ± 0.10
pH	4.62 ± 0.07	4.54 ± 0.04
Specific growth rate	0.2 h^{-1}	0.2 h^{-1}
Phase 4:		
OD_{600}	5.41 ± 0.04	4.32 ± 0.29
pH	4.11 ± 0.06	4.06 ± 0.07
Specific growth rate	~0 h^{-1}	0 h^{-1}

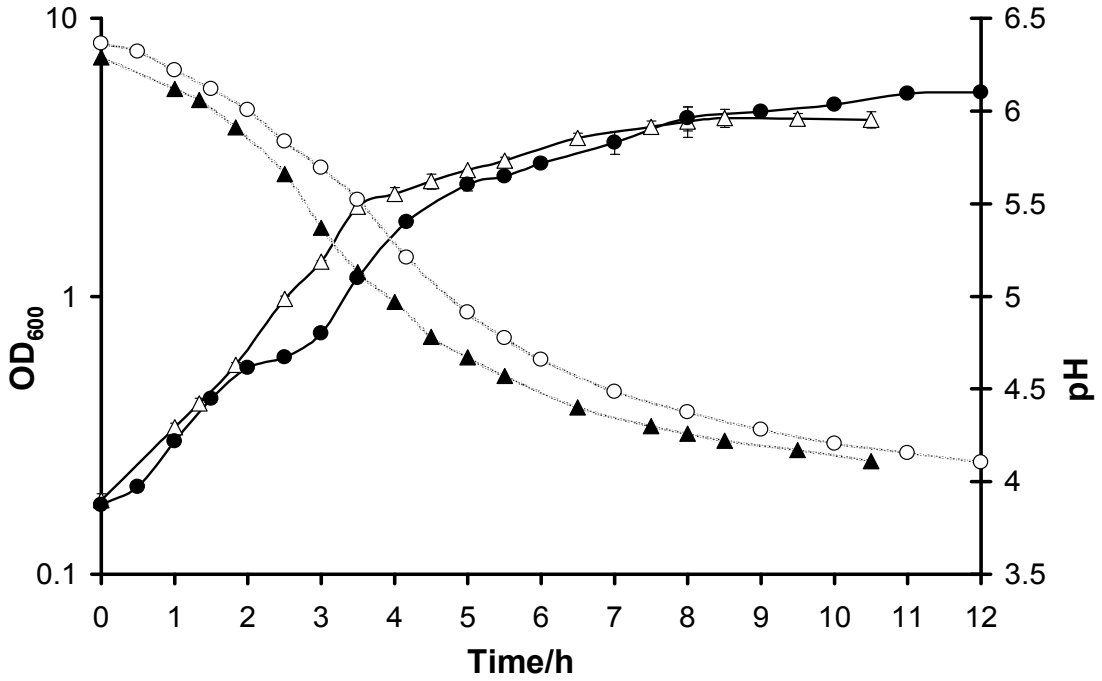


Figure 1: Growth characteristics of L. plantarum in batch fermentation under anaerobic (triangles) and aerobic (circles) conditions. The optical density of the anaerobic culture (open triangles) and acidification (closed triangles). The optical density of the aerobic culture (closed circles) and acidification (open circles).

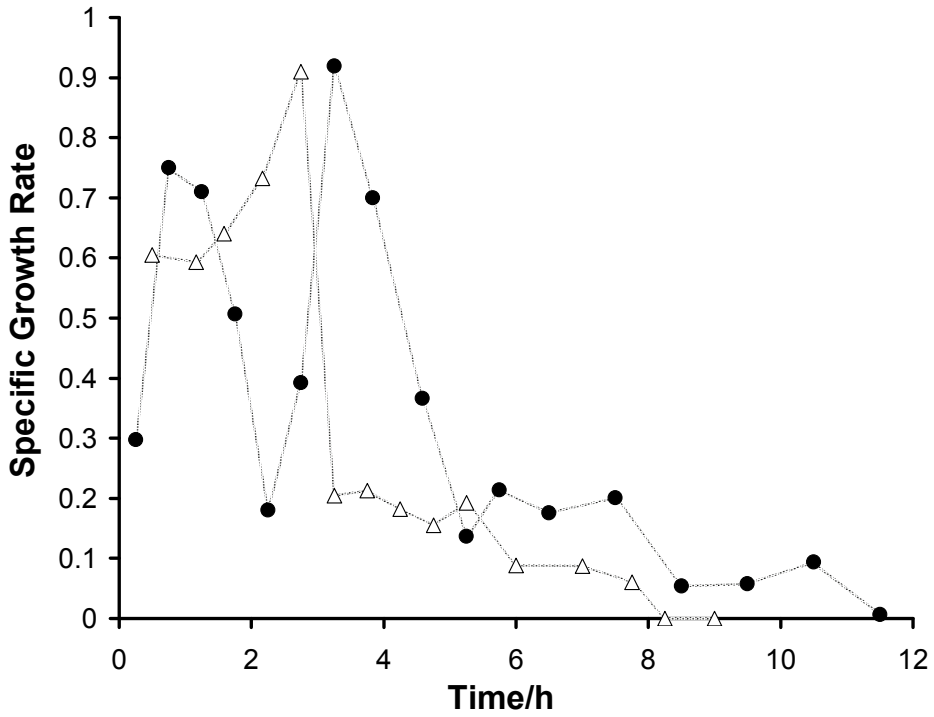


Figure 2: Specific growth rate of the anaerobic (open triangles) and aerobic (closed circles) culture during batch growth. In the early growth phases rate is high and decreases dramatically. The temporary growth stagnation in the aerobic culture after 2 h is clearly visible.

Growth under aerobic conditions resulted in a higher final optical density as compared to the anaerobic culture. Concomitantly, the relative acidification (corrected for cell density) appeared comparable in both cultures until stationary phase (Table 1), suggesting that aeration led to differences in central carbon metabolism and possibly resulted in different fermentation end-products. To determine fermentation end-products at various time points, organic acid and alcoholic end-product composition was measured in culture supernatant samples taken at the 4 time points (P1 to P4). Both cultures appeared to produce mainly lactate up to P3 in approximately equal amounts under both conditions (Table 2). However, in P4 the production of concentration of lactate reached in the anaerobic culture is higher (108 mM) as compared with the aerobic culture (87 mM) (Table 2). Accurate assessment of acetate production levels was complicated by the presence of this compound in the initial medium at a relatively high level (83 mM). Nevertheless, acetate production was estimated to be 6 mM in the aerobic fermentation in the early stationary phase, while the acetate production was 3-fold lower during anaerobic fermentation (Table 2).

This detailed analysis of aerobic and anaerobic growth curves, combined with determination of fermentation end-products clearly indicated a significantly altered metabolism of *L. plantarum* WCFS1 as a consequence of oxygen presence.

Table 2: Concentration of end-fermentation products in the supernatant of the aerobic and anaerobic culture. Values are given in mM, ND means not detected.

	Early log.	Mid log.	Transition	Stationary
Anaerobic				
Lactate	2.2 ± 0.9	11.3 ± 0.6	57.4 ± 1.2	109.8 ± 5.6
Acetate	n.d	n.d	n.d	2.0 ± 1.2
Aerobic				
Lactate	7.2 ± 0.3	15.2 ± 0.7	62.5 ± 1.0	87.4 ± 1.4
Acetate	0.8 ± 0.2	1.0 ± 0.4	1.5 ± 1.0	5.5 ± 1.0

Full genome transcriptional analyses of aerobic and anaerobic cultures

To elucidate the processes that cause the differences between aerobic and anaerobic cultures, genome wide transcription analyses were performed using amplicon-based DNA-microarrays. The transcriptome experiments revealed that 67 genes were significantly differentially expressed when comparing aerobic to anaerobic cultures throughout the whole growth curve (Table 3). A hierarchical cluster analysis divides these differentially expressed genes into three groups, i.e., two large groups containing up- or down regulated genes as a consequence

of aeration, irrespective of the growth phase analyzed, and a third group containing genes of which regulation also appeared highly dependent on the growth phase (Figure 3). This latter group of genes includes for example the purine biosynthesis gene cluster, which was higher expressed in the early growth phase (P1) during aerobic conditions, and higher expressed in the other three phases (P2-P4) during anaerobic conditions

Table 3: Genes differently expressed due to aeration of the culture with. The functional classification of genes used here was described before (19). The ^{2}log values of the ratio of gene expression under aerobic over anaerobic conditions are shown for each of the four phases (P1-P4). The p-value (p) is calculated for overall aerobic versus anaerobic conditions.

ORF	Name	Product	*p*	P1	P2	P3	P4
Amino acid biosynthesis							
lp_0957	*asnA*	aspartate--ammonia ligase	0.05	1.2	1.4	1.2	0.4
lp_2551	*hisC*	histidinol-phosphate aminotransferase	0.05	-1.5	-0.7	-1.0	-1.5
lp_2563	*hisK*	histidinol-phosphatase	0.04	1.0	0.6	1.6	-0.5
Biosynthesis of cofactors, prosthetic groups, and carriers							
lp_1253	*gshR2*	glutathione reductase	0.01	2.1	2.2	2.7	2.8
lp_1715		prenyltransferase	0.05	2.7	-1.1	-0.3	-0.2
lp_2633	*trxH*	thioredoxin H-type	0.05	0.2	0.4	2.2	0.9
Cell envelope							
lp_0297		extracellular protein	0.04	0.9	1.8	1.9	0.9
lp_1935		cell surface hydrolase (putative)	0.04	-1.7	-2.3	-0.8	-1.6
lp_3154	*acm3-C*	muramidase, C-terminal fragment	0.05	1.6	1.4	0.8	2.0
Cellular processes							
lp_0426	*plnU*	integral membrane protein PlnU, membrane-bound protease CAAX fam	0.03	1.3	1.2	1.6	0.8
lp_3578	*kat*	Catalase	0.03	0.9	2.5	2.1	-0.6
lp_0066	*pgmB2*	beta-phosphoglucomutase	0.05	0.9	1.3	1.6	0.8
Energy metabolism							
lp_0055		fumarate reductase, flavoprotein subunit precursor	0.04	1.4	0.9	1.3	1.5
lp_2629	*pox3*	pyruvate oxidase	0.00	1.8	3.0	3.8	2.1
lp_3033		short-chain dehydrogenase/oxidoreductase	0.04	1.7	1.3	1.1	1.2
lp_3449	*nox5*	NADH oxidase	0.01	1.5	2.3	2.7	0.5
lp_3483	*lacL*	beta-galactosidase, large subunit	0.01	2.8	1.6	1.5	2.2
lp_3556	*araB*	L-ribulokinase (putative)	0.04	1.7	1.8	1.0	1.5
Fatty acid and phospholipid metabolism							
lp_0075		acyl carrier protein phosphodiesterase	0.02	1.6	1.6	1.5	1.1
lp_3273	*cls*	cardiolipin synthetase 2	0.03	-0.7	-0.6	-2.0	-0.3
Hypothetical proteins							
lp_0151		Unknown	0.04	1.24	1.68	1.48	1.4
lp_0170	*dak3*	dihydroxyacetone phosphotransferase, phosphoryl donor protein	0.03	-2.6	0.2	-0.4	-0.1
lp_0190		Oxidoreductase	0.04	1.6	1.2	1.1	1.4
lp_0248		Unknown	0.04	1.0	1.6	1.7	0.7
lp_0823		protein containing diguanylate cyclase/phosphodiesterase domain 2	0.03	-1.9	-1.1	-1.1	-1.0
lp_1290		integral membrane protein	0.00	-2.1	-2.0	-4.3	-1.6
lp_1766		Unknown	0.02	-0.6	-0.7	-2.9	-2.8

ORF	Name	Product	*p*	P1	P2	P3	P4
lp_1813		Unknown	0.04	-1.2	-0.4	-1.4	-0.7
lp_1901		Unknown	0.03	-1.6	-1.2	-1.1	-2.5
lp_1916		integral membrane protein	0.00	1.8	2.2	3.37	3.28
lp_2113		Unknown	0.03	1.0	1.8	1.9	0.7
lp_2275		Unknown	0.05	1.5	2.6	1.0	0.1
lp_2624		Unknown	0.02	2.4	2.1	1.1	1.0
lp_2987		Unknown	0.05	-1.2	-0.2	-1.2	-0.8
lp_3057		Unknown	0.00	-1.7	-4.8	-3.9	-2.0
lp_3275		Unknown	0.02	-1.4	-0.4	-1.9	-1.3
lp_3350		Unknown	0.02	2.1	2.9	1.4	1.7
Other categories							
lp_0683		prophage P1 protein 60	0.03	2.3	1.5	0.5	1.6
lp_0689		prophage P1 protein 66, lipoprotein precursor	0.01	3.4	1.8	1.5	2.9
lp_0917		prophage P1 protein 8	0.01	2.5	2.2	1.3	1.3
lp_2413		prophage P2a protein 44	0.02	2.4	2.3	1.1	2.4
lp_3165		transposase, fragment	0.03	-1.9	-1.3	-1.1	-1.3
Protein fate							
lp_3272	*ptp2*	protein-tyrosine phosphatase	0.01	-1.7	-0.1	-2.3	-1.1
Purines, pyrimidines, nucleosides and nucleotides							
lp_2720	*purH*	bifunctional protein: phosphoribosylaminoimidazolecarboxamide formyltransferase; IMP cyclohydrolase	0.02	-1.7	1.1	-1.71	-0.4
lp_2723	*purF*	amidophosphoribosyltransferase precursor	0.03	-0.9	1.3	-2.0	-0.2
lp_2724	*purL*	phosphoribosylformylglycinamidine synthase II	0.03	-0.8	2.0	-2.2	0.3
lp_2725	*lpurQ*	phosphoribosylformylglycinamidine synthase I	0.05	-1.0	1.3	-1.4	0.3
lp_2727	*purC*	phosphoribosylaminoimidazole-succinocarboxamide synthase	0.02	-1.2	2.0	-1.9	-0.2
lp_2728	*purK1*	phosphoribosylaminoimidazole carboxylase, ATPase subunit	0.05	-0.7	2.1	-1.8	-0.1
lp_3269	*purB*	adenylosuccinate lyase	0.01	-1.2	-0.8	-3.0	-0.6
lp_3270	*purA*	adenylosuccinate synthase	0.01	-1.7	0.5	-2.5	-1.6
lp_3271	*guaC*	GMP reductase	0.00	-1.9	0.6	-3.5	-1.9
Regulatory functions							
lp_0845		transcription regulator	0.04	0.9	0.8	1.7	0.2
lp_0889		transcription regulator	0.00	2.5	2.4	2.9	1.8
lp_1116	*mleR1*	malolactic regulator	0.00	3.9	2.4	1.6	3.0
Transport and binding proteins							
lp_0439	*pts8C*	cellobiose PTS, EIIC	0.02	2.0	1.4	1.6	1.2
lp_0747	*pstD*	phosphate ABC transporter, permease protein	0.03	-2.2	-1.4	-0.7	1.6
lp_1261	*oppA*	oligopeptide ABC transporter, substrate binding protein	0.04	1.2	0.7	1.4	-0.7
lp_1326		glycerol-3-phosphate ABC transporter, permease protein (putative)	0.03	1.9	2.0	1.1	2.8
lp_1393		ABC transporter, ATP-binding and permease protein	0.03	1.6	0.7	1.1	2.3
lp_3103	*fhuD*	iron chelatin ABC transporter, substrate binding protein	0.03	-1.7	-1.6	-1.3	-2.5
lp_3104	*fhuC*	iron chelatin ABC transporter, ATP-binding protein	0.03	-1.0	-1.8	-1.9	0.1

ORF	Name	Product	*p*	P1	P2	P3	P4
lp_3600	*pts36B*	galactitol PTS, EIIB	0.04	1.2	1.2	1.4	-0.6
lp_3642		sugar ABC transporter, substrate binding protein	0.01	3.1	2.6	1.4	2.4
lp_3643		sugar ABC transporter, permease protein	0.04	1.9	1.5	0.8	1.5

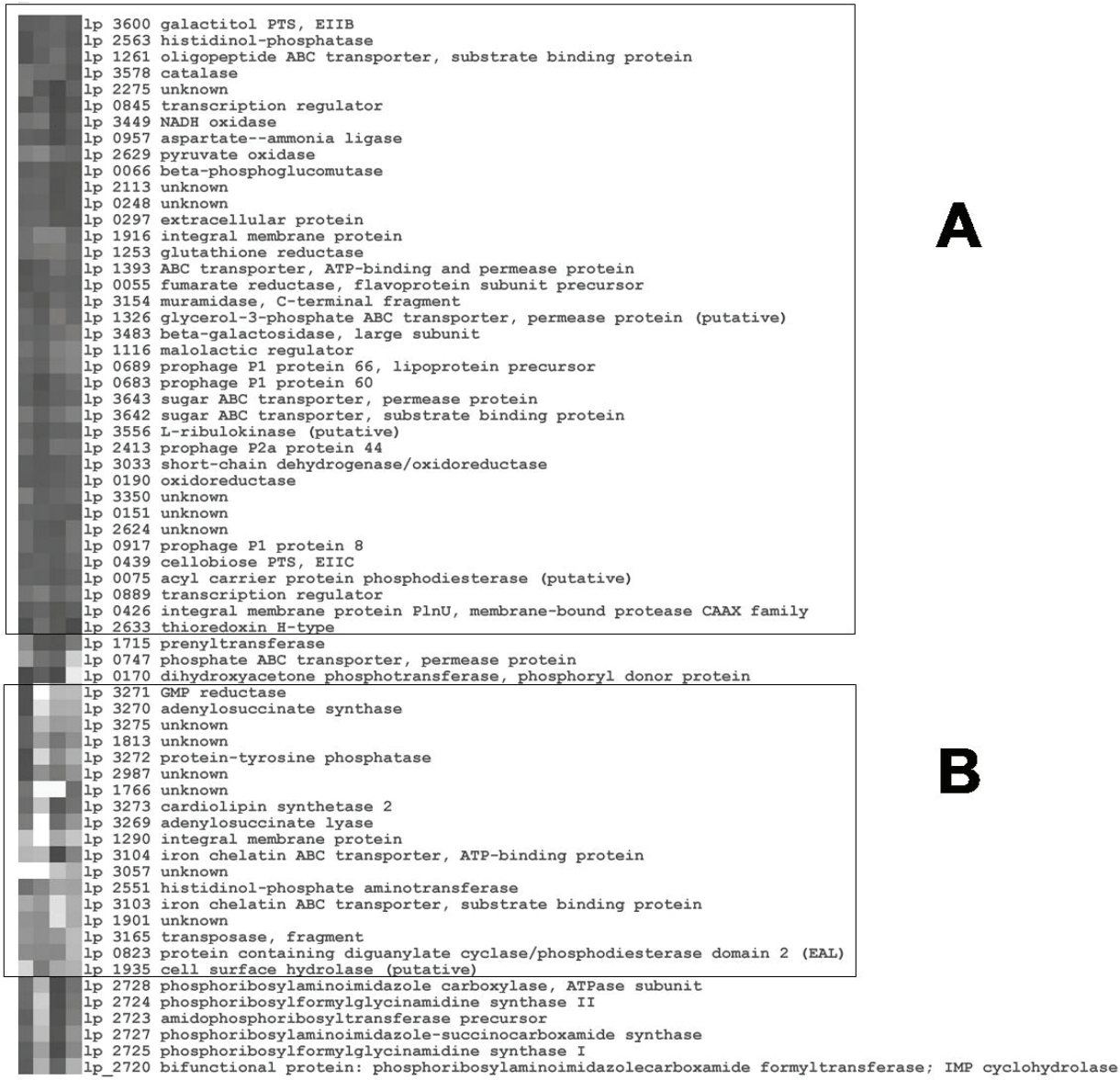


Figure 3: Hierarchical clustering of genes that are significantly differentially regulated between aerobic and anaerobic cultures. Three major groups occur of which two major groups contain genes that are up- (group A) and down-regulated (group B) due to the presence of oxygen, but hardly regulated by the growth phase, and a minor group in which the genes are also highly regulated by the growth phase.

Among the genes that were up-regulated under aerobic growth there appeared to be some that may be involved in a rudimentary respiratory chain, including a fumarate reductase, a NADP-dependent quinone oxidoreductase, and a prenyltransferase. The latter enzyme was predicted to contain a 4-dihydroxy-2-naphthoate octaprenyl transferase domain (NCIB conserved domain search, e-score $3*10^{-33}$, (22)), an enzyme activity needed for the production of menaquinone (vitamin K2), which can act as an electron carrier in a respiratory chain (44).

Aerobic conditions also induced an elevated expression level of genes encoding proteins and enzymes involved in the protection against reactive oxygen species (ROS), including genes predicted to encode a NADH-oxidase, glutathione reductase, and thioredoxin. Interestingly, the latter two genes have recently been implicated in hydrogen peroxide stress response in *L. plantarum* WCFS (38). Furthermore, the catalase gene was higher expressed under aerobic conditions and found to encode a protein that displays a high level of identity (99% identical amino acids) with an experimentally verified catalase in *L. plantarum* CNRZ 1228 (1).

The *L. plantarum* WCFS1 genome is predicted to encode four glutathione reductases, of which only one appeared to be up-regulated during aerobic growth. Analogously, NADH-oxidase (*nox; 4 paralogues*) and pyruvate oxidase (*pox; 5 paralogues*) encoding genes appear to be strikingly redundant in *L. plantarum* WCFS1, of which only one paralogue (*pox3*, *poxF* in strain Lp80 (21)) appeared to be regulated by aeration. Notably, recent studies indicated that only two of the *pox* genes actually encode an active pyruvate oxidase (PoxB and PoxF), both regulated in response to oxygen exposure (14, 21).

A notable observation is the regulation of the transcriptional regulator encoded by *lp_0889*, which belongs to the MarR-family of regulators, which was previously also shown to be induced by hydrogen peroxide stress (38). The activation of expression of this gene by oxygen and hydrogen peroxide exposure strongly supports a role for this protein in the regulation of the defense against ROS.

Some differentially regulated genes are involved in lipid metabolism and could influence membrane composition. Aerobic growth leads to increased expression of genes predicted to encode an integral membrane protein PlnU, which probably functions in acetyl transfer (NCBI conserved domain search (22)), and an acyl carrier protein phosphodiesterase, both involved in lipid metabolism. In contrast, anaerobic growth appeared to induce the cardiolipin synthetase 2 gene, predicted to be involved in the formation of cardiolipin, an anionic membrane lipid involved in initiation of DNA replication and protein translocation (24).

A number of genes encoding sugar transporters were found to be expressed at a higher level under aerobic growth conditions (lp_3600, lp_3643, lp_0439). Genes for an iron transport

system (lp_3103-3104) appeared to be expressed at lower level under aerobic conditions, possibly to reduce the cellular concentration of iron, a key player in fenton's reaction that produces ROS.

Overall, comparative transcriptome profiling of aerobically and anaerobically grown cultures of *L. plantarum* WCFS1 revealed changes in the expression of genes involved in lactate metabolism, lipid metabolism, stress response, transport capacities, and regulatory function.

Changes during the early growth phase of an aerobic culture

As described above, *L. plantarum* WCFS1 displays temporary growth stagnation between sample point P1 and P2 in the early aerobic growth phase. Since the pre-cultures used to inoculate these aerobic fermentation cultures were already adapted to aerobic growth, it seems likely that a limitation in the growth medium and not the presence of oxygen per se caused the growth stagnation. Notably, the growth stagnation phenomenon always appeared to occur approximately 100 to 120 min after the start of the fermentation, and was found to be independent of the initial culture density (Fig. 4). Furthermore, the duration of the growth stagnation appeared to inversely correlate with the inoculum size, since higher density inoculation led to shorter stagnation periods (Fig. 4). These characteristics suggest that the growth stagnation observed is due to limitation of a specific medium compound that is flushed out by aeration during the initial phase of the fermentation.

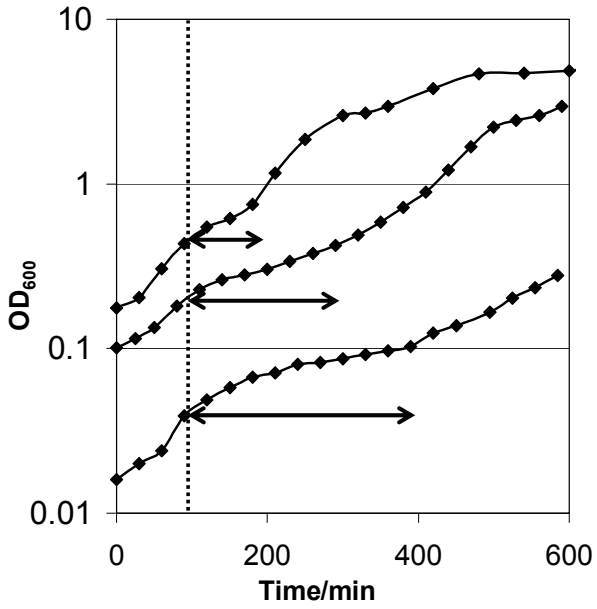


Figure 4: Temporary inhibition of the growth rate in the early aerobic growth phase. Three cultures are shown, inoculated at OD_{600} of 0.2, 0.1, and 0.02. Clearly, the duration of this phase increases with decreasing initial OD (Arrows). Representative graphs of experiments performed in triplicate are shown.

The transcriptome profiles obtained prior (P1) and after (P2) the growth stagnation were investigated in detail and the projection of these data on a metabolic map using the metabolic model of *L. plantarum* WCFS1 (42), revealed a number of reactions in the pyruvate metabolism to be expressed at a higher level in P2 relative to P1 (figure 5A). The transcription data suggest that cells harvested in P2 convert citrate to pyruvate, as the citrate lyase gene is higher expressed (3-fold higher in P3 than in P1), possibly via malate since the malate dehydrogenase gene is also 3-fold induced. Additionally, genes for enzymes involved in the conversion of pyruvate into compounds like acetate, acetaldehyde, and actetyl-CoA were up-regulated in P2 (Fig. 5). Interestingly, all these induced pathways include a reaction that produces CO_2. CO_2 is essential in purine biosynthesis during the production of the intermediate 5-amino-1-ribosylimidazole 5-phosphate. Moreover, the reaction catalyzed by carbamoyl-phosphate synthase, which is involved in pyrimidine synthesis requires the dissolved form of carbon dioxide (HCO_3^-). Intriguingly, the genes for enzymes involved in these pyrimidine and purine biosynthesis reactions also appeared to be expressed at a higher level during P2, the carbamoyl phosphate synthase gene 3-fold higher and the phosphoribosyl-amino-imidazole carboxylase gene 2-fold higher. These data suggest that the limiting CO_2 concentrations lead to the growth stagnation by their impact on the biosynthesis rates of nucleotides. Moreover, the data suggest that no CO_2 limitation is apparent during the early growth phase (P1), since high growth rates are sustained without the induction of the CO_2-producing pathways. Time-dependent CO_2-limitation could occur by flushing-out of the CO_2 contained in the medium as a consequence of the high gas-flush rate imposed by aeration conditions. The induction of carbon dioxide producing pathways appears to indicate that the bacterium initiates CO_2 production to compensate for its limiting environmental availability.

Figure 5A: Transcriptome data of aerobic P1 versus P2 projected on a metabolic map of pyruvate metabolism. Black triangles indicate reactions corresponding to up regulated genes in P2, grey boxes indicate no significant regulation, and a cross indicates that no data are available. Abbreviations: 2PG = 2-phosphoglycerate, Pep = Phosphoenolpyruvate, Pyr = Pyruvate, L/D-Lac = L/D-lactate, Mal = Malate, Fum = Fumarate, OAA = Oxaloacetate, Cit = Citrate, EtOH = Ethanol, AcAld = Acetaldehyde, Ac = Acetate, Ac-P = Acetyl phosphate, AcCoA = Acetyl CoA, For = Formiate.

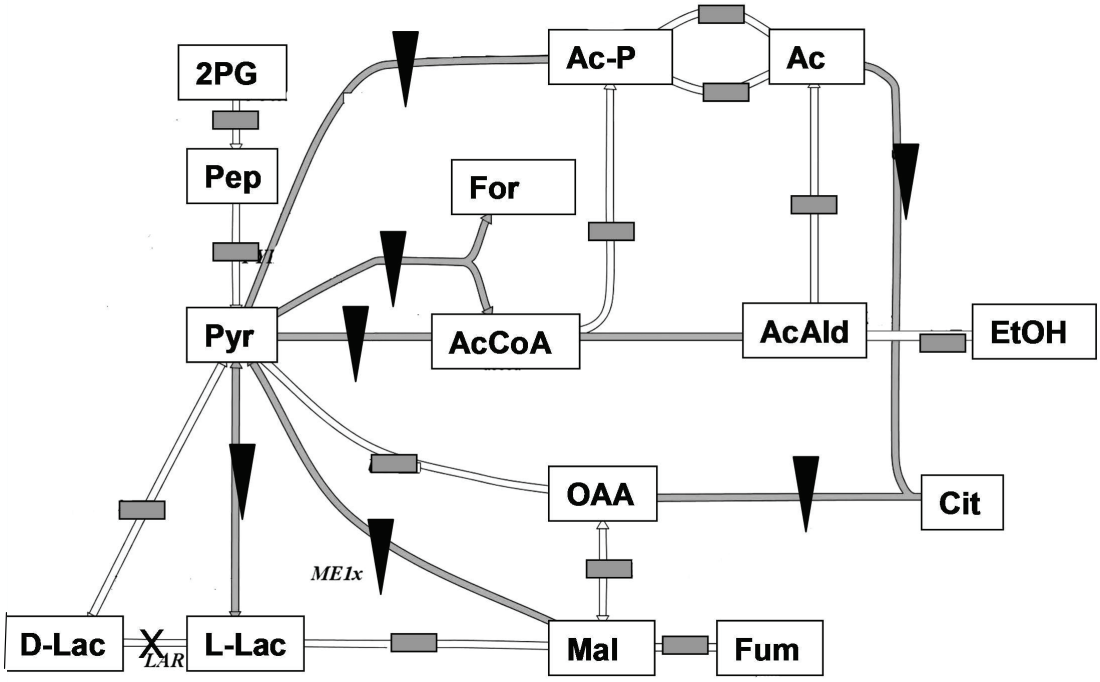
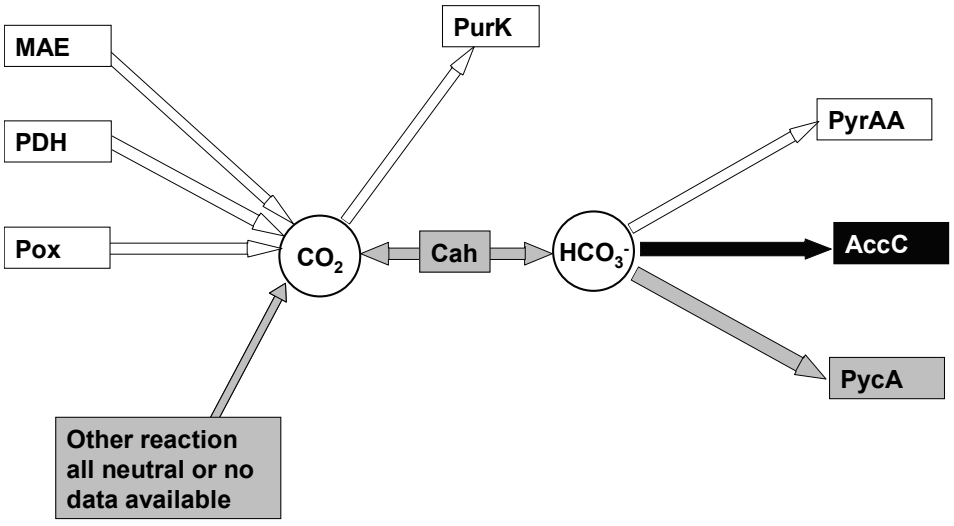


Figure 5B: Carbon dioxide consuming and producing reactions in L. plantarum WCFS1. Enzymes are differentially expressed between the time points P1 and P2 in aerobic cultures. White arrows indicate reactions corresponding to up regulated genes in P2, grey arrows indicate no significant regulation, and black arrows indicate down regulation in P2. MAE = Malic enzyme, PDH =Pyruvate dehydrogenase, Pox = Pyruvate oxidase, PurK = Phosphoribosylamino-imidazole carboxylase, Cah = Carbonate anhydrase, PyrAA = Carbamoyl-phosphate synthase, AccC = acetyl-CoA carboxylase, PycA = pyruvate carboxylase



To investigate whether CO_2 limitation causes the observed growth stagnation during the early logarithmic growth phase, *L. plantarum* WCFS1 was cultured aerobically and flushed with standard air or with air enriched with 1% CO_2. To emphasize the growth stagnation, the culture was inoculated at an initial inoculum OD_{600} of approximately 0.1, which led to an extended stagnation period as compared to initial inoculum OD_{600} of 0.2 (see above and Fig. 4). In the culture flushed with normal air the growth stagnation occurred as usual after approximately 100 min (Fig. 6) and due to the lower initial OD_{600} lasted for at least 2.5 h. In contrast, the culture flushed with 1% CO_2 enriched air displayed no growth stagnation (Fig. 6), confirming that CO_2 limitation hampers continuous growth of aerobically cultured *L. plantarum*, which can readily be compensated by the supplementation with an additional CO_2 supply.

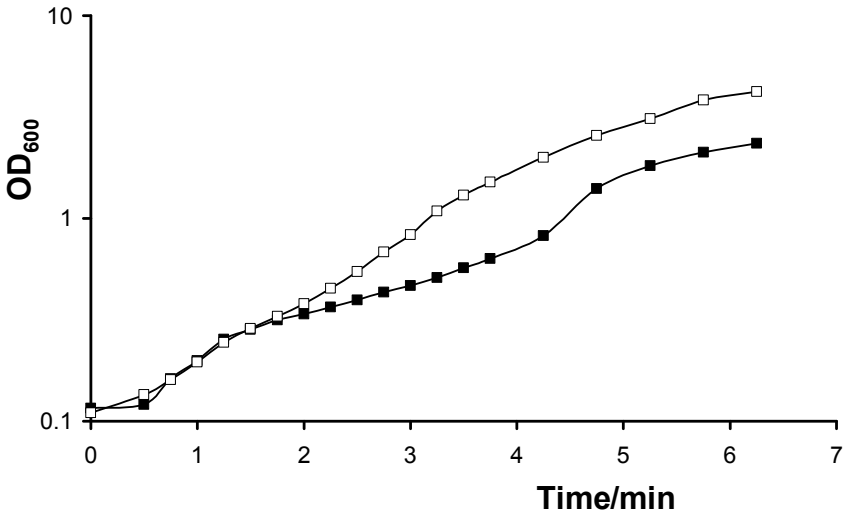


Figure 6: Abolishment of the temporary growth inhibition by increased CO_2 concentration. The culture flushed with air shows a temporary decrease of the growth rate (closed squares) whereas the growth inhibition is abolished in the culture flushed with air mixed with 1% CO_2 (open squares).

Differences in the later growth phase

Previous studies have indicated that there are no major differences between aerobic and anaerobic *L. plantarum* cultures when they are provided with an excess of glucose (26) and that enzyme activities typically responsible for aerobic metabolism mainly are produced during the later growth phases (14, 21, 25). Therefore, we had a closer look at the differences in gene expression between the two cultures at sample point P4.

In total 306 genes showed higher expression and 271 genes lower expression in the aerobic growth phase P4 as compared to the anaerobic growth phase P4 (complementary data, Table 1). Aerobic growth conditions appeared to induce a number of genes that are predicted to be involved in typical growth related activities, which is in agreement with the more pronounced stagnation of growth in anaerobic fermentations after reaching the stationary phase of growth. The genes include 33 ribosomal genes, genes involved in cell shape determination and cell-division, and genes encoding proteins involved in DNA-processing, transcription, and translation as, e.g., DNA-directed RNA polymerase, DNA-helicase, elongation factors, and peptide chain release factors. Remarkable is the higher level of expression of 4 genes for universal stress proteins of the UspA family in the later growth phase (P4) of cultures grown under anaerobic conditions, suggesting a more pronounced stress response in the anaerobic culture. Also, two genes encoding enzymes annotated as L-2-hydroxyisocaproate dehydrogenases are higher expressed. These enzymes oxidize α-ketoacids into α-hydroxyacids with a preferred carbon bone of 5-6 atoms (33).

Altogether, the transcriptional analysis of the later growth phases indicates that there are indeed differences between aerobic and anaerobic cultures in the later growth phases, as shown by the high number of genes differentially expressed between the cultures.

Discussion

L. plantarum is a lactic acid bacterium which is frequently encountered in environmental niches that are commonly characterized by anaerobic or micro-aerobic conditions, such as plant fermentations and the mammalian GI-tract (2, 32). However, *L. plantarum* is capable of oxygen consumption, but only when glucose is limited (15). The comparison between an aerobic and an anaerobic *L. plantarum* batch culture described in this study revealed several physiological differences between aerobic and anaerobic growth. Both cultures started growing at high rates and acidified the medium rapidly, apparently irrespective of the presence or absence of oxygen, confirming previously described results (15, 16). However, the early expression of the gene for pyruvate oxidase (*poxF*) gene and the production of acetate during early aerobic growth are in apparent contradiction with previous observations of glucose repression of *pox* (21). The difference can be explained by the temporary growth stagnation observed in the early phases of the aerobic growth, as glucose mediated catabolite repression is probably relieved in slowly growing cells. Moreover, Pox is a CO_2-producing

enzyme and the regulation due to CO_2-limitation is possible, although the underlying mechanism of CO_2-sensing remains unclear, not only in *L. plantarum* but in all organism (30).

In the lactic acid bacterium *Lactococcus lactis* an electron transfer chain (ETC) system was shown to generate a proton motive force under aerobic conditions depending on medium composition, leading to an almost doubled biomass yield (5, 13). The transcriptome analysis revealed expression of a rudimentary ETC system in *L. plantarum* during aerobic growth, suggesting oxidative phosphorylation capacities under some conditions. However, as only a slight increase in biomass was observed in the aerobic culture, it is probably not-functional under the applied growth conditions.

The regulation of a gene encoding for a potential transcription factor (lp_0889) was already observed during peroxide stress, indicating a key-role for this regulator in oxidative stress response. An advanced annotation of the regulatory proteins in the genome of *L. plantarum* WCFS1 revealed 28% amino acid homology between this transcription regulator and OxyR (43), a key-player of redox regulation in bacteria (18), and the experimental data now confirm this prediction.

The increased expression of genes encoding hydrogen peroxide producing enzymes as e.g. Pox and NADH-oxidase during early growth phases in aerobic cultures could lead to accumulation of hydrogen peroxide (7), a reactive oxygen species which can damage DNA, proteins, and membranes (12). This clearly explains the aerobic expression of genes encoding enzymes that contribute to hydrogen peroxide detoxification: catalase, GSH-reductase, and a thioredoxin. Additionally, the differential expression of genes encoding enzymes involved in lipid metabolism and several membrane proteins suggests changes in the membrane composition, which are possibly related to the membrane-damaging effects of peroxide and other ROS.

In the later growth phases the differences between aerobic and anaerobic cultures become more pronounced, also due to the apparent different growth charateristics of the cultures, which is illustrated by the differential expression of genes probably directly related to growth. Growth continues in the aerobic culture at a very low rate, probably due to additional gain of energy from the conversion of lactate to acetate via POX-activity (25, 34).

Lactate inhibits lactate dehydrogenase leading to an imbalanced NAD^+/NADH-ratio (31). The up-regulation of two L-2-hydroxyisocaproate dehydrogenase genes in the anaerobic culture is of interest, since these enzymes catalyze the same type of reaction as lactate dehydrogenases (conversion of α-ketoacids as e.g. pyruvate into α-hydroxyacids as e.g. lactate). Although their activity on pyruvate is low (33) and their physiological function is unknown (4), the

higher expression of these genes at high lactate concentrations suggests they take over the function of LDH and function in restoring the NAD^+/NADH balance. Another candidate-substrate for L-2-hydroxyisocaproate dehydrogenase is 2-oxoisocaproate which is produced from leucine by a branched chain amino acid aminotranferase. The corresponding gene (lp_2390) is significantly higher expressed (2.7-fold) in later phase anaerobic cells compared to mid-log anaerobic cells.

Peroxide production, as side-product of the acetate forming pathway (35), can result in reduction of the growth rate in the early growth phase of the aerobic culture (7, 25). However, *L. plantarum* has sophisticated defense systems that protect it against hydrogen peroxide (39) and the low amounts of peroxide formed (theoretically equimolar with that of acetate) should not lead to severe stress for the bacterium.

The metabolic model for *L. plantarum* WCFS1 predicts usage of bicarbonate for the production of purines and pyrimidines (41). The solubility of carbonate at 37° C and at pH < 6.5 is low and intense flushing of media with air will lead to a decrease of carbonate concentration, ultimately, causing growth stagnation in cultures flushed with air (figure 1B) or nitrogen (data not shown). The dependency of growth on the CO_2 or carbonate concentration could be confirmed by the restoration of growth when the culture flushed with 1% CO_2.

Our analyses give new insight in the growth of *L. plantarum* under aerobic conditions. Effects directly related to oxygen occur in the later growth phases, when growth rate is very low. Furthermore, our analyses show the power of comprehensive transcriptome interpretation in identifying the cause of growth inhibition in the early growth phase and in devising an approach to overcome this inhibition.

Acknowledgments

This work was supported by Grant No. IGE1018 from the Dutch IOP-Genomics Program.

We thank Roelie Holleman for performing the HPLC-analysis.

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Complementary data

Complementary table 1A: Genes higher expressed in the stationary aerobic phase compared to the stationary anaerobic phase. Ratio shown is the absolute ratio

Gene	Product	ph4
lp_0002	DNA-directed DNA polymerase III, beta chain	2.59
lp_0010	single-strand binding protein	2.14
lp_0013	ribosomal protein L9	2.43
lp_0018	ABC transporter, substrate binding protein	2.54
lp_0027	beta-phosphoglucomutase	3.33
lp_0031	cold shock protein CspL	2.85
lp_0047	aldehyde dehydrogenase	3.43
lp_0050	p-nitrobenzoate reductase	2.01
lp_0055	fumarate reductase, flavoprotein subunit precursor	2.87
lp_0072	unknown	3.00
lp_0075	acyl carrier protein phosphodiesterase (putative)	2.10
lp_0077	unknown	3.23
lp_0078	transport protein	2.57
lp_0080	acetyltransferase (GNAT) family (putative)	3.45
lp_0081	intracellular protease/amidase (putative)	3.91
lp_0085	cation efflux protein (putative)	2.40
lp_0088	prolyl aminopeptidase	3.23
lp_0102	cobalamin biosynthesis protein	7.08
lp_0105	NCAIR mutase, PurE-related protein	2.41
lp_0116	purine-cytosine transport protein	2.58
lp_0120	amino acid transport protein	3.06
lp_0134	transport protein	2.05
lp_0149	ABC transporter, ATP-binding protein	2.03
lp_0151	unknown	2.58
lp_0160	ABC transporter, ATP-binding protein	2.07
lp_0182	mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase	2.41
lp_0190	oxidoreductase	2.60
lp_0203	phosphoglycerate dehydrogenase	2.23
lp_0208	unknown	3.52
lp_0214	integral membrane protein	2.59
lp_0217	ABC transporter, permease protein	9.76
lp_0218	ABC transporter, ATP-binding protein	7.27
lp_0219	ABC transporter, permease protein	2.29
lp_0250	unknown	4.19
lp_0253	2-keto-3-deoxygluconate kinase	2.55
lp_0258	unknown	2.46
lp_0268	teichoic acid biosynthesis protein	2.03
lp_0272	unknown	5.27
lp_0302	extracellular protein	2.40
lp_0307	unknown	5.33
lp_0315	spermidine/putrescine ABC transporter, substrate binding protein	2.26
lp_0317	spermidine/putrescine ABC transporter, permease protein	2.59
lp_0337	biotin--[acetyl-CoA-carboxylase] ligase and biotin operon repressor	2.01
lp_0349	ammonium transport protein	2.24
lp_0367	glycine betaine/carnitine/choline ABC transporter, substrate binding and permease protein	2.18
lp_0368	glycine betaine/carnitine/choline ABC transporter, ATP-binding protein	2.70
lp_0370	glycerol kinase	3.73
lp_0373	cell surface protein precursor (putative)	2.09
lp_0377	integral membrane protein	4.62
lp_0404	immunity protein PlnL	2.30
lp_0432	DNA helicase (putative)	3.00
lp_0434	tryptophan--tRNA ligase	2.45
lp_0439	cellobiose PTS, EIIC	2.32
lp_0443	transcription regulator (putative)	2.06
lp_0463	zinc/iron ABC transporter, ATP-binding protein	2.36
lp_0464	zinc/iron ABC transporter, permease protein	3.50
lp_0476	hydrolase (putative)	2.20
lp_0490	unknown	3.30
lp_0511	transcription terminator factor Rho	2.54
lp_0512	ribosomal protein L31	6.59
lp_0520	ATP-dependent RNA helicase	2.37
lp_0528	N-acetyl-gamma-glutamyl-phosphate reductase	2.33
lp_0529	glutamate N-acetyltransferase	4.98
lp_0540	integral membrane protein	2.44
lp_0541	unknown	3.16
lp_0559	transport protein	2.28
lp_0604	Deoxyuridine 5'-triphosphate nucleotidohydrolase	2.61
lp_0619	ribosomal protein L11	4.12
lp_0624	prophage P1 protein 1, integrase	3.91
lp_0625	prophage P1 protein 2	2.71
lp_0628	prophage P1 protein 5	2.80
lp_0645	prophage P1 protein 22	2.15
lp_0683	prophage P1 protein 60	2.93
lp_0688	prophage P1 protein 65, DNA entry nuclease	3.67

lp_0689	prophage P1 protein 66, lipoprotein precursor	7.23
lp_0692	ribonucleoside-diphosphate reductase, beta chain	2.25
lp_0693	ribonucleoside-diphosphate reductase, alpha chain	2.05
lp_0718	ribosomal-protein-alanine N-acetyltransferase	2.42
lp_0741	peptide chain release factor 2, C-terminal fragment	2.47
lp_0746	phosphate ABC transporter, substrate binding protein	2.32
lp_0747	phosphate ABC transporter, permease protein	3.10
lp_0775	argininosuccinate synthase	2.58
lp_0776	argininosuccinate lyase	2.23
lp_0802	polar amino acid ABC transporter, substrate binding and permease protein	2.34
lp_0815	Na(+)/H(+) antiporter (putative)	2.28
lp_0818	unknown	2.55
lp_0819	unknown	3.35
lp_0822	glutamine-fructose-6-phosphate transaminase (isomerizing)	3.07
lp_0889	transcription regulator	3.43
lp_0892	transcription regulator (putative)	2.30
lp_0893	multidrug transport protein	2.30
lp_0898	GroEL supressor protein SugE	2.14
lp_0906	6-phospho-beta-glucosidase	2.14
lp_0910	DNA helicase (putative)	2.34
lp_0917	prophage P1 protein 8	2.41
lp_0941	integrase	2.46
lp_0955	acyl carrier protein phosphodiesterase	2.91
lp_0962	ATP-dependent DNA helicase RecQ	2.85
lp_0997	cold shock protein CspC	3.33
lp_1001	phosphatase (putative)	2.03
lp_1008	lysine transport protein	3.64
lp_1021	DNA-directed RNA polymerase, beta subunit	2.77
lp_1022	DNA-directed RNA polymerase, beta' subunit	2.22
lp_1025	ribosomal protein S12	6.23
lp_1026	ribosomal protein S7	4.45
lp_1027	elongation factor G	3.23
lp_1032	ribosomal protein S10	2.15
lp_1033	ribosomal protein L3	5.01
lp_1034	ribosomal protein L4	5.94
lp_1035	ribosomal protein L23	7.69
lp_1036	ribosomal protein L2	3.93
lp_1038	ribosomal protein S19	3.42
lp_1039	ribosomal protein L22	2.86
lp_1040	ribosomal protein S3	2.75
lp_1041	ribosomal protein L16	2.45
lp_1043	ribosomal protein L29	2.08
lp_1044	ribosomal protein S17	3.37
lp_1045	ribosomal protein L14	2.96
lp_1046	ribosomal protein L24	3.78
lp_1047	ribosomal protein L5	2.74
lp_1050	ribosomal protein S8	3.07
lp_1051	ribosomal protein L6	3.73
lp_1052	ribosomal protein L18	2.68
lp_1053	ribosomal protein S5	4.19
lp_1055	ribosomal protein L15	4.79
lp_1056	preprotein translocase, SecY subunit	2.71
lp_1058	adenylate kinase	4.45
lp_1059	translation initiation factor IF-1	3.29
lp_1059 a	ribosomal protein L36	4.74
lp_1060	ribosomal protein S13	3.99
lp_1061	ribosomal protein S11	3.37
lp_1062	DNA-directed RNA polymerase, alpha subunit	5.20
lp_1064	transposase	2.50
lp_1067	integral membrane protein	2.22
lp_1077	ribosomal protein L13	2.14
lp_1086	3-dehydroquinate synthase	3.20
lp_1088	unknown	2.04
lp_1093	2-(5"-triphosphoribosyl)-3'-dephosphocoenzyme-A synthase	2.04
lp_1116	malolactic regulator	8.19
lp_1120	amino acid transport protein	2.70
lp_1151	RNA methyltransferase	3.18
lp_1179	repeat unit transporter	3.39
lp_1180	glycosyltransferase	2.40
lp_1181	acyltransferase/acetyltransferase	2.76
lp_1182	exopolysaccharide biosynthesis protein	3.10
lp_1183	glycosyltransferase	2.16
lp_1204	polysaccharide polymerase	3.85
lp_1225	polysaccharide biosynthesis protein (putative)	3.97
lp_1226	O-acetyltransferase	2.23
lp_1230	transcription regulator	2.40
lp_1239	unknown	2.44
lp_1253	glutathione reductase	7.04
lp_1255	peptide chain release factor 3	4.71
lp_1292	acetyltransferase (putative)	2.36
lp_1310	poly (glycerol-phosphate) alpha-glucosyltransferase (putative)	3.93
lp_1316	leucine--tRNA ligase	3.16
lp_1326	glycerol-3-phosphate ABC transporter, permease protein (putative)	6.82

lp_1328	glycerophosphodiester phosphodiesterase	2.08
lp_1336	ABC transporter, ATP-binding protein	2.25
lp_1357	extracellular protein, membrane-anchored (putative)	2.77
lp_1388	unknown	3.40
lp_1393	ABC transporter, ATP-binding and permease protein	4.86
lp_1411	arginine catabolic regulator	2.27
lp_1443	transcription regulator	2.74
lp_1493	molybdopterin-guanine dinucleotide biosynthesis protein MobB	2.05
lp_1519	1 segregation helicase (putative)	2.71
lp_1535	unknown	2.71
lp_1546	prenyltransferase	2.63
lp_1549	exonuclease SbcD	2.04
lp_1636	ribosomal protein S16	2.49
lp_1640	ribosomal protein L19	3.13
lp_1652	anthranilate synthase, component I	2.82
lp_1653	anthranilate synthase, component II	3.41
lp_1672	acyl carrier protein	5.65
lp_1673	[acyl-carrier protein] S-malonyltransferase	7.34
lp_1675	3-oxoacyl-[acyl-carrier protein] synthase II	4.93
lp_1676	acetyl-CoA carboxylase, biotin carboxyl carrier protein	3.68
lp_1677	(3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase	3.50
lp_1678	acetyl-CoA carboxylase, biotin carboxylase subunit	4.70
lp_1679	acetyl-CoA carboxylase, carboxyl transferase subunit beta	2.49
lp_1682	phosphopantetheinyltransferase	11.4
lp_1684	integral membrane protein	3.17
lp_1685	transcription regulator	2.72
lp_1686	acyl-CoA thioester hydrolase (putative)	2.64
lp_1687	GTPase	3.25
lp_1688	transcription regulator (putative)	2.06
lp_1689	transport protein	2.19
lp_1695	integral membrane protein	2.56
lp_1708	unknown	4.16
lp_1716	potassium transport protein	2.55
lp_1722	amino acid transport protein	2.87
lp_1730	maltose phosphorylase	2.38
lp_1731	aldose 1-epimerase	2.36
lp_1734	diphosphomevalonate decarboxylase	3.20
lp_1735	mevalonate kinase	2.56
lp_1751	penicillin binding protein 1A	2.55
lp_1795	transcription regulator	2.69
lp_1796	DegV family protein	2.82
lp_1797	acetyltransferase (putative)	3.61
lp_1811	amino acid transport protein	2.31
lp_1835	protein-methionine-S-oxide reductase	5.09
lp_1842	integral membrane protein	2.01
lp_1850	DNA topoisomerase	2.12
lp_1852	DNA processing protein	2.11
lp_1855	unknown	2.32
lp_1864	carboxy-terminal processing proteinase	2.46
lp_1867	unknown	2.95
lp_1883	cytidylate kinase	2.04
lp_1915	lipoprotein precursor	2.05
lp_1916	integral membrane protein	9.68
lp_1980	aspartate--tRNA ligase	2.10
lp_2022	transposase, fragment	2.30
lp_2054	elongation factor TS	2.24
lp_2055	ribosomal protein S2	2.33
lp_2058	endonuclease containing a URI domain (putative)	2.02
lp_2103	glycosyltransferase	2.01
lp_2105	UDP-glucose 4-epimerase	3.13
lp_2111	glutamine ABC transporter, substrate binding and permease protein	5.08
lp_2118	trigger factor	2.67
lp_2125	ribosomal protein S15	2.19
lp_2146	GTP-binding protein TypA	2.61
lp_2158	metallo-beta-lactamase superfamily protein (putative)	2.30
lp_2162	extracellular protein, gamma-D-glutamate-meso-diaminopimelate muropeptidase (putative)	3.16
lp_2222	inorganic polyphosphate/ATP-NAD kinase (putative)	2.04
lp_2244	unknown	3.57
lp_2315	septum site-determining protein MinD	2.06
lp_2319	cell shape determining protein MreB	2.29
lp_2335	transcription regulator	2.36
lp_2342	unknown	2.33
lp_2350	D-Methionine ABC transporter, substrate binding protein (putative)	2.28
lp_2351	D-Methionine ABC transporter, permease protein (putative)	2.13
lp_2357	unknown	2.43
lp_2363	H(+)-transporting two-sector ATPase, epsilon subunit	2.12
lp_2364	H(+)-transporting two-sector ATPase, beta subunit	3.22
lp_2365	H(+)-transporting two-sector ATPase, gamma subunit	2.07
lp_2366	H(+)-transporting two-sector ATPase, alpha subunit	2.37
lp_2367	H(+)-transporting two-sector ATPase, delta subunit	2.60
lp_2368	H(+)-transporting two-sector ATPase, B subunit	2.36
lp_2370	H(+)-transporting two-sector ATPase, A subunit	2.56

lp_2402	prophage P2a protein 55	2.12
lp_2413	prophage P2a protein 44	5.34
lp_2418	prophage P2a protein 39	2.03
lp_2419	prophage P2a protein 38	2.14
lp_2423	prophage P2a protein 34	2.60
lp_2434	prophage P2a protein 23	2.41
lp_2436	prophage P2a protein 21	2.02
lp_2440	prophage P2a protein 17	2.94
lp_2441	prophage P2a protein 16	2.91
lp_2444	prophage P2a protein 13	3.25
lp_2445	prophage P2a protein 12	3.89
lp_2446	prophage P2a protein 11	2.60
lp_2464	prophage P2b protein 17, portal protein	2.66
lp_2485	unknown	2.34
lp_2559	histidinol dehydrogenase	2.51
lp_2629	pyruvate oxidase	4.14
lp_2631	lipase/esterase (putative)	2.43
lp_2666	unknown	3.81
lp_2716	glycosyltransferase (putative)	2.11
lp_2721	phosphoribosylglycinamide formyltransferase	3.01
lp_2722	phosphoribosylformylglycinamidine cyclo-ligase	3.54
lp_2743	ABC transporter, ATP-binding protein	2.06
lp_2753	unknown	2.04
lp_2793	unknown	2.74
lp_2797	unknown	2.08
lp_2824	integral membrane protein	3.43
lp_2872	oxidoreductase	2.64
lp_2878	maltose O-acetyltransferase	2.25
lp_3019	unknown	2.41
lp_3021	protein containing diguanylate cyclase/phosphodiesterase domain 1 (GGDEF)	2.49
lp_3033	short-chain dehydrogenase/oxidoreductase	2.37
lp_3072	cell surface protein precursor	2.54
lp_3075	cell surface protein (putative)	2.04
lp_3102	transcription regulator	3.52
lp_3107	transcription regulator (putative)	2.32
lp_3108	oxidoreductase	2.21
lp_3133	beta-glucosides PTS, EIIBCA	2.45
lp_3154	muramidase, C-terminal fragment	4.12
lp_3178	extracellular protein	2.45
lp_3204	pyrimidine nucleoside transport protein	2.24
lp_3209	polar amino acid ABC transporter, substrate binding protein	2.55
lp_3214	polar amino acid ABC transporter, substrate binding protein	3.38
lp_3217	integral membrane protein (putative)	2.19
lp_3227	unknown	2.30
lp_3310	integral membrane protein	2.09
lp_3350	unknown	3.32
lp_3358	transport protein	3.42
lp_3368	multidrug transport protein, N-terminal fragment	2.90
lp_3393	cell surface hydrolase, membrane-bound (putative)	2.53
lp_3421	extracellular protein, gamma-D-glutamate-meso-diaminopimelate muropeptidase (putative)	4.33
lp_3425	unknown	8.32
lp_3436	glycerol uptake facilitator protein	4.45
lp_3458	transcription regulator (putative)	2.39
lp_3482	galactokinase	2.04
lp_3483	beta-galactosidase, large subunit	4.55
lp_3540	transport protein	2.01
lp_3546	galacitol PTS, EIIC	3.23
lp_3551	phosphoketolase	2.62
lp_3556	L-ribulokinase (putative)	2.83
lp_3568	N-acetylneuraminate lyase	2.12
lp_3571	N-acetylmannosamine-6-phosphate 2-epimerase	4.56
lp_3593	L-rhamnose isomerase	2.12
lp_3642	sugar ABC transporter, substrate binding protein	5.11
lp_3643	sugar ABC transporter, permease protein	2.78
lp_3688	ribonuclease P	2.03

Complementary table 1B: Genes down regulated in the stationary aerobic phase compared to the stationary anaerobic phase. Ratio shown is the absolute ratio.

gene	product	ph4
lp_0059	arsenate reductase	0.47
lp_0063	unknown	0.40
lp_0073	unknown	0.18
lp_0096	unknown	0.27
lp_0111	oxidoreductase	0.47
lp_0137	oxidoreductase	0.47
lp_0138	unknown	0.41
lp_0141	extracellular protein	0.16
lp_0154	unknown	0.40
lp_0156	unknown	0.27
lp_0158	unknown	0.49
lp_0176	maltose/maltodextrin ABC transporter, permease protein	0.45
lp_0183	integral membrane protein	0.24
lp_0184	fructokinase	0.33
lp_0226	glucosamine-6-phosphate isomerase	0.40
lp_0228	dipeptidase	0.48
lp_0240	unknown	0.49
lp_0260	unknown	0.44
lp_0261	integral membrane protein	0.30
lp_0264	PTS system, trehalose-specific IIBC component	0.47
lp_0266	unknown	0.29
lp_0283	response regulator	0.44
lp_0286	cellobiose PTS, EIIC	0.39
lp_0310	acetate kinase	0.44
lp_0329	acetaldehyde dehydrogenase	0.16
lp_0355	cell division protein SufI	0.22
lp_0357	integral membrane protein	0.37
lp_0358	transcription regulator (putative)	0.41
lp_0376	integral membrane protein	0.49
lp_0387	unknown	0.30
lp_0409	immunity protein PlnM	0.48
lp_0415	plantaricin A precursor peptide, induction factor	0.22
lp_0418	response regulator PlnD, repressor	0.42
lp_0419	immunity protein PlnI, membrane-bound protease CAAX family	0.39
lp_0535	unknown	0.36
lp_0601	cysteine aminopeptidase	0.47
lp_0602	ribose 5-phosphate epimerase	0.49
lp_0631	prophage P1 protein 8	0.43
lp_0642	prophage P1 protein 19	0.20
lp_0653	prophage P1 protein 30	0.38
lp_0654	prophage P1 protein 31	0.45
lp_0655	prophage P1 protein 32	0.41
lp_0723	ABC transporter, ATP-binding protein	0.37
lp_0737	ribosomal protein S30EA	0.43
lp_0753	integral membrane protein	0.49
lp_0778	integral membrane protein	0.46
lp_0780	unknown	0.47
lp_0823	protein containing diguanylate cyclase/phosphodiesterase domain 2 (EAL)	0.49
lp_0840	transport protein	0.47
lp_0868	metal uptake regulator	0.27
lp_0874	bifunctional protein: amino acid aminotransferase; 2-hydroxyacid dehydrogenase	0.49
lp_0875	unknown	0.32
lp_0899	unknown	0.37
lp_0900	phosphoglycerate mutase	0.25
lp_0907	integral membrane protein of the dedA family	0.37
lp_0908		0.31
lp_0929	alkaline shock protein	0.48
lp_0946	cell surface protein precursor, GY family	0.16
lp_0951	transcription regulator	0.49
lp_0981	integral membrane protein	0.31
lp_0982	amino acid transport protein	0.31
lp_0995	unknown	0.34
lp_1020	transcription regulator (putative)	0.29
lp_1069	NADH dehydrogenase	0.41
lp_1070	lipoprotein precursor	0.29
lp_1101	L-lactate dehydrogenase	0.23
lp_1102	cation transport protein	0.30
lp_1105	malic enzyme, NAD-dependent	0.43
lp_1107	citrate lyase, acyl carrier protein	0.49
lp_1125	cytochrome D ubiquinol oxidase, subunit I	0.41
lp_1161	oxidoreductase	0.49
lp_1166	unknown	0.48
lp_1168	unknown	0.24
lp_1236	unknown	0.43
lp_1237	unknown	0.33
lp_1245	L-2-hydroxyisocaproate dehydrogenase	0.48

lp_1290	integral membrane protein	0.34
lp_1295	cation transport protein	0.39
lp_1299	poly(glycerol-phosphate) alpha-glucosyltransferase	0.29
lp_1346	aspartate-semialdehyde dehydrogenase	0.39
lp_1347	unknown	0.47
lp_1348	unknown	0.38
lp_1350	oxidoreductase	0.17
lp_1351	unknown	0.30
lp_1352	transcription regulator (putative)	0.43
lp_1362	unknown	0.21
lp_1363	unknown	0.33
lp_1364	excinuclease ABC, subunit A	0.46
lp_1375	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase	0.43
lp_1394	unknown	0.37
lp_1395	unknown	0.34
lp_1403	cell surface protein, ErfK family	0.46
lp_1419	unknown	0.22
lp_1450	extracellular protein	0.18
lp_1459	unknown	0.47
lp_1477	flavodoxin	0.32
lp_1480	molybdopterin precursor synthase MoaA	0.41
lp_1481	nitrite extrusion protein	0.25
lp_1487	response regulator	0.46
lp_1524	glycosyltransferase	0.49
lp_1544	response regulator	0.20
lp_1552	integral membrane protein	0.24
lp_1557	transcription regulator (putative)	0.48
lp_1574	unknown	0.45
lp_1584	unknown	0.17
lp_1590	integral membrane protein	0.40
lp_1598	transcription termination factor NusB	0.39
lp_1642	unknown	0.41
lp_1662	acetyltransferase (putative)	0.39
lp_1700	sensory protein	0.33
lp_1701	nucleotide-binding protein, universal stress protein UspA family	0.24
lp_1703	unknown	0.35
lp_1721	4-aminobutyrate aminotransferase	0.25
lp_1747	nucleotide-binding protein, universal stress protein UspA family	0.36
lp_1750	ABC transporter, ATP-binding protein	0.32
lp_1762	unknown	0.24
lp_1766	unknown	0.14
lp_1767	lysin	0.49
lp_1873	polynucleotide adenylyltransferase	0.41
lp_1901	unknown	0.18
lp_1923	succinyl-diaminopimelate desuccinylase	0.46
lp_1929	unknown	0.46
lp_1933	galactoside O-acetyltransferase	0.47
lp_1935	cell surface hydrolase (putative)	0.34
lp_2205	integral membrane protein	0.45
lp_2226	competence protein	0.49
lp_2272	unknown	0.32
lp_2349	L-2-hydroxyisocaproate dehydrogenase	0.35
lp_2384	mannose-6-phosphate isomerase	0.47
lp_2456	prophage P2a protein 1	0.42
lp_2483	unknown	0.35
lp_2499	ABC transporter, ATP-binding and permease protein	0.43
lp_2521	transcription regulator, AsnC-type	0.36
lp_2522	unknown	0.29
lp_2532	2-dehydropantoate 2-reductase	0.34
lp_2550	maltose O-acetyltransferase	0.42
lp_2551	histidinol-phosphate aminotransferase	0.35
lp_2560	ATP phosphoribosyltransferase	0.43
lp_2567	transcription regulator	0.35
lp_2568	short-chain dehydrogenase/oxidoreductase	0.47
lp_2586	cell surface hydrolase, membrane-bound (putative)	0.37
lp_2596	formate acetyltransferase activating enzyme	0.35
lp_2597	transcription regulator	0.41
lp_2598	formate C-acetyltransferase	0.36
lp_2600	transaldolase	0.36
lp_2614	ABC transporter, ATP-binding protein	0.42
lp_2625	acetyltransferase (putative)	0.45
lp_2635	integral membrane protein	0.39
lp_2651	transcription regulator	0.36
lp_2653	pseudouridylate synthase	0.43
lp_2668	small heat shock protein	0.34
lp_2669	unknown	0.36
lp_2674	Na(+)/H(+) antiporter	0.37
lp_2676	transcription regulator	0.48
lp_2683	aminotransferase with N-terminal regulator domain	0.27
lp_2690	unknown	0.38
lp_2694	ATP-dependent nuclease, subunit B	0.36
lp_2698	orotidine-5'-phosphate decarboxylase	0.40
lp_2701	carbamoyl-phosphate synthase, pyrimidine-specific, small	0.45

lp_2703	aspartate carbamoyltransferase	0.20
lp_2708	purine transport regulator	0.47
lp_2733	oxidoreductase	0.33
lp_2734	lipoate-protein ligase	0.49
lp_2745	nucleotide-binding protein, universal stress protein UspA family	0.16
lp_2747	teichoic acid glycosylation protein	0.33
lp_2755	integral membrane protein (putative)	0.41
lp_2758	threonine synthase	0.49
lp_2766	unknown	0.42
lp_2780	cellobiose PTS, EIIA	0.25
lp_2781	cellobiose PTS, EIIB	0.27
lp_2788	2-dehydropantoate 2-reductase	0.29
lp_2798	3-dehydroquinate dehydratase	0.48
lp_2799	amino acid transport protein	0.33
lp_2812	unknown	0.41
lp_2813	unknown	0.40
lp_2814	unknown	0.45
lp_2820	integral membrane protein	0.47
lp_2825	purine nucleosidase	0.30
lp_2833	transport protein	0.41
lp_2841	integral membrane protein	0.37
lp_2842	transcription regulator	0.15
lp_2857	ABC transporter, ATP-binding protein, C-terminal fragment	0.23
lp_2858	ABC transporter, ATP-binding protein, N-terminal fragment	0.23
lp_2863	signal peptidase I	0.49
lp_2877	nucleotide-binding protein, universal stress protein UspA family	0.25
lp_2879	hydrolase, HAD superfamily	0.28
lp_2882	cell growth regulatory protein MazF	0.43
lp_2896	transport protein, N-terminal fragment	0.15
lp_2897	transport protein, C-terminal fragment	0.44
lp_2901	integral membrane protein	0.46
lp_2902	transcription regulator	0.40
lp_2926	unknown	0.25
lp_2927	PTS, EIIA	0.46
lp_2939	unknown	0.20
lp_2945	3-octaprenyl-4-hydroxybenzoate carboxy-lyase	0.32
lp_2952	unknown	0.44
lp_2956	unknown	0.46
lp_2964	transcription regulator (putative)	0.41
lp_2965	sugar kinase and transcription regulator	0.27
lp_2967	transcription regulator	0.43
lp_2968	nitroreductase	0.48
lp_2975	extracellular protein	0.28
lp_2976	cell surface protein precursor (putative)	0.13
lp_2977	cell surface protein precursor	0.20
lp_2978	extracellular protein	0.04
lp_2989	teichoic acid glycosylation protein (putative)	0.41
lp_2991	transcription regulator	0.24
lp_2992	manganese transport protein	0.32
lp_2993	unknown	0.21
lp_2996	transcription regulator, C-terminal fragment	0.28
lp_2998	integral membrane protein	0.29
lp_3003	unknown	0.39
lp_3024	transcription regulator	0.48
lp_3048	unknown	0.49
lp_3051	1,3-propanediol dehydrogenase	0.32
lp_3057	unknown	0.24
lp_3065	cell surface protein precursor	0.32
lp_3077	extracellular protein (putative)	0.21
lp_3085	asparagine synthase (glutamine-hydrolysing)	0.40
lp_3092	succinate-semialdehyde dehydrogenase (NAD(P)+)	0.45
lp_3103	iron chelatin ABC transporter, substrate binding protein	0.18
lp_3165	transposase, fragment	0.40
lp_3172	xylose operon regulator	0.40
lp_3243	unknown	0.44
lp_3246	unknown	0.47
lp_3247	ferric uptake regulator	0.35
lp_3251	3',5'-cyclic-nucleotide phosphodiesterase	0.48
lp_3256	DegV family protein	0.49
lp_3270	adenylosuccinate synthase	0.32
lp_3271	GMP reductase	0.28
lp_3272	protein-tyrosine phosphatase	0.48
lp_3275	unknown	0.40
lp_3280	cation efflux protein	0.47
lp_3323	unknown	0.36
lp_3359	integral membrane protein	0.22
lp_3380	prophage P3 protein 10	0.24
lp_3402	transport protein	0.08
lp_3416	transcription regulator (putative)	0.14
lp_3453	extracellular protein	0.49
lp_3454	cell surface protein (putative	0.48
lp_3466	branched-chain amino acid transport protein	0.40
lp_3469	beta-galactosidase I	0.25
lp_3471	alpha-L-rhamnosidase (putative)	0.36

lp_3473	alpha-L-rhamnosidase (putative)	0.37
lp_3495	transcription regulator	0.36
lp_3497	transport protein	0.45
lp_3499	shikimate 5-dehydrogenase	0.49
lp_3502	transcription regulator	0.42
lp_3503	transport protein	0.30
lp_3507	cellobiose PTS, EIIC	0.33
lp_3519	PTS, EIIA	0.16
lp_3520	glucose PTS, EIIA	0.43
lp_3524	unknown	0.20
lp_3525	6-phospho-beta-glucosidase	0.42
lp_3526	6-phospho-beta-glucosidase	0.46
lp_3530	maltose phosphorylase	0.33
lp_3548	galacitol PTS, EIIA	0.45
lp_3552	unknown	0.41
lp_3577	integral membrane protein	0.31
lp_3597	transcription regulator	0.47
lp_3622	sorbitol operon transcription regulator	0.43
lp_3629	beta-glucosidase	0.48
lp_3655	sorbitol operon activator	0.33
lp_3658	ribose transport protein	0.32
lp_3662	bifunctional protein: alcohol dehydrogenase; acetaldehyde dehydrogenase	0.46
lp_3673	cysteine aminopeptidase	0.44
lp_3675	signal peptidase I	0.44
lp_3676	extracellular protein	0.11
lp_3677	cell surface protein precursor	0.30
lp_3678	cell surface protein precursor	0.07
lp_3679	extracellular protein	0.10

Chapter 5

Comparative Transcriptome and Proteome Analyses of the Growth Phase dependent CcpA-regulon in *Lactobacillus plantarum* WCFS1

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ABSTRACT

CcpA, the major regulator of carbon catabolite repression in low GC Gram-positive bacteria, regulates gene expression by binding to so-called *cre* elements, commonly located in the proximity of promoters. We describe the genetic and physiological characterization of the CcpA-regulon in *Lactobacillus plantarum*. A *Δccpa* derivative (NZ7304), growing on glucose containing media displayed reduced growth and acidification rates, but reached higher biomass yields when compared with the wild type strain. Furthermore, composition of fermentation end-products shifted from homo lactate fermentation in the wild type to a mixed fermentation in NZ7304. Whole genome transcriptome analyses were performed throughout the growth curves and revealed main CcpA activity in the early- and mid-log phase, regulating the expression of approximately 250 genes. The transcriptome analyses followed the same trend as observed in a regulon prediction based on putative *cre*-sites: massive regulation in genes of the transporters, energy metabolism, and regulatory functions classes. The availability of transcriptome as well as proteome data sets of the same samples allowed us to compare the results of both omics approaches. These comparative analyses revealed that while 80% of the proteins identified displayed the same direction of regulation as the corresponding transcript, only approximately 30% appeared to be affected to a quantitatively similar extend at the level of transcript and protein abundance. In conclusion, this study showed that combined transcriptome and proteome analyses provide a powerful approach to study novel (post-translational) regulatory networks in the cell.

Introduction

In contrast to eukaryotic cells, were fundamental biological differences between transcription and translation (e.g. splicing) cause complication to predict cell-proteomes from transcriptome data (19), bacterial transcription and translation are more directly coupled , suggesting a more straightforward translation between these two levels of function regulation (reviewed in (17)). However, studies in which transcriptomic data are confirmed by proteomic analyses are rare, due to biological processes (e.g. differential protein and mRNA turnover) and technical limitations (19). In a study to identify glucose repressed genes in *B. subtilis* transcriptomic data were not confirmed by proteomic data as the number of identified proteins was too low (54). Another study revealed low correlation between the data sets due to differential mRNA and extracellular protein stability (29). However, a study in the Gram-positive bacteria *Staphylococcus aureus*, gene expression and protein analyses data were found to be similar if evaluated on functional classes, but low in similarity on single gene level, the aim of a combined study (40). Despite the difficulties to correlate transcriptomic and proteomic data, integration of these data is highly desirable as it leads to a more comprehensive and complete view of cellular processes, host-microbe interaction, and genotype and phenotype correlation.

Carbon catabolite repression (CCR), the phenomenon that the presence of a favorable sugar represses the uptake and utilization of less-favorable sugars, has been studied in a variety of micro-organisms since many years. The carbon catabolite protein A (CcpA) is the canonical regulator of CCR in Gram-positive bacteria with low G+C-content, playing a key-role in a mechanism that differs fundamentally from that in Gram-negative bacteria (20). CcpA is a DNA-binding protein belonging to the LacI/GalR-family of transcriptional regulators (52) and binds to a catabolite response element (*cre*) located in the proximity of promoters, thereby blocking or enhancing transcription of downstream genes and/or operons (53). The nucleotide sequence of c*re*-sites has been characterized in a range of bacteria, including *L. plantarum* (30), and is conserved, allowing a prediction of *cre*-sites present in a genome sequence (32). CcpA mediated carbon catabolite repression, or preferably carbon catabolite control, in *Bacillus subtilis* and *Lactococcus lactis* involves regulation of genes from a range of functional classes, confirming a global regulatory role for CcpA (33, 55). In addition, recent studies in *B.subtilis* and *L. lactis* revealed growth-phase dependent variation of CcpA-mediated gene expression control (28, 55)

CcpA is activated by the histidine containing protein HPr, a phosphate carrier protein which belongs to the phosphotransferase system (PTS) for sugar uptake. HPr is phopshorylated at residue His15 by the generic enzyme-I of the PTS-phospho-cascade, which in its turn is phosphorylated at the expense of the glycolytic intermediate phosphoenolpyruvate (PEP). HPr-His-P transfers its phosphate to the sugar specific PTS-enzyme IIA enzyme that energizes carbohydrate-transport via the PTS-IIC transporter. In addition, a HPr-kinase/phosphatase activated phosphorylation of HPr at residue Ser46 leads to CcpA activation and thereby regulation of gene expression (for a review see (48)).

Here we describe the *in silico* prediction of the CcpA-regulon in *Lactobacillus plantarum* WCFS1 by detection of putative *cre* sites in the genome. Experimental validation of this prediction included comparative analysis of the wild-type strain and its *ccpA*-mutant derivative, using physiological, transcriptomic and proteomic approaches, performed in different growth phases. The *ccpA*-mutant display slower growth as compared to the wild-type strain in early growth phases, while it appears to grow faster at later stages of growth. Moreover, the mutant displayed a mixed acid (acetate and lactate) fermentation pattern while the wild-type is essentially homolactic under the conditions employed here. Transcriptome analyses exemplified the dynamics of *ccpA* mediated gene expression control at various growth phases and confirmed its central role in catabolite control in *Lactobacilus plantarum* WCFS1. The availability of proteome reference maps of *L. plantarum* WCFS1 (7) allowed us to compare transcriptome data with proteomic analysis of the same samples. Parallel regulation at the transcription and protein level could be confirmed for approximately 30% of the proteins identified on a 2D-gel, whereas approximately 25% of the identified proteins displayed discrepancy between proteome and transcriptome data. This study illustrates the strength of combined proteomic-transcriptomic approaches to elucidate differential regulation in bacteria.

Materials and Methods

Bacterial strain and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strain DH5α was used as a cloning host and grown aerobically in TY-medium (38). When appropriate, ampicillin (50 μg/ml) was added to the medium. *Lactobacillus plantarum* WCFS1 wild type and its *ccpA* derivative NZ7304 (*ΔccpA*) were grown anaerobically in

duplicate in nitrogen flushed (0.1 volume per minute) MRS broth (prepared without Tween 80) (8) in a non pH-controlled batch culture with stirring at 125 rpm. Glucose was added to a final concentration of 2% (w/v) and the initial pH of the medium was set at 6.5. Growth took place in a Biocontroller ADI 1030 (Applikon BV, Schiedam, the Netherlands). Cell growth was monitored by measuring the optical density at 600 nm in a spectrophotometer (Ultrospec 3000, Pharmacia Biotech BV, Roosendaal, the Netherlands). Two independent biological duplicates were used for all data presented here and mean values were plotted in the curves.

Table 1: Bacterial strains and plasmids used in this study.

Material	***Relevant properties***	***Reference***
Strain		
Escherichia coli DH5α	Cloning host	
Lactobacillus plantarum WCFS1	Wild type: single colony isolate from human saliva isolate NCIMB8826	(23)
Lactobacillus plantarum NZ7304	CcpA::Ery, WCFS1 derivative	This work
Plasmids		
pUC18-Ery	Mutagenesis vector: Amp^R, Em^R, 3.8 kb derivative of pUC19 containing 1.1 kb HinPI fragment of pIL253 carrying the EryR gene	(49)
pNZ7305	Amp^R, Em^R, pUC18-Ery derivative containing 1.1 kb 5'-flanking region of *ccpA*.	This work
pNZ7304	Amp^R, Em^R, pUC18-Ery *ccpA::ery* replacement derivative containing pNZ7305 and pNZ7306 derived 5'- and 3'-flanking regions of *ccpA*.	This work

DNA manipulations

Molecular cloning and DNA manipulations were essentially performed as described by Sambrook et. al. (38). Restriction enzymes and the proof reading enzyme *Pwo* DNA polymerase were obtained from Promega (Leiden, The Netherlands). T4-ligase was obtained from Boehringer GmbH (Mannheim, Germany). Large scale plasmid DNA isolations were performed using a Jet Star Maxiprep Kit (Genomed GmbH, Bad Oberhausen, Germany). Primers were purchased from Proligo France SAS (Paris, France). *L. plantarum c*hromosomal DNA was isolated using a cell-lyses method followed by protease K-treatment and phenol-chloroform extraction, as described previously (22).

Cloning strategy and gene disruption

For construction of the *ccpA* gene deletion mutagenesis vector pNZ7304 the upstream region of the *ccpA*-gene was amplified using the primers 5'-CCGGAATTCGCCTTCCTTAGTAA CGACCCC-3' and 5'-CGCGGATCCGGACCCAAGACAATCACGTTGACG-3', using chromosomal DNA of WCFS1 as template. The resulting 1.1 kb fragment was digested with *EcoRI* and *BamHI* (restriction site introduced by the primers; underlined) and cloned upstream of an erythromycin resistant gene in a similar digested pUC18 derivative (49), resulting in pNZ7305. Subsequently, a 1.1 kb downstream region of the *ccpA*-gene was amplified using the set of primers: 5'-AAACTGCAGCGGTTGTCTGCCAGCTAGTGACG-3', containing a *Pst*1 restriction site (underlined), and 5'- CCCAAGCTTGGCCG TTGCGACCTTAGCCGGC-3', containing a *Hind*III restriction site (underlined). The resulting fragment was digested with the respective restriction enzymes and cloned downstream of the erythromycin resistant gene in the similar digested pNZ7305. The resulting plasmid, designated pNZ7304, contains the 5'- and 3'-flanking regions of *ccpA* separated by a erythromycin resistance gene and can be used for double cross over gene replacement mutagenesis in *L. plantarum* (11). The resulting plasmid was transformed into *L. plantarum* by electroporation as described previously (11) and primary plasmid-integrants were selected on MRS-plates with 5 µg/ml erythromycin at 37° C. Integrants were grown in MRS supplemented with erythromycin (5 µg/ml) for 40 generations to force a second homologous recombination event leading to excision of the plasmid and to *ccpA*::*ery*. The anticipated mutagenesis-plasmid integration upstream of the *ccp*-gene was checked by PCR using a universal-primer annealing in the erythromycin resistance gene (5'-CACGAAC CGTCTTATCTCCC-3') and a site-specific primer annealing upstream of the chromosomal region used for homologous recombination (5'-CGGATTATGTCGTGACGGCC-3'). Integration downstream of the *ccpA*-gene was checked using the primer 5'-ACGAAC CGTCTTATCTCCC-3' in the erythromycin resistance gene and the primer 5'-GCGGTAA CGTCTGTTGTAATGGC-3' downstream of the chromosomal region used for homologous recombination. The gene deletion strain NZ7304 was additionally check for its β-glucosidase activity on glucose, indicating correct *ccpA*-deletion, by determining its capacity to produce saligenin out of salicin ad described previously (35).

Sampling

To minimize biological error introduction due to sampling, cells were quenched in a -40º C 60% methanol-HEPES buffer as described by Pieterse (37). After sampling, cells were harvested by centrifugation (13182 * g, -20º C, Sorvall RC5B plus centrifuge; Sorvall,

Newton, US) and cell-pellets were transferred immediately with a pre-cooled spatula to a screw-crap tube containing 500 mg zirconium beads, 500µl Phenol/Chloroform-mix 1:1, 30 µl 3 M Na-Acetate (pH 5.2), 30 µl 10% SDS, and 400 µl MRS-medium (Merck, Darmstadt, Germany), carefully avoiding the cells to thaw. The tubes containing the cells were shaken vigorously, frozen in liquid nitrogen and stored at -80º C.

Samples for HPLC-analysis were taken from the culture, cells were harvested in an Eppendorf table-top centrifuge (20800 * g; Eppendorf 5417C, Hamburg, Germany) and culture supernatants were transferred to a new tube and stored at -20ºC until further analysis. For protein analyses, samples were taken from the culture and cells were harvested at 3360 x g for 5 minutes at 37°C. The supernatant was discarded and the cells were stored at -20 °C.

RNA extraction and quality control

RNA was isolated as described previously (45), using a phenol-chloroform extraction followed by a purification using the High Pure RNA isolation kit (Roche, Manheim, Germany). The yield and purity of the RNA were determined by measurement of the absorption at 260 nm and 280 nm (Ultrospec 3000, Pharmacia Biotech, Roosendaal, The Netherlands). The RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent technologies, Palo Alto, Ca, USA) following the manufacturer's instructions. Only RNA samples displaying 16S/23S-rRNA ratios of 1.6 or higher, were labeled and used for micro array experiments.

cDNA-synthesis, labeling, and hybridization

The Cyscribe Post-labeling kit (Amersham Biosciences, Amersham, UK) was used to synthesize and to label cDNA. Labeled cDNAs were hybridized as described previously on amplicon based microarrays containing fragment of approximately 97% of the genes of *L. plantarum* WCFS1 (45).

Scanning, Data extraction, and analyses

The slides were scanned with a Scan Array Express 4000 scanner (Perkin Elmer, Wellesley, USA) and the images were analyzed with Imagene software 4.2 (BioDiscovery, El Segundo, USA). The overall mutation effect was determined by taken the genes with a significant differential expression (p-value< 0.05) throughout whole growth. Differential expression was determined using R (http://www.r-project.org/) and the linear models for microarray data library (Limma), which includes a LOESS fit of the data (6) and allows comparisons between many RNA targets simultaneously in arbitrary complicated designed experiments (43).

Significantly differential expressed genes in the phases were determined as follows: Background corrected spot intensities in both channels (I1 and I2) were converted to M-A

coordinates, where M=log2 (I1/I2) and A=log2 (I1/I2)/2 and subsequently normalized using a LOESS fit (6), assuming that, on average, M is independent of A and centered around a value of zero (43). Ratios of the normalized intensities were used for further analysis with a cut-off value of an absolute ^{2}log value greater than 1 (which equals two times differential expression). Experiments were performed in duplicate and genes displaying consistent, reproducible direction and approximate amplitude of regulation were taken into account.

Bio-informatics methods

To identify putative *cre*-sites the algorithm for fitting a mixture model by expectation maximization (MEME) (1) was used on 22 experimentally verified *cre*-site promoters from *B. subtilis* (32). MEME-parameters were set as follows: one motif per sequence should be found (OOPS) and only the given strand should be searched. The MEME-based motif was used to search the complete genome of *L. plantarum* WCFS1, using the motive alignment and search tool (MAST) (2). MAST parameters were a set at a cut-off E-value of $1.00x10^{-6}$, only motifs lying between the start codon and 300 base pairs upstream of the start codon were selected, and overlap with other genes was allowed.

Classification of genes

L. plantarum WCFS1 genes were classified based on the classification in 16 main classes proposed by Kleerebezem (23) using the latest updated version of the *Lactobacillus plantarum* WCFS1 database (https://bamics3.cmbi.kun.nl/plantdb).

Determination of organic compounds in the supernatant

Lactate, acetate, ethanol, and acetoin concentrations were determined using a high-performance liquid chromatography (HPLC) as described previously, with an HPX-87P anion exchange column (Bio-Rad, Inc.) using 0.01 M H_2SO_4 as elution fluid (44).

Preparation of soluble protein fraction

Pelleted cells were washed with PBS and double distilled water (ddH_2O), respectively, and centrifuged at 5000 x *g* for 10 min. The supernatant was discarded and the cells were resuspended in buffer containing 8 M urea (Bio-Rad, Hercules, CA, USA), 2% w/v CHAPS (Sigma, St Louis, MO, USA) 65 mM DTT (Sigma), 0.5% v/v IPG buffer (pH 3-10 NL; Amersham Pharmacia Biotech, Uppsala, Sweden). The bacteria were lysed mechanically by beating with zirconium beads (diameter 0.8 mm) using a mini bead-beater (Biospec Products, Bartlesville, OK, USA) for 5 x 1 min with 1 min intervals on ice. The cell debris was removed by centrifugation at 9000 x *g* and the supernatant was collected and centrifuged at 20,000 x *g* for 30 min at 4°C. The supernatant was collected and stored at -80°C until further use.

Prior to gel electrophoresis, the protein concentration was determined using a Bradford-based DC-protein assay (Bio-Rad), using BSA as the standard. SDS-PAGE was used to visualize the cytosolic proteins and also to confirm protein quality of all protein extracts (32). Briefly, 20 μg of protein was loaded on a 12.5% SDS-PAGE gel that was run constantly at 110 V until the bromophenol blue front was run off. Afterwards, the gels were stained with silver nitrate according to Shevchenko *et al.* (41). Gel images were obtained using a GS-800 calibrated densitometer (Bio-Rad).

Iso-electric focusing and two-dimensional electrophoresis

L. plantarum cytosolic proteins were first separated by IEF. Proteins (60 μg) were loaded on Immobiline Dry Strips (pH range 3-10, non-linear, 24 cm long; Amersham). IEF was performed on an IPGphor electrophoresis unit (Amersham), operating at a constant temperature of 20°C. The strips, containing the samples, were first actively rehydrated at 30 volts for 12 h. The IEF program was as follows: 250 V for 1h, 500 V for 1 h, 1000 V for 1 h, 1,000-8,000 V for 2 h and 8,000 V for 52,000 Vh. Prior to running the second dimension, the strips were equilibrated for 15 min in 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 1% DTT and for 15 min in 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 2.5% iodoacetamide. The strips were placed on 12.5% SDS-PAGE gels and covered with 0.5% agarose containing a trace of bromophenol blue. The gels were run at a constant voltage of 200 V in a Dodeca Cell system (Bio-Rad) until the marker dye had reached the base of the gel. The gels were stained with Ag-nitrate as described above. Images were obtained with a GS-800 calibrated densitometer (Bio-Rad).

Protein identification

Image analysis was performed using the PDQuest software (version 7.2) (Bio-Rad). Three gels were produced for every growth condition and were used for comparison of the obtained spots. Proteins were considered differentially produced when spot intensities passed the threshold of at least a 2-fold difference in up- or down-regulation in combination with a student's t-test using a statistical 95% reliability score. Proteins were identified using a proteome reference map (PRM) of the wild type *L. plantarum* WCFS1 (7). Using the PDQuest software, protein spots from the proteome reference map were matched with the protein gels containing protein fraction from the wild type strain and ΔCcpA-strain used in this study.

Results

Characterization of a *ccpa*-mutant strain NZ7304

To elucidate CcpA-function in *L. plantarum* WCFS1, a *ccpA*-deficient strain was constructed using a double-cross-over gene replacement strategy. The strain, designated NZ7304, was grown in rich medium (MRS) supplemented with excess glucose, conditions under which the wild type strain displayed a characteristic bi-phasic growth (46). Growth and acidification rates in the wild type were high during early stages of batch-growth (doubling time 50 minutes), and decreased during later growth phases, reaching a final OD_{600} of approximately 4.5 and a final pH of 4.0 (Fig. 1). In contrast, NZ7304 (Δc*cpA*) reached a higher final density (OD_{600} = 8.0), and did not display the characteristic biphasic growth observed for the wild type strain (Fig. 1). The relative growth rate of NZ7304 was lower during the early growth phases (D of 80 minutes), but continued at an approximately constant rate until entering the stationary phase (Fig. 1). Additionally, NZ7304 displayed reduced acidification rates as compared to the wild-type leading to higher pH values despite higher cell-densities (exemplified by the measurements after 7.5 hours of growth; Fig. 1), indicating an altered ratio between cell density and acidification as a consequence of the *ccpA* deletion.

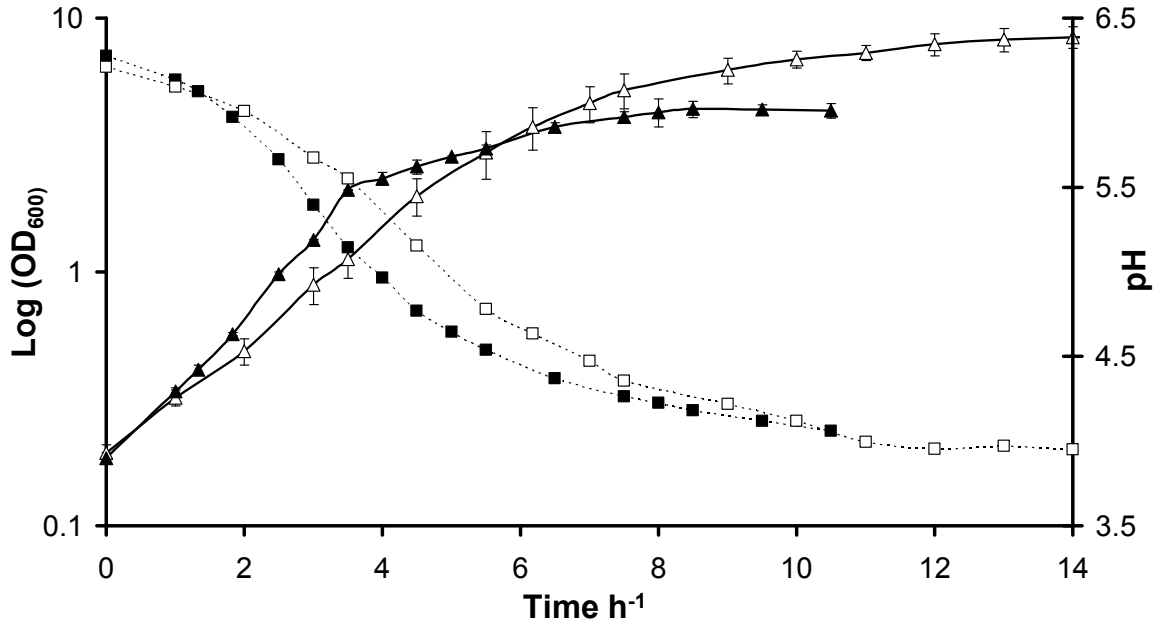

Figure 1: Anaerobic growth curves (triangles, left Y-axis) and acidification (squares, right Y-axis) of the wild type strain WCFS1 (closed symbols) and the ccpA-mutant strain NZ7304 (open symbols).

Under the conditions employed here, *L. plantarum* wild-type cultures have been shown to display a virtually homolactic fermentation pattern, producing D- and L-lactate in approximately equimolar amounts (11), so the reduced acidification rate observed for the *ccpA* mutant suggests altered fermentation characteristics in this strain. Therefore, concentrations of different end-products in culture supernatants of *L. plantarum* wild-type and its *ccpA* mutant were determined. The lactate concentration that was ultimately reached in the stationary phase was similar in both strains (Table 2). However, the clearly higher cell densities reached by the *ccpA*-mutant lead to a drastically changed ratio between biomass and lactate production as compared to the wild type. Accurate measurement of acetate formed during growth is hampered by the presence of considerable amounts of acetate in the medium used (60 mM), which could inhibit the formation of this metabolite as a fermentation end-product (25). Nevertheless, acetate formation of 2 mmol was observed during the stationary phase for the wild-type strain (Table 2). In contrast, acetate production reached up to 4.6 mmol in the culture media of NZ7304, where this metabolite appeared to be produced both during logarithmic and stationary phases of growth (Table 2). A similar, acetate producing phenotype has previously also been observed for wild-type *L. plantarum* cultures when grown under aerobic conditions (16, 25, 46).

Taken together, the *ccpA*-deletion in *L. plantarum* WCFS1 resulted in altered growth characteristics and fermentation end-products. The *ccpA* mutant reached higher cell densities in batch cultures, produced reduced amounts of lactate per cell, and produced acetate already in the early growth curve.

Table 2: Analyses of end-fermentation products in the four growth phases of the wild type and the ccpA-mutant. Values are given in mM. n. d. means not detected.

	Early log.	*Mid log.*	*Transition*	*Stationary*
WCFS1				
Lactate	2.2 ± 0.9	11.3 ± 0.6	57.4 ± 1.2	109.8 ± 5.6
Acetate	n.d	n.d	n.d	2.0 ± 1.2
NZ7304				
Lactate	2.2 ± 0.9	14.1 ± 0.1	49.3 ± 0.8	139.7 ± 4.3
Acetate	0.3 ± 0.1	0.7 ± 0.2	0.6 ± 0.1	4.6 ± 0.1

Identification of *cre*-sites in *Lactobacillus plantarum* WCFS1

CcpA is a renowned global regulator and exerts its regulation by binding to a conserved 14 bp nucleotide sequence (*cre*-site) (53). We identified putative *cre*-sites in the genome of *L. plantarum* WCFS1, using a motif based on 22 experimentally verified *cre*-sites from *B. subtilis* (32). Using the MEME and MAST-algorithms (1, 2), a total of 500 putative *cre*-sites were identified within 300 base pairs upstream of the startcodon of predicted protein encoding genes in the *L. plantarum* genome. Genes belonging to all main functional classes are represented in the *ccpA*-regulon prediction (Table 3) but a relative enrichment of *cre*-sites was found in the upstream regions of genes predicted to encode transporters (16%), regulatory functions (11%), and enzymes involved in energy metabolism (9%) (Table 3). A refined subclasses analysis of these three main functional categories revealed high prevalence of *cre*-sites upstream of genes encoding sugar transporting phosphotransferase systems (PTS) (27%) and transporters of carbohydrates, organic alcohols and acids (18%), which is in agreement with the assumed catabolite control function of CcpA. Relative enrichment in the regulatory class was found for the LacI-family (22%, including the *ccpA* gene itself, the GntR-family (15%), and the AraC-family (7%), regulators families involved in general metabolism (GntR) (18), sugar degradation (AraC)(13), and carbon catabolite control (LacI)(20). The high number of *cre*-sites in the class of LacI-regulators was anticipated, since most LacI-regulators appear to be autoregulatory and recognize sequence motifs that are highly similar to the *cre* site (12). In the class of energy metabolism preferences for genes in involved in fermentation capacities (20%, all encoding pyruvate dissipating enzymes), and electron transport (11%) was found.

This *ccpA* regulon prediction confirms the suggested global role for CcpA in regulation of carbohydrate utilization by *Lactobacillus plantarum* WCFS1 with emphasis at the levels of transport, energy metabolism, and specific regulatory functions, which clearly parallels the CcpA-regulons reported for *B. subtilis* and *L. lactis* (33, 55).

Table 3: Analysis of the prediction of catabolic response elements (cre) in the genome sequence of Lactobacillus plantarum WCFS1. Columns represent the total number of genes in the functional category (N-Genome) and the number of predicted genes in the functional category (N-prediction).

Main Classes	*N-Genome*	*N-prediction*
Regulatory functions	267 (9%)	55 (11%)
Transport and binding proteins	417 (14%)	79 (16%)
Protein fate	57 (2%)	19 (4%)
Energy metabolism	239 (8%)	46 (9%)
Biosynthesis of cofactors, prosthetic groups, and carriers	72 (2%)	17 (3%)
Central intermediary metabolism	59 (2%)	13 (3%)
Purines, pyrimidines, nucleosides and nucleotides	89 (3%)	17 (4%)
Fatty acid and phospholipids metabolism	61 (2%)	10 (2%)
Cell envelope	234 (8%)	38 (8%)
sugar metabolism	2 (0.1%)	0 (0%)
DNA metabolism	82 (3%)	13 (3%)
Transcription	28 (1%)	3 (1%)
Cellular processes	120 (4%)	18 (4%)
Amino acid biosynthesis	82 (3%)	8 (2%)
Protein synthesis	144 (5%)	16 (3%)
Hypothetical proteins	906 (30%)	137 (27%)
Other categories	206 (7%)	11 (2%)
Total	3065 (100%)	500

Growth phase specific transcriptome profiling

Recent studies in *L. lactis* and *B. subtilis* revealed differences in CcpA mediated control of gene expression in different phases of growth (28, 55). To investigate growth phase dependent, *ccpA*-mediated regulation of gene expression in *L. plantarum*, full genome transcriptomes at various time points during batch fermentation were determined using an amplicon-based micro array. The analyses showed that 34 genes were expressed at a higher level in the mutant (NZ7304), indicating CcpA-mediated repression, while only 4 genes appeared to be subjected to consistent CcpA activation as could be concluded from their higher level of expression in the wild type strain (Table 4). Among these was the *ccpA*-gene itself, confirming once more the correct deletion of the gene.

The observed acetate producing phenotype of the mutant was reflected by the higher expression of genes putatively involved in acetate production in *L. plantarum.* Pyruvate oxidase (Pox) converts pyruvate to hydrogen peroxide, carbon dioxide, and acetyl-P and the latter compound can be further converted to acetate. The *pox1*-gene (*poxD*-gene in strain

Lp80 (15)) harbors a putative *cre*-site was higher expressed in the mutant throughout the whole growth curve, whereas expression of the other *pox*-genes was apparently not affected by *ccpA*-deletion. Additionally, the pyruvate dehydrogenase operon, which encodes the enzymatic function of pyruvate conversion to acetyl-CoA and carbon dioxide, was higher expressed in the mutant. Subsequently, acetyl-CoA can be converted to acetate but the genes encoding for these to activities were not higher expressed in the mutant.

Acetate production under anaerobic conditions is possible by the formate acetyltransferase pathway (24). The formate acetyltransferase (*pflB*) gene is absent on the array employed and no data of its expression were available. Nevertheless, it seems likely that *pflB* is subject to CcpA mediated repression, as it is preceded by a predicted *cre*-site and its activator positioned downstream *pflB* was shown to be repressed by CcpA (Table 4).

Differences in gene expression were more pronounced in pair wise comparisons between individual time points (Table 5). In the early and mid logarithmic phases of growth transcription profiles were comparable. Approximately 45 and 200 genes are expressed at a higher and lower level, respectively, in the wild type strain, indicating a more prominent role of CcpA in repression as compared to activation of gene expression. The most predominant regulation was found in the functional categories of energy metabolism, regulatory functions, and transporters.

Table 4A: Genes expressed at a lower level in the wild-type strain WCFS1 compared to its ccpA-mutant derivative NZ7304 at all growth phases.

ORF	*name*	*Product*	*p-value*
Biosynthesis of cofactors, prosthetic groups, and carriers			
lp_0115	*thiE*	thiamine-phosphate pyrophosphorylase	0.040
Cell envelope			
lp_1763	*lp_1763*	Glycosyltransferase	0.023
Central intermediary metabolism			
lp_1730	*map3*	maltose phosphorylase	0.001
lp_3530	*map4*	maltose phosphorylase	0.008
Energy metabolism			
lp_0849	*pox1*	pyruvate oxidase	0.002
lp_0874	*lp_0874*	bifunctional protein: amino acid aminotransferase; 2-hydroxyacid dehydrogenase	0.008
lp_1250	*gntK*	Gluconokinase	0.046
lp_1731	*galM2*	aldose 1-epimerase	0.001
lp_2152	*pdhC*	pyruvate dehydrogenase complex, E2 component; dihydrolipoamide S-acetyltransferase	0.001
lp_2153	*pdhB*	pyruvate dehydrogenase complex, E1 component, beta subunit	0.001
lp_2154	*pdhA*	pyruvate dehydrogenase complex, E1 component, alpha subunit	0.001
lp_3314	*pflA2*	formate acetyltransferase activating enzyme	0.001
lp_3487	*galM3*	aldose 1-epimerase	0.001

ORF	*name*	*Product*	*p-value*
lp_3526	*pbg10*	6-phospho-beta-glucosidase	0.001
lp_3551	*xpk2*	Phosphoketolase	0.001
lp_3555	*araD*	L-ribulose 5-phosphate 4-epimerase	0.034
lp_3607	*iolE*	inositol catabolism protein IolE	0.004
Fatty acid and phospholipid metabolism			
lp_0067	*bsh2*	choloylglycine hydrolase	0.002
lp_0370	*glpK1*	glycerol kinase	0.001
Hypothetical proteins			
lp_0098	*lp_0098*	adenylyl transferase (putative)	0.011
lp_1518	*lp_1518*	Unknown	0.003
lp_1566	*lp_1566*	Unknown	0.023
lp_2787	*lp_2787*	hydrolase, HAD superfamily, Cof family	0.004
lp_2993	*lp_2993*	Unknown	0.002
lp_3100	*lp_3100*	Oxidoreductase	0.032
lp_3552	*lp_3552*	Unknown	0.040
Regulatory functions			
lp_0172	*lp_0172*	transcription regulator	0.001
lp_3234	*lp_3234*	transcription regulator	0.032
lp_3523	*lp_3523*	glucokinase regulatory protein	0.049
Transport and binding proteins			
lp_0265	*pts5ABC*	PTS system, trehalose-specific IIBC component	0.026
lp_0286	*pts6C*	cellobiose PTS, EIIC	0.004
lp_2780	*pts20A*	cellobiose PTS, EIIA	0.001
lp_3635	*msmK2*	multiple sugar ABC transporter, ATP-binding protein	0.001

Table 4B: Genes up regulated in the WCFS1 compared to the ccpA-mutant

ORF	name	*Product*	*p-value*
Hypothetical proteins			
lp_0111	lp_0111	Oxidoreductase	0.049
lp_0927	lp_0927	Unknown	0.022
Regulatory functions			
lp_2256	ccpA	catabolite control protein A	0.001
lp_3579	spx5	regulatory protein Spx	0.001

In the mutant 208 and 246 genes were expressed at a higher level in early and mid-log respectively (Table 5) of which 61 shared. Among these genes, putatively repressed as a consequence of the *ccpA* mutation, a strong enrichment is observed for genes encoding sugars transporters (40 genes) and other functions related to sugar metabolism. Combined, these functional classes present a coherent response that includes transport and degradation functions for sugars, the pentose phosphate pathway, the tricarboxylic acid cycle (TCA-cycle), and pyruvate metabolism. In the other classes only a few genes were regulated, including two putative bile salt hydrolases (*bsh2, bsh4*) and some cell envelope proteins with unidentified function.

In *L. lactis* activation of the glycolytic genes phosphofructokinase (PFK), pyruvate kinase (PYK) and the pyruvate dissipating enzyme L-lactate dehydrogenase (L-LDH) by CcpA has been described (27). A refined look at the regulation during exponential growth of genes encoding these functions in *L. plantarum* revealed higher expression of one, *cre*-sequence harboring, L-lactate/malate dehydrogenase (lp_1101) in the mutant and no differential expression of other LDHs (*ldhL1, ldhD*), probably the only functional two LDHs in *L. plantarum* (11). Only slightly higher expression (1.8 fold) was observed in the wild type for the putative operon containing the *pfk*-gene and the *pyk*-gene (data not shown).

Table 5: Differential expressed genes categorized in functional classes in the wild type (up) compared to the mutant (down) strain in the four phases.

Class	*Phase 1*		*Phase 2*		*Phase 3*		*Phase 4*	
	up	down	up	down	up	down	up	down
Amino acid biosynthesis	2	1	0	1	5	2	5	2
Biosynthesis of cofactors, prosthetic groups, and carriers	0	2	1	2	1	2	1	8
Cell envelope	4	7	3	7	12	3	21	17
Cellular processes	4	3	4	3	3	4	11	3
Central intermediary metabolism	1	14	0	11	1	9	1	11
DNA metabolism	2	1	1	0	1	0	6	2
Energy metabolism	1	51	2	60	5	40	11	42
Fatty acid and phospholipid metabolism	0	5	0	7	0	5	5	2
Hypothetical proteins	9	45	13	53	31	30	71	53
Other categories	2	1	1	2	2	15	4	8
Protein fate	0	2	0	3	3	1	8	2
Protein synthesis	0	1	0	3	1	24	11	5
Purines, pyrimidines, nucleosides and nucleotides	1	2	2	5	7	6	8	4
Regulatory functions	8	21	8	21	12	7	17	16
sugar metabolism	0	0	0	0	0	0	1	0
Transcription	0	0	0	0	0	1	0	1
Transport and binding proteins	11	52	10	68	15	34	16	46
Total	**45**	**208**	**45**	**246**	**99**	**183**	**197**	**222**
CRE sites	4	76	6	91	11	42	32	45

During the later growth phases approximately 200 genes are expressed at a lower level in the wild type compared to NZ7304. Among these genes the functional class related to cell envelope functions appeared to be represented prominently as compared to other phases. In addition, and analogous to the logarithmic phases of growth, energy metabolism, regulatory functions, and transporter functional classes are among the most prominently affected. Higher

expression of 23 ribosomal proteins is observed in the mutant strain compared to the wild-type during the later growth phase (protein synthesis genes in table 3). Similar expression was observed in wild-type cells growing exponentially (OD_{600} = 1.0) compared to the wild type in the transition state (data not shown), suggesting growth rate dependent regulation of ribosomal genes. In contrast, differential expression of these genes was not observed in the *ccpA* versus wild type comparison at mid-log phase.

The relative number of regulated genes in the later growth phases preceded by a predicted *cre*-site is two times lower compared to early and mid logarithmic phase, suggesting that the role of CcpA during the later phases of growth may be more indirect.

Transcriptional analyses throughout the growth curve showed that CcpA mutation affects mainly genes encoding regulatory functions, energy metabolism, and transporters, similar to the classes already predicted to be regulated by CcpA.

Comparison of transcriptome and proteome

The availability of transcriptome as well as proteome data sets allowed us to compare the results of both omics approaches to get potentially a complete overview of the biological system underlying CcpA-mediated transcriptional regulation. The availability of transcriptome as well as proteome data sets allowed us to compare the results of both 'omics approaches to get potentially a complete overview of the biological system underlying CcpA-mediated transcriptional regulation. Therefore differentially regulated spots in the proteomic analysis of the wild type versus *ccpA*-mutant were analyzed for their transcriptional regulation as determined by micro array analysis (Table 6). When simply applying a straightforward analysis based on direction of regulation, ("+ or –" score), without taking the amplitude of regulation into account, 81% of the proteins (17 out of 21) showed a similar direction of regulation during logarithmic growth (OD_{600} 1.0) (Table 6A), whereas 80% (15 out of 18) showed a similar direction of regulation during late logarithmic growth (OD_{600} 3.0) (Table 6B). Upon inclusion of the level of regulation as a similarity criterion, maximally allowing a two-fold difference between transcript and protein level change (difference in ^{2}log ratio smaller or equal than 1; Table 6, last column), revealed less similarity in the transcriptome versus proteome. Of the identified protein spots, 33% (7 out of 21; Table 6A) displayed similar expression differences as observed at the transcriptome level during the mid-exponential growth phase. Such analyses in the late logarithmic growth phase revealed similar differential transcript levels between wild-type and mutant of 27% of the identified protein spots (4 out of 15).

Dissimilarity was observed for the phopshoglycerate kinase (PGK) an enzyme in the *gap* operon, an operon encoding glycolytic genes. Control of this operon is possibly in part achieved via increased mRNA stability as has been found both in the Gram-negative bacterium *Zymomonos mobilis* (10) and the Gram-positive model organism *Bacillus subtilis* (26, 31). The discrepancy between the RNA and protein abundance indicates a high need for this enzyme in the *ccpA*-mutant compared to the other genes in the operon. Analogous results were found for D-Lactate dehydrogenase, which is the last gene in a putative operon containing a hypothetical gene and a endonuclease.

High similarity between the data was found for the mannose *IIAB* gene, the first gene of the mannose PTS, a by σ^{54}-regulated sugar transporter (chapter 2) indicating a direct relation between transcription and the transport activity.

These comparison analyses of proteome and transcriptome data revealed that 80% of the data appeared similar on basis of direction of expression changes, while this percentage was drastically lower (30%) when quantitative expressions were compared at compared at transcript and protein level.

Table 6: Proteomics results (ratio-P) of the wild type (+) versus the ccpA-mutant (-) (WT/ccpA) of samples taken at mid log phase (A) and late log phase (B). The regulation is given as ^{2}log ratio. The transcriptome data are compared at sample level (Ratio-T), similar magnitudes of expression were calculated by subtracting the Ratio-P from Ratio-T (last column, T minus P).

A

ORF	*Name*	*Product*	*Ratio-P*	*Ratio-T*	*T minus P*
lp_0233	*mtlD*	mannitol-1-phosphate 5-dehydrogenase	-3.3	-3.6	0.3
lp_0330	*fba*	fructose-bisphosphate aldolase	-1.7	0.1	-1.8
lp_0369	*gshR1*	glutathione reductase	1.0	1.1	- 0.1
lp_0575	*pts9AB*	mannose PTS, EIIAB	3.3	3.6	-0.3
lp_0622	*rplL*	ribosomal protein L12/L7	1.0	*missing*	-
lp_0786	*clpP*	ATP-dependent Clp protease proteolytic subunit	-1.0	-0.5	-0.5
lp_0929	*asp1*	alkaline shock protein	2.3	2.9	-0.6
lp_1321/lp_2193	*pepV* /*ftsZ*	Dipeptidase/ cell division proteins FtsZ	-2.3	0.9/ 1.3	-3.2/ -3.6
lp_1563	*greA2*	transcription elongation factor GreA	1.7	0.5	1.2
lp_1872		hypothetical protein	-3.3	-1.6	-1.7
lp_1874	*dapB*	dihydrodipicolinate reductase	3.3	-0.9	4.2
lp_1874	*dapB*	dihydrodipicolinate reductase	2	-0.9	2.9
lp_2057	*ldhD*	D-lactate dehydrogenase	-2	0.2	-2.2
lp_2119/lp_0790	*Tuf* /*pgk*	elongation factor Tu/ phosphoglycerate kinase	-1.7	0.1/ -0.2	-1.8/ -1.5
lp_2267	*xtp1*	xanthosine triphosphate pyrophosphatase	1	-0.3	1.3
lp_2652		hypothetical protein	1	*missing*	-
lp_2697	*pyrE*	orotate phosphoribosyltransferase	3.3	3.7	-0.4
lp_2702	*pyrC*	Dihydroorotase	3.3	3.6	-0.3

B

ORF	*name*	*Product*	*Ratio-P*	*Ratio-T*	*T minus P*
lp_0233	*mtlD*	mannitol-1-phosphate 5-dehydrogenase	-3.3	-1.5	1.8
lp_0244	*lp_0244*	oxidoreductase (putative)	-1.7	-1.2	0.5
lp_0622	*rplL*	ribosomal protein L12/L7	2.3	*missing*	-
lp_0727	*groES*	GroES co-chaperonin	3.3	0.8	2.5
lp_0792	*enoA1*	phosphopyruvate hydratase	-1	0.7	-0.3
lp_0929	*asp1*	alkaline shock protein	2	1.4	0.6
lp_0930	*asp2*	alkaline shock protein	3.3	0.3	3.0
lp_1163	*lp_1163*	nucleotide-binding protein	2	-0.5	2.5
lp_1321/ 2193	*pepV/ ftsZ*	Dipeptidase/ cell division proteins FtsZ	-1.7	-0.3/ 1.3	-1.4/ -3.0
lp_1872	*lp_1872*	hypothetical protein	-2.3	-1.9	-0.4
lp_2340	*lp_2340*	hypothetical protein	-3.3	-0.8	-2.5
lp_2360	*lp_2360*	ribosomal protein acetylating enzyme	2.3	3.4	-1.1
lp_2364	*atpD*	H(+)-transporting two-sector ATPase	1.0	-0.5	1.5
lp_2652	*lp_2652*	hypothetical protein	1.7	*missing*	-
lp_2697	*pyrE*	orotate phosphoribosyltransferase	1.7	*missing*	-
lp_2823	*lp_2823*	ABC transporter, ATP-binding protein	1.7	*missing*	-
lp_2877	*lp_2877*	hypothetical protein	- 1.0	0.2	1.2

Discussion

CcpA is a global regulator in *L. plantarum* WCFS1 as transcriptome analyses revealed approximately 8% of all genes to be affected by deletion of the *ccpA* gene. Emphasis of CcpA-involved transcription control is found in carbon utilization control, whereas a link to nitrogen or nucleotide metabolism, as observed in other Gram-postive bacteria (51, 55), could not be confirmed.

CcpA is activated by HPr-Ser46, which is formed as a response to the energy status of the cell (47). Wild-type *L. plantarum* cultures produced high amounts of lactate during the early growth phase, indicating a high glycolytic flux and corresponding high energy status under the conditions applied here (48), explaining the major impact of CcpA activity during these early phases of growth. Nevertheless, *ccpA*-dependent differential regulation of genes was also observed during the later growth phases, albeit that these genes appeared to be less frequently preceded by a *cre*-site. This observation could imply a less direct role of CcpA in the regulation of these genes, and might suggest an indirect effect via growth rate differences observed for the mutant relative to its parental strain. This effect is especially prominent during the later stages of growth.

Growth conditions that provide excess glucose concentrations lead to an almost homolactic fermentation pattern in the wild-type cells of *L. plantarum* (11), which through the glycolytic

pathway generate two molar equivalents of ATP per glucose consumed. However, analogous to what has been described for a Δ*ccpA* derivative of the homolactic LAB *Lactococcus lactis* MG1363 (27), several pathways leading to acetate appear to be present in this organism, i.e., anaerobic conversion via pyruvate formate lyase (PFL) (24), aerobic conversion via pyruvate oxidase (POX) (16), and via pyruvate dehydrogenase (PDH). The genes encoding the latter enzyme appeared to be tightly controlled by CcpA as revealed by the transcriptome analysis, suggesting that this pathway is involved in the acetate production observed in the *ccpA* mutant strain. However, several studies suggested that PDH activity is lacking in *L. plantarum* (9, 21, 34), indicating that acetate production via this pathway remains to be established. Pyruvate oxidase (*pox*) expression has previously been shown to be under control of CcpA (25), which is confirmed in our transcriptome analysis, although apparently a paralogous gene is regulated (see below). However, due to the anaerobic growth conditions, acetate production via the POX-dependent pathway is a very unlikely explanation for the observation of acetate production in the *ccpA* mutant strain. On basis of the L. plantarum WCFS1 genome sequence annotation, acetate production is also possibly through the pyruvate format lyase (PFL), phosphotransacetylase (PTA), and acetate kinase (ACK) pathway (24). The *pta*-gene has no putative *cre*-site and is not differential expressed, but the *pfl*-gene and the *ack*-gene harbor putative *cre*-sites, suggesting CcpA-dependent regulation, and acetate production is likely to occur via this pathway, which is in agreement with earlier observations.

Acetate production provides an additional ATP gain compared to lactate production and thereby the *ccpA* mutant produces more energy from the same amount of glucose consumed. Growth rate of *L. plantarum* decreases rapidly below pH-values of 5.0 (14), which possibly explains the bi-phasic growth of observed in the wild type culture. The mutant acidifies slower and the higher cell density reached by the mutant is most likely related to the reduced acid stress as well as to the increased energy gain.

L. plantarum WCFS1 possesses five *pox*-genes (*poxB-poxF*) (15, 23), of which only *pox1* was affected by *ccpA*-mutation. The *pox1* is homologous to *poxD* of *L. plantarum* strain Lp80 (15) of which no transcript could be detected under condition where carbon catabolite repression is released (15). The active forms of Pox in strain Lp80 PoxB and PoxF are both under catabolite control and are homologous to Pox5 and Pox3 of strain WCFS1 (15). The differential expression *poxD* in a WCFS derived *ccpA*-mutant suggests different regulation of the *pox*-genes in these strains.

The full genome transcription analyses show that the main function of CcpA in *L. plantarum* is carbon catabolite repression and regulation of sugar metabolism, which display a clear parallel with the role of CcpA in *B. subtilis* and *L. lactis* (33, 55). However, in *L. lactis* and *B. subtilis* CcpA also appeared to control specific functions related to nitrogen metabolism and nucleotide transport (51, 55), while this observation is not confirmed in *L. plantarum* where such linkages between different domains of metabolism apparently are not controlled by CcpA. Activation of glycolytic enzymes by CcpA as describe in *L. lactis* (27) does not occur in *L. plantarum*. A slightly higher expression of operon encoding *pyk* and *pfk* is observed in the wild type, but apparently not as high as in *L. lactis* (27). As the operon is not preceded by a *cre*-sequence, it is assumable that no activation by CcpA occurs for this operon. This is more in agreement with the mechanism in *Lactobacillus casei* in which no CcpA-mediated activation was observed for the operon (50).

Nevertheless, our studies revealed a large set of genes that are subject to CcpA mediated control in *L. plantarum* that are located throughout the genome. These genes include several intriguing ones, like the up regulation of two *cre*-site in the promoter containing bile salt hydrolases, suggesting a role for CcpA in the GI-tract. Notably, relief of catabolite repression in the GI-tract has already been suggested on basis of a R-IVET screening procedure revealing *L. plantarum* genes that were specifically induced during intestinal transit in mice (4). In addition, a putative role of CcpA in niche-adaptation was also suggested by its role in the regulation of expression of putative oligo- and polysaccharide degrading proteins (42). Moreover, since the CcpA regulon also encompasses a large number of transcriptional regulators, possibly providing this central regulator with putative two/step control of an even wider range of genes and pathways in *L. plantarum*, where in general its regulatory focus is targeted to sugar metabolism and transport of sugars and organic compounds.

The transcriptional units in bacteria, the operons, contain frequently several, co-expressed genes. However, under differential circumstances, the need of specific proteins encoded within one operon might change, leading to dissimilarity between protein and mRNA abundance due to differential turnover of the two types of molecules (19). The appearance of Clp-proteases on the proteomic analysis of the *ccpA* mutant might indicate differential turn-over as these proteases are involved in protein degradation (5). The stability of mRNA in bacteria is low and previous analyses showed that mRNA in *Escherichia coli* have half-lives between 3 and 8 minutes (3) and huge changes in the abundance of mRNA's in the Gram positive bacterium *B. subtilis* occur over very narrow intervals of time (39). In contrast, the

half-life of proteins is relatively high, reaching up to 60 min during growth and /or starvation (36). These biological phenomena could explain the discrepancy between the proteome and transcriptome data.

The apparent higher abundance of PGK and D-LDH in the *ccpA*-mutant compared to the other genes in their specific operons, as indicated by the proteome-transcriptome comparison, indicates a different control of glucose to lactate conversion, maybe due to the lower growth rate.

The mannose PTS in *L. plantarum* is regulated by σ^{54}, a sigma factor that allows rapid and strict regulation of expression (46). The similarity between mannose PTS protein and mRNA abundance indicates a strict coupling of transcription and translation that enables rapidly transporter synthesis when glucose is present in the medium.

Due to the short life time of mRNA molecules, the activity of a microbe can be measured more accurately by proteome analyses than transcriptome analyses. In contrast, transcriptome analyses provide a methodology that enables a more accurate evaluation of the dynamics and regulatory networks underlying microbial responses to environmental changes. However, comparison of transcriptome and proteome data shows that a combined approach is a powerful way to determine abundance of molecules in the cell. Ultimately, combining the techniques might lead to new targets which are possibly regulated via mRNA and/or protein stability and thus identification of new regulatory networks in the cell.

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Chapter 6

Regulation of Carbohydrate Transport and Utilization in *Lactobacillus plantarum* WCFS1

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Abstract

L. plantarum WCFS1 possesses a relative high number of predicted sugar uptake and utilization systems, allowing the bacterium to grow on a wide range of carbohydrates. In order to gain insight into carbohydrate metabolism and regulation, transcriptome analyses of *L. plantarum* grown on 4 carbohydrates (mannose, fructose, sucrose, and lactose) were performed and interpreted by comparison to the transcriptome profile obtained for cells grown on glucose. The significantly regulated genes could be grouped into 2 categories: genes involved in sugar transport and conversion, and genes with no direct link to the specific sugar. The majority of these additional responses could be explained by the relief of catabolite repression. Our data confirm the annotation of specific genes and provide further insight in catabolite control and sugar utilisation in *L. plantarum*.

Introduction

Uptake and utilization of carbohydrates are essential processes for all heterotrophic organisms to generate energy and to produce precursors required for growth. Various regulatory effects that create hierarchical preference of specific carbohydrate uptake and utilization and its consequences on the general metabolism of the cell have been described already in the early days of microbiological research. Nowadays, many of these effects have been clustered in the regulation network related to carbon catabolite control (4). The first step in carbohydrate utilization is transport into the cell. Bacterial carbohydrate transport is mediated by three main mechanisms: energy (ATP) driven transport (e.g. ABC-transporters), chemical-osmotic driven transport (e.g. symporters, antiporters), and group translocation in which chemical modification is coupled to transport (25). The best-known examples of the latter group are the phosphotransferase systems (PTS), in which transport is coupled to phosphorylation of the substrate (26). PTSs are the most efficient mode of carbohydrate transporter (9) and therefore represent the commonly preferred transport mechanism for sugar uptake (28). PTSs consist of the common, not sugar-specific proteins Enzyme I (EI) and phosphocarrier protein HPr that act in combination with the sugar specific proteins Enzyme IIA, IIB, involved in a phosphate cascade, and the permease unit IIC (26). EI transfers a phosphate from the glycolytic intermediate phosphoenolpyruvate (PEP) to the His-15 residue of HPr. Subsequently, the phosphate is transferred via a cascade involving the IIA and IIB enzymes to the incoming sugar (26).

During catabolism of readily fermentable sugars such as glucose, the synthesis of enzymes involved in the catabolism of other sugars is repressed, a global regulatory phenomenon commonly termed carbon catabolite repression (CCR). The dominant mechanism of global carbon control in low G+C Gram-positive bacteria is one involving HPr, the bifunctional HPr kinase/phosphatase (HPrK) and the catabolite control protein A, CcpA (12). CcpA is a member of the LacI-GalR family of bacterial regulator proteins and regulates transcription of genes at global level by binding to a *cis*-acting DNA sequence designated catabolite responsive element (*cre*) (13, 20). Catabolite control by CcpA involves both transcriptional activation and repression and the CcpA regulon is commonly scattered throughout the entire bacterial genome, which was confirmed by the comparative whole-genome transcriptome analyses in the Gram-positive species *B. subtilis*, *L. lactis*, and *L. plantarum* and their *ccpA* mutant derivatives (22, 32, 37).

Lactic acid bacteria (LAB) represent a group of Gram-positive microorganisms that produce lactate during homo- or hetero-fermentative metabolism. Knowledge of sugar utilization by LAB is of importance for the industry, as the involved enzymatic processes contribute to flavor, texture, and preservative qualities of fermented food products (14). *Lactobacillus plantarum* is a facultative hetero-fermentative lactic acid bacterium encountered in a variety of environmental niches, including dairy, meat, and many vegetable or plant fermentations (30). Furthermore, it is often found as a natural inhabitant of the human gastrointestinal (GI) tract (1) and some *L. plantarum* strains are considered to have probiotic activity (8, 14). The complete genome sequence of the *L. plantarum* strain WCFS1 was determined and encoded all enzymes required for the sugar metabolizing glycolysis and phosphoketolase pathways (15). Moreover, *L. plantarum* is predicted to encode a large pyruvate-dissipating enzyme arsenal, leading to various possible end-products of fermentation (15). The ability of *L. plantarum* WCFS1 to grow on a wide variety of carbohydrates is reflected in its genomic content, which contains a relative high number of putative sugar transporters (15). The genome encodes 25 complete PTSs, several incomplete PTSs, and 30 alternative transport systems that were predicted to be involved in the transport of carbon sources. Interestingly, a large proportion of these genes encoding sugar transport and utilization appear to be clustered in a 600-kb region near the origin of replication, the so-called sugar island (15, 21).

In this work gene expression profiles of *Lactobacillus plantarum* cells grown on four different carbon sources (mannose, fructose, sucrose, and lactose) were compared to expression profiles cells growing on glucose. The results confirm the annotation of a range of genes that were predicted to be involved in the transport and metabolic pathways related to these sugars routes. Furthermore, the results provide additional insight in carbon catabolite control during growth on different carbon sources.

Materials and Methods

Bacterial Strains and Growth conditions

Lactobacillus plantarum strain WCFS1, a single-colony isolate of the human-sioltae NCIMB8826, was pre-cultured anaerobically at 37°C in a chemical defined medium (CDM) (36) supplemented with 1% (w/v) glucose for 24 hours. Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}) in an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Roosendaal, The Netherlands). To ensure carbohydrate specific growth, pre-cultured cells were washed twice in CDM without carbon source and inoculated at OD_{600}

of 0.05 in CDM containing 2% (w/v) of the specific carbohydrates. Maximum growth rate was determined after growth had clearly been initiated (starting at OD_{600} of at least 0.2). Carbohydrates were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Sampling

Cultures grown on different carbohydrate substrates were harvested during the logarithmic phase of growth (OD_{600} equals 1.0 for all cultures) by quenching metabolic and transcriptional activity instantly using a quenching protocol that has been described previously by Pieterse et. al. (24). To this end, 1 volume of culture was quenched in 4 volumes of -40º C 60% methanol-HEPES buffer as described by Pieterse (24). After quenching, cells were harvested by centrifugation (13182 * g, 10 min, -20º C) using a Sorvall RC5B plus centrifuge (Sorvall, Newton, US). Immediately after harvesting, cell pellets were rapidly transferred with a pre-chilled spatula to a screw-crap tube containing 500 mg zirconium beads, 500µl Phenol/Chloroform-mix 1:1, 30 µl 3 M Na-Acetate (pH 5.2), 30 µl 10% SDS, and 400 µl MRS-medium (Merck, Darmstadt, Germany), carefully avoiding thawing of the cell pellet material. The tubes containing the cells were shaken, frozen in liquid nitrogen and stored at -80º C.

Culture supernatant samples were taken isolated by removal of the cells by centrifugation (20800 * g, 1 min), in an Eppendorf table centrifuge 5417C (Eppendorf, Hamburg, Germany). The supernatant was transferred to a new tube and stored at -20º C until further use.

RNA extraction and quality control

RNA was isolated as described previously (34), using a phenol-chloroform extraction followed by a purification using the High Pure RNA isolation kit (Roche, Manheim, Germany). The yield and purity of the RNA were determined by measurement of the absorption at 260 nm and 280 nm (Ultrospec 3000, Pharmacia Biotech, Roosendaal, The Netherlands). The RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent technologies, Palo Alto, Ca, USA) following the manufacturer's instructions. Only RNA samples displaying 16S/23S-rRNA ratios of 1.6 or higher, were labeled and used for micro array experiments.

cDNA-synthesis, labeling, and hybridization

The Cyscribe Post-labeling kit (Amersham Biosciences, Amersham, UK) was used to synthesize and to label cDNA. Labeled cDNAs were hybridized as described previously on amplicon based microarrays containing fragment of approximately 97% of the genes of *L. plantarum* WCFS1 (34).

Scanning, data extraction, and analyses

The slides were scanned with a Scan Array Express 4000 scanner (Perkin Elmer, Wellesley, USA) and the images were analyzed with Imagene software 4.2 (BioDiscovery, El Segundo, USA). Microarray data were stored in the BioArray Software Enviroment BASE (http://base.thep.lu.se/), installed at a local institute server. Data were normalized by a mean background correction and Lowess normalization (locally weighted scatterplot smoother) (6), and subsequently transported to Excel XP (Microsoft, Redmond, USA). Significant differential gene expression was determined using a t-test with two-tailed distribution and two-sample equal variance. Cut-off for significantly differential expression of genes was set at a p-value ≤ 0.05 and a mean intensity ratio of at least 2-fold ($\log^2$ ratio<-1 or $\log^2$ >1).

HPLC analyses

Sugar concentration in culture supernatant was determined by HPLC a described previously (16), using a HPX87-P cation exchange column (Biorad, Herculus, USA) for separation and water as elution fluid. A 16 min runtime was used and sugars were detected using a refractive index detector.

Results

Growth characteristics of L. plantarum WCFS1 on different carbohydrates

The capability of *L. plantarum* to grown on a variety of carbohydrates (23) is reflected in the relative high amount of carbohydrate transporters encoded in the genome (15). However, growth characteristics on carbohydrates are not available for the sequenced strain WCFS1 and therefore we initially set out to grow this strain on a range of carbohydrates and determined maximum growth rate. The highest maximal growth rates were measured for *L. plantarum* WCFS1 cultures in CDM media containing glucose and cellobiose as the sole carbon sources, while only slightly lower growth rates were observed in maltose, mannose, sucrose, and N-acetylglucosamine containing CDM (Table 1). In contrast, maximal growth rates observed on fructose and lactose containing media were clearly lower as compared to glucose-medium, while no growth could be detected on arabinose, ribose, and sorbitol in the first 24 h. Growth defect of *L. plantarum* on the 3 latter carbohydrates is surprising, as transporters and utilization routes for these sugars are encoded in the genome (15). So far, no inuline transporter has been identified in *L. plantarum* and although plant-derived oligosaccharides degrading complexes have been proposed in this bacterium (31), growth

deficiency on inulin is in agreement with earlier publications (23). Previously, we have reported growth initiation on galactose after supplementing the medium with a small amount of glucose (34), but performing the same methodology on the "no-growth"-sugars did not lead to growth initiation.

Glucose is the most favorable sugars for most organisms and is known to prevent uptake of other sugars, a phenomenon known as inducer exclusion (27). To evaluate inducer exclusion phenomena in *L. plantarum*, the bacterium was grown on CDM media containing glucose as well as one additional sugar (maltose, sucrose, fructose, or lactose) and followed carbon utilization by the growing bacteria as a function of time The results clearly established that only fructose can be co-metabolized with glucose (Fig. 1), suggesting that uptake and utilization of the other sugars is repressed by glucose, which is in good agreement with glucose mediated catabolite control dogma. Unfortunately, mannose and glucose could not be distinguished by our sugar analysis method that was based on HPLC. However, the observed expression of the mannose PTS in glucose growing cells and results indicating that glucose and mannose are imported by this transport system (34), suggest that *L. plantarum* actually co-metabolizes mannose and glucose, which remained undetected in this study due to technical limitations.

The growth characteristics of *L. plantarum* WCFS1 on media containing these sugars confirmed that glucose can be considered as the most favorable carbohydrate for this bacterium. Furthermore, the results suggest that predicted transport functions on basis of genomic annotation are not always compatible with the experimentally observed growth characteristics. Moreover, biochemical assays (API 50) showed positive reaction on those sugars on which no growth was detected (15), suggesting growth is possible. Prolonged incubations of cells pre-grown on glucose led to growth on sorbitol and ribose, albeit with long lag phases and slow growth (data not shown).

Table 1: Growth of L. plantarum WCFS1 on a range of carbon sources. Given are the doubling times in hours, NG = No growth (less than one doubling in the first 24 hours).

Carbon source	***Doubling time in*** hours
glucose	1.01 ±0.14
cellobiose	1.02 ±0.04
fructose	2.20 ±0.71
lactose	3.72 ±1.94
maltose	1.20 ±0.01
mannose	1.07 ±0.26
sucrose	1.08 ±0.09
N-Acetylglucosamine	1.22 ±0.04
arabinose	NG
inulin	NG
ribose	NG
sorbitol	NG

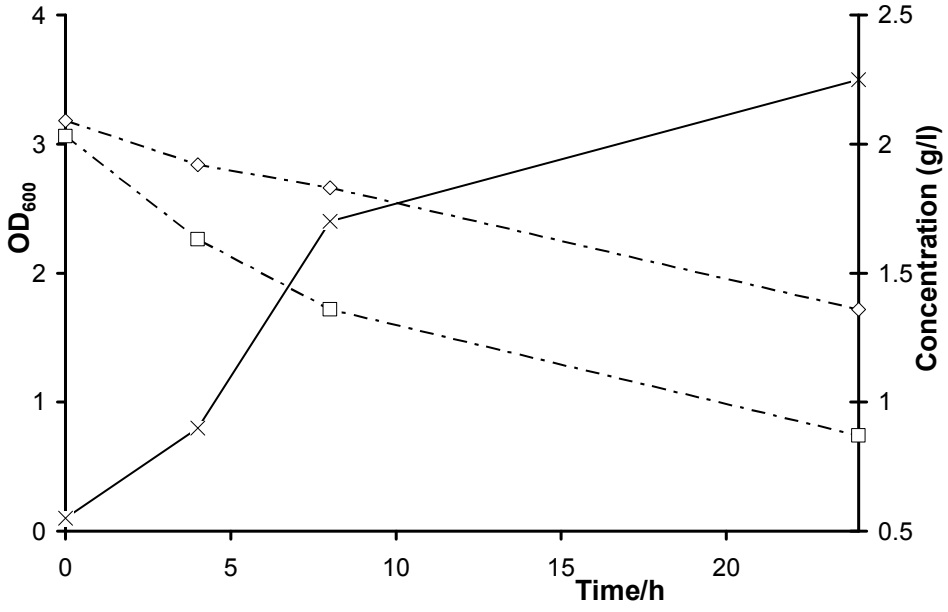


Figure 1: Co-metabolization of carbohydrates by L. plantarum. The optical density is plotted on the left Y-axis and cell density is indicated with crosses. Sugar concentrations are plotted on the right Y-axis: squares: glucose; diamantes: fructose.

Transcriptional analysis of WCFS1 grown on different carbohydrates

To gain insight in the control of carbohydrate uptake and utilization, the transcriptome of *L. plantarum* grown on mannose, sucrose, fructose, and lactose was compared to the transcriptome on glucose using full-genome amplicon based microarrays (34). Additionally, the regulated genes were compared with an existing CcpA binding site (*cre*-site) prediction in the genome of *L. plantarum* WCFS1 that was based on evidenced *cre*-sites of *Bacillus subtilis* (32).

Previous work showed that mannose uptake in *L. plantarum* is exclusively mediated via the mannose PTS, which also acts as the main glucose and mannose transporter and has additional functions in controlling sugar metabolism at a more global level (34). In total 97 genes were significantly higher expressed in mannose grown cells compared to glucose and 34 of these genes harbored one or more putative *cre*-sites (table 3). No differential expression was observed for the genes of the mannose PTS in cells grown on either glucose or mannose, which is in agreement with previous results (34). Among the genes that appeared to be expressed at a higher level in mannose growing cells, was the gene encoding mannose-6-phospate isomerase (*pmi*), which was expected since this enzyme catalyzes the reaction of mannose-6P to glucose-6P, the essential step between mannose uptake and glycolysis. Mannose growth led to higher levels of expression of the mannitol operon, consisting of a mannitol PTS (*mtlABC*), transcription regulator (*mtlR*) and mannitol dehydrogenase (*mtlD*). Furthermore, three cellobiose PTS were up regulated in the presence of mannose as were two pyruvate oxidases (*pox2* and *pox3),* which convert pyruvate into acetyl-phosphate to generate acetate under aerobic conditions. Other genes involved in primary metabolism such as acetate kinase (*ack*) and the pyruvate dehydrogenase complex (*pdh*) were expressed at a higher level in mannose growing cultures (supplementary material table 1B), genes already described to be under control of catabolite control mediated by CcpA (32).

Fructose was co-metabolized with glucose by *L. plantarum*, while growth on fructose as sole carbon source in CDM was two times slower as compared to growth on glucose. Transcriptome analysis of *L. plantarum* growing on fructose containing CDM medium revealed the higher expression of 72 genes on fructose as compared to glucose and enrichment in the functional classes of transport and cell envelope functions was observed (Table 2). The operon annotated as the fructose locus, consisting of genes predicted to encode a PTS, a 1-phosphofructokinase and a regulator was higher expressed. This observation strongly supports uptake of fructose via this PTS, generating intracellular fructose-1 phosphate, which is subsequently converted to fructose-1,6 biphosphate by the 1-

phosphofructokinase, (supplementary material table 4B). Analogous to what was seen in cells growing in mannose containing CDM, fructose led to the higher expression of 2 cellobiose PTS components and three pyruvate oxidases. Further a small heat shock protein and a thioredoxin reductase were up regulated on fructose, both proteins involved in stress response (3, 18), indicating a triggered stress-response in fructose grown cells.

Table 2: Number of genes differentially expressed in functional categories and the number of putative cre-sites found in the upstream fragments of the genes.

	Mannose		*Sucrose*		*Fructose*		*Lactose*	
Functional class	up	down	up	down	up	down	up	down
Amino acid biosynthesis	0	3	0	3	0	14	2	9
Biosynthesis of cofactors, prosthetic groups, and carriers	1	0	2	0	1	1	0	1
Cell envelope	4	4	5	4	14	12	1	0
Cellular processes	2	1	3	1	2	2	1	0
Central intermediary metabolism	2	2	2	0	3	0	3	0
DNA metabolism	1	0	0	0	0	2	0	0
Energy metabolism	27	1	31	2	4	18	19	5
Fatty acid and phospholipid metabolism	1	0	0	1	4	0	0	0
Hypothetical proteins	35	5	25	7	23	14	11	6
Other categories	1	3	0	3	3	10	0	0
Protein fate	0	0	0	1	0	3	1	0
Protein synthesis	0	0	0	2	1	30	0	2
Purines, pyrimidines, nucleosides and nucleotides	1	4	0	6	1	14	1	6
Regulatory functions	8	0	7	3	4	9	2	1
Transcription	0	0	0	0	0	2	0	0
Transport and binding proteins	14	6	16	7	12	23	15	12
Total	97	29	91	40	72	153	56	42
cre-sites	34	5	38	6	11	15	17	4

The *L. plantarum* genome contains a locus putatively involved in sucrose utilization that is shown to be repressed during growth on glucose compared to growth on fructo-oligosaccharides (29). Transcriptional analysis of cells growing on sucrose containing CDM revealed higher expression of 92 genes, of which 39 were preceded by a putative promoter region that contains a predicted *cre*-site (table 3). Overall, comparative analysis of the sucrose-growth affected genes with those modulated by *ccpA* mutation, revealed a 71% parallel between these responses. The genes of the sucrose operon, which encode a sucrose PTS, a β-fructofuranosidase, a fructokinase, a sucrose operon repressor and a α-glucosidase were higher expressed during growth on sucrose. Additionally, six phospho-β-glucosidases were higher expressed on sucrose, enzymes that are probably able to hydrolyze sucrose-6P.

Other carbohydrate transporters were modulated by growth on sucrose relative to growth on glucose, including a lactose and fructose transporter, and N-acetylglucosamine and 4 cellobiose PTS. Interestingly, ORF lp_3611, which encodes a sugar transporter, is in our data set only expressed in sucrose-growing cells, suggesting a role in sucrose transport capacity for this protein. Regulation of pyruvate oxidases and acetate kinases is similar to mannose and fructose. Further, a small heat shock protein and a thioredoxin reductase are regulated, parallel as seen on fructose and suggesting fructose growth elicits a specific stress response. The observation of an NADH-dehydrogenase and an NADH-oxidase could suggest an imbalanced NADH/NAD-ratio.

L. plantarum WCFS1 is predicted to encode two putative lactose transporters (*lacS1* and *lacS2*) that are both genetically linked to β-galactosidase (15). Global transcriptional analysis revealed significant up regulation of 42 genes and down regulation of 56 genes on lactose as sole carbon source in comparison to glucose. Both lactose transporters were induced, as were the genes of the Leloir-pathway, suggesting parallel utilization of the glucose and the galactose moiety of lactose. Comparative analysis of regulated expression with other sugar transcriptome profiles identified common regulation of the cellobiose PTSs and pyruvate oxidases. In contrast, growth on lactose affected expression of a sorbitol and a maltose transporter and regulation of NADH-peroxidase, NADH oxidase (*nox5*, identical as on sucrose) and a protein-methionine-S-oxide reductase, the latter three genes known to be involved in the response of *L. plantarum* to oxidative stress (33).

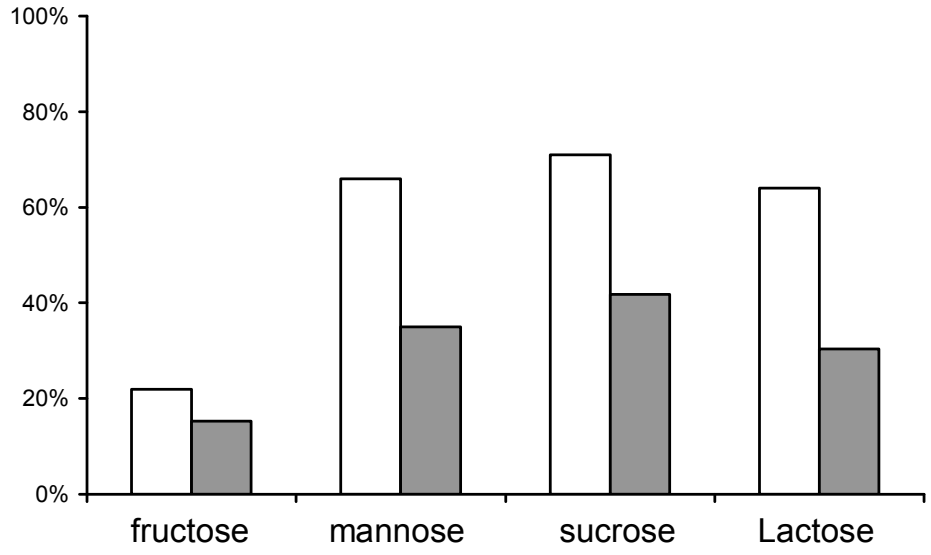
Taken together, the response of *L. plantarum* when grown on other sugars can be divided into to two groups: the first group consists of genes which are directly related to transport and utilization of the carbon source; the second group consists of genes with a putative *cre*-sites, and thus seem to be regulated due to carbon-source mediated modulation of CcpA activity. This latter group encompasses genes that have no clearly apparent relation to the carbon source provided during growth.

Analyses of genes exhibiting higher expression on all tested sugars

All four tested sugars exhibited a higher expression of a large amount of genes harboring putative *cre*-sites in their upstream regions (Table 2). These *cre*-sites are the binding domains of the catabolite control protein CcpA (12), and as CcpA regulates a large set of genes in *L. plantarum* (32), we compared the data sets with a data set of an exponential growing *ccpA*-mutant compared to an exponential growing wild-type strain. This comparison revealed that the response to the sugars was partially identical to the response to *ccpA*-mutation. A

considerable number of genes higher expressed on mannose, sucrose and lactose were also found to be higher expressed in the *ccpA*-mutant (Fig. 2), whereas the similarity was less pronounced on fructose, indicating CcpA-mediated repression is partial relieved in non-glucose grown cells.

Figure 2: Putative CcpA regulation of the genes higher expressed on one of the four tested sugars compared to glucose. White bars: percentage of identity between the sugar response and the ccpA-wild-type comparison. Grey bars: percentage of putative cre-sites found in front of genes higher expressed on the specific sugar.



L. plantarum contains 9 putative cellobiose PTS, although correct annotation of these genes is debatable (M. Kleerebezem, personal communication). In *Listeria monocytogenes* orphan cellobiose PTS IIC-components have a role in signaling leading to virulence gene expression (17) and the higher expression of the cellobiose IIC-gene *pts6C* on all four tested sugars suggests such a signaling role in *L. plantarum* during growth on less favorable sugars.

Apart from the cellobiose PTS, pyruvate oxidase encoding genes were higher expressed on all tested sugar: *pox3* (designated *poxF* in strain LP80 (10, 19)) on all tested sugar, *pox5* (*poxB*) on fructose and lactose and *pox2* (*poxE*) on mannose fructose and sucrose. The latter, *poxE*, is higher expressed in a *ccpA*-mutant compared to the wild-type during exponential growth (32). This indicates a glucose mediated repression of pox genes, which is in agreement with previous studies (10).

Discussion

L. plantarum is a versatile Gram-positive bacterium with the ability to grow on a large number of carbohydrates, which is reflected by the relatively high number of carbohydrate transporter encoding genes predicted in its genome (15). Using a transcriptomic approach we could elucidate the genetic response to different carbohydrate availability, thereby confirming known pathways and identifying additional, unknown responses. The majority of these additional responses can be explained by the relief of catabolite repression.

Our analyses revealed minimal differences between glucose and mannose in transport and utilization. Analogously, an identical transporter was previously shown to be involved in import of both these sugars and mannose utilization requires only one additional enzyme step compared to glucose (Fig. 3). Nevertheless, a decrease of CcpA activity seems to occur, since the differential responses to mannose and *ccpA* deletion displayed a high degree of similarity (Fig. 2), indicating a glucose specific activation of CcpA in *L. plantarum.* CcpA is activated by the PTS enzyme HPr, phosphorylated at the additional residue Ser-46 by a HPr-kinase/phophatase (HPrK/P), which kinase activity is stimulated by fructose 1,6-biphoshate (FBP) (35). Intracellular levels and ratio's of glycolytic intermediates modulate the balance between P-His15-HPr and P-Ser46-HPr, thereby mediating CcpA-activity (35). Accordingly, the decreased CcpA-activity in mannose grown cells is possibly due to differences in glycolytic intermediate levels. The mannose PTS has a high affinity for HPr (9) and drains the PEP-pool in resting *L. plantarum* cells (34). Furthermore, phosphorylation studies in the mannose PTS in *Streptocoocus thermophilus* revealed preferential glucose phosphorylation by the mannose PTS relative to mannose (7). Slower phosphorylation of mannose compared to glucose could lead to accumulation of PEP during growth on mannose, as it might happen that the PEP-derived phosphoryl group can not be transferred to an incoming mannose as efficient as to an incoming glucose. This is a possible explanation how modulation of the PEP-pool via the PTS-transport efficiency could lead to reduced HPrk/P-activity and thus to decreased CcpA activity during growth on mannose.

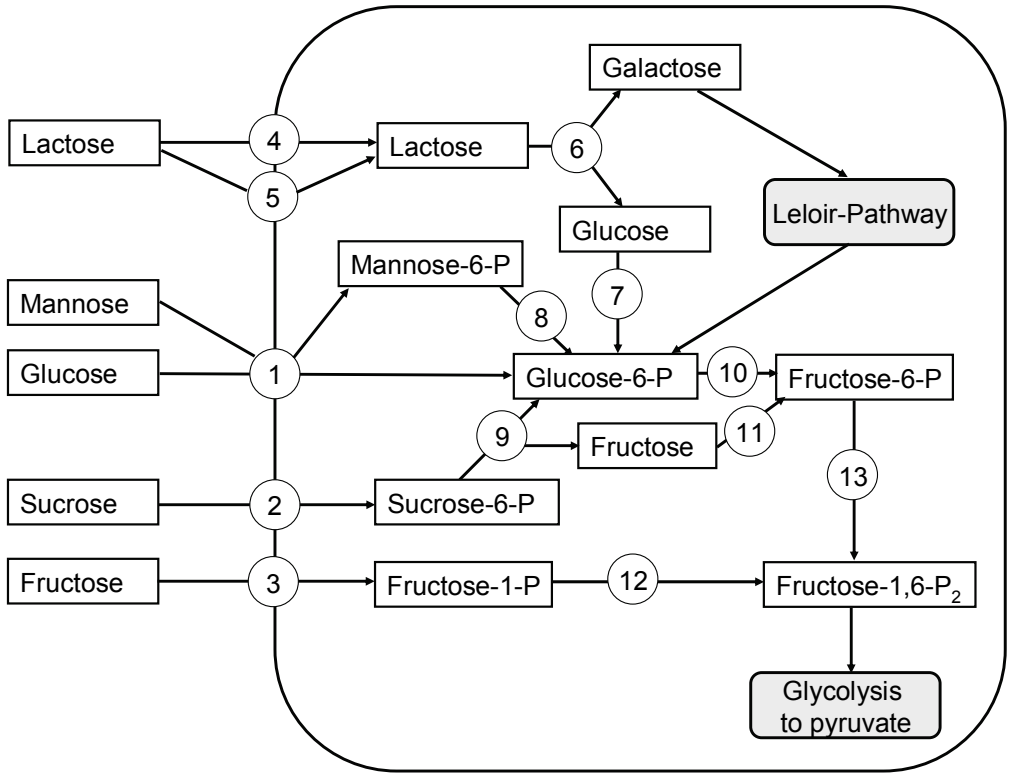
Sucrose is transported by a PTS system and intracellular degraded to glucose 6-phopshate and fructose. Expression of the fructose PTS during growth on sucrose suggests sucrose transport by this PTS, which has previously also been suggested for S*treptococcus mutans* (2). Growth rates on sucrose and glucose were almost identical (Table 1), suggesting a similar energy state of the cells. However, CcpA mediated repression seemed to be abolished in cells growing on sucrose, again suggesting a glucose specific activation of CcpA.

The number of genes differentially expressed on fructose harbouring putative *cre*-sites in their upstream region is remarkably lower as on the other tested sugars, indicating maintenance of CCR, this despite the slower growth observed on fructose relative to glucose. Fructose is transported by a PTS and the resulting fructose-1-P is known to be the inducer of the fructose operon (5), and this inducer activation leads to high expression of the operon, notwithstanding (partially) maintained CcpA-activity. Although expression of the fructose operon independently of glucose mediated CCR is in apparent contradiction with findings described for *Lactococcus lactis* (5), it explains the co-metabolization of fructose and glucose (Fig 1). Furthermore, analyses of the CcpA-regulon in *L. plantarum* revealed that repression of the fructose operon is maintained in a *ΔccpA* background (32) which supports CcpA-independent regulation of fructose utilization.

The results indicate lactose transport by two transporters and parallel utilisation of the two monosaccharide moieties of lactose (glucose and galactose) via the glycolysis and the Leloir-pathway (Fig. 3). However, our data only reflect the situation during logarithmic growth at OD_{600} of 1.0 and utilisation might be different at earlier or later stages of growth, as has previously been described for *S. thermophillus* (11).

Our results demonstrate that *L. plantarum* possesses inducible transporters for specific sugars (Fig. 3), whereas additionally unspecific transporters are induced. Regulation of these transporters might occur via dedicated transcriptional regulators independent of CcpA. Glucose mediated catabolite repression via CcpA is relieved by growth on most sugars, whereas additional sugar-specific regulation occurs. These differential responses probably allow the bacterium to rapidly adapt to changes in the availability of carbohydrates in the environment. This capacity is crucial for maintenance of a competitive advantage in a variety of environmental conditions and especially relevant for niches where the carbohydrate availability might change over time, which could be expected in complex, multispecies fermentations like silage and other plant-material fermentations or the mammalian gastro-intestinal tract, which are niches that have been shown to suit *L. plantarum* well.

Figure 4: Carbohydrate utilisation of mannose, fructose, sucrose, and lactose in L. plantarum as revealed by the transcriptome analyses in this paper. The sugar specific pathways presented in this picture were higher expressed in cells grown on the specific sugar. 1= Mannose PTS, 2 = Sucrose PTS, 3 = Fructose PTS, 4 = LacS1, 5 = LacS2, 6= β-galactosidase, 7= glusokinase, 8= mannose-6P isomerase, 9= β-fructofuranosidase, 10= Glucose-6P isomerase, 11= fructokinase, 12= 1-phosphofructokinase, 13= 6-phosphofructokinase.



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Complementary data:

Table 1A: Genes significantly up regulated in cells grown on glucose compared to cells grown on mannose. Ratio's (R) are in ^{2}log scale.

ORF	*Name*	*Product*	*R*	*p-value*
lp_0226	*gnp*	glucosamine-6-phosphate isomerase	1.05	3.26E-02
lp_0295	*lp_0295*	transport protein	1.27	2.01E-02
lp_0385	*lp_0385*	hypothetical protein	1.45	1.24E-02
lp_0388	*lp_0388*	hypothetical protein	1.23	5.97E-05
lp_0529	*argJ*	glutamate N-acetyltransferase	1.21	1.86E-02
lp_0624	*lp_0624*	prophage P1 protein 1, integrase	1.00	4.56E-02
lp_0686	*lp_0686*	prophage P1 protein 63	1.06	3.55E-02
lp_0699	*lp_0699*	hypothetical protein	1.12	1.79E-02
lp_0710	*phnW*	(2-aminoethyl)phosphonate--pyruvate aminotransferase	1.09	8.54E-03
lp_0733	*pstF*	phosphate ABC transporter, substrate binding protein	1.22	2.55E-03
lp_0848	*lp_0848*	transport protein	1.25	5.61E-03
lp_0934	*lp_0934*	hypothetical protein	2.16	1.75E-02
lp_0988	*lp_0988*	lipoprotein precursor	1.51	6.23E-03
lp_1197	*cps2A*	exopolysaccharide biosynthesis protein	1.67	7.44E-05
lp_1200	*galE2*	UDP-glucose 4-epimerase	1.46	2.56E-03
lp_1219	*glf2*	UDP-galactopyranose mutase	1.17	6.54E-04
lp_1420	*nsr*	nisin resistance protein (putative)	1.23	2.23E-03
lp_1654	*trpD*	anthranilate phosphoribosyltransferase	1.23	5.70E-02
lp_1745	*lp_1745*	amino acid ABC transporter, permease protein	1.77	5.77E-02
lp_1748	*lp_1748*	ABC transporter, permease protein	1.75	2.99E-02
lp_2011	*lp_2011*	transposase, fragment (putative)	1.71	3.53E-02
lp_2488	*lp_2488*	unknown	1.97	3.78E-02
lp_2558	*hisB*	bifunctional protein: histidinol-phosphatase; imidazoleglycerol-phosphate dehydratase	1.16	4.28E-02
lp_2726	*purS*	conserved purine biosynthesis cluster protein	1.83	3.67E-02
lp_2727	*purC*	phosphoribosylaminoimidazole-succinocarboxamide synthase	1.41	5.67E-02
lp_2768	*lp_2768*	transport protein	1.00	1.01E-02
lp_2925	*lp_2925*	cell surface protein precursor	1.32	1.46E-02
lp_3153	*acm3M*	muramidase, middle fragment	1.02	2.66E-02
lp_3355	*lp_3355*	oxidoreductase	1.23	3.20E-02

Table 1B: Genes significantly down regulated in cells grown on glucose compared to cells grown on mannose. Ratio's are in ^{2}log scale.

ORF	*Name*	*Product*	*R*	*p-value*
lp_0063	*lp_0063*	hypothetical protein	-2.84	3.57E-05
lp_0096	*lp_0096*	hypothetical protein	-1.64	4.84E-03
lp_0118	*lp_0118*	hypothetical protein	-1.21	3.38E-03
lp_0155	*lp_0155*	hypothetical protein	-1.19	1.28E-03
lp_0156	*lp_0156*	hypothetical protein	-1.72	5.26E-03
lp_0170	*dak3*	phosphoryl donor to dihydroxyacetone	-1.64	2.83E-02
lp_0171	*dhaP*	glycerol uptake facilitator protein	-1.47	6.81E-03
lp_0173	*lp_0173*	transcription regulator	-1.37	2.10E-02
lp_0174	*agl1*	alpha-glucosidase	-1.75	5.05E-06
lp_0179	*amy2*	alpha-amylase	-1.65	9.69E-03
lp_0224	*lp_0224*	integral membrane protein	-1.09	2.69E-02
lp_0231	*mtlR*	transcription regulator, mannitol operon	-3.14	2.48E-02
lp_0232	*pts2A*	mannitol PTS, EIIA	-2.94	1.34E-03
lp_0233	*mtlD*	mannitol-1-phosphate 5-dehydrogenase	-2.78	5.84E-05
lp_0240	*lp_0240*	hypothetical protein	-2.02	1.60E-03
lp_0242	*ndk*	nucleoside-diphosphate kinase	-1.25	2.92E-04
lp_0277	*lp_0277*	extracellular protein	-1.25	3.35E-02
lp_0286	*pts6C*	cellobiose PTS, EIIC	-2.90	4.43E-05
lp_0310	*ack2*	acetate kinase	-1.77	4.00E-05
lp_0331	*lp_0331*	sugar transport protein	-1.06	1.18E-02
lp_0355	*sufI*	cell division protein SufI	-1.17	3.14E-02
lp_0370	*glpK1*	glycerol kinase	-1.07	4.11E-03
lp_0394	*lp_0394*	transport protein	-2.14	2.33E-04
lp_0395	*lp_0395*	hydrolase, HAD superfamily, Cof family	-1.27	3.40E-03
lp_0396	*lp_0396*	transcription regulator	-1.63	2.11E-03
lp_0397	*lp_0397*	hydrolase, HAD superfamily, Cof family	-1.27	1.36E-02
lp_0440	*pbg1*	6-phospho-beta-glucosidase	-1.93	2.64E-04
lp_0472	*lp_0472*	integral membrane protein	-1.46	2.23E-02
lp_0574	*lp_0574*	hypothetical protein	-1.48	1.60E-03
lp_0636	*lp_0636*	prophage P1 protein 13	-1.84	8.64E-03
lp_0753	*lp_0753*	integral membrane protein	-1.54	1.70E-02
lp_0838	*lp_0838*	integral membrane protein	-1.05	1.19E-02
lp_0849	*pox1*	pyruvate oxidase	-1.12	3.40E-03
lp_0852	*pox2*	pyruvate oxidase	-1.47	4.34E-03
lp_0899	*lp_0899*	hypothetical protein	-1.61	1.29E-02

lp_1102	*lp_1102*	cation transport protein	-1.64	2.94E-03
lp_1242	*lp_1242*	extracellular protein, gamma-D-glutamate-meso-diaminopimelate muropeptidase (putative)	-1.09	7.75E-03
lp_1353	*lp_1353*	hydrolase, HAD superfamily, Cof family	-1.21	6.02E-07
lp_1500	*narI*	nitrate reductase, gamma chain	-2.45	2.34E-04
lp_1546	*lp_1546*	prenyltransferase	-1.27	1.19E-03
lp_1566	*lp_1566*	hypothetical protein	-1.27	2.33E-02
lp_1660	*lp_1660*	oxidoreductase	-1.01	2.61E-02
lp_1763	*lp_1763*	glycosyltransferase	-1.19	4.76E-03
lp_1850	*topA*	DNA topoisomerase	-1.19	1.39E-02
lp_1974	*lp_1974*	transcription regulator of gluconeogenic genes	-1.52	1.31E-03
lp_2151	*pdhD*	pyruvate dehydrogenase complex, E3 component; dihydrolipoamide dehydrogenase	-1.55	2.07E-04
lp_2152	*pdhC*	pyruvate dehydrogenase complex, E2 component; dihydrolipoamide S-acetyltransferase	-1.70	2.26E-04
lp_2153	*pdhB*	pyruvate dehydrogenase complex, E1 component, beta subunit	-2.28	6.78E-03
lp_2154	*pdhA*	pyruvate dehydrogenase complex, E1 component, alpha subunit	-2.89	9.52E-05
lp_2219	*lp_2219*	hypothetical protein	-1.22	2.68E-02
lp_2340	*lp_2340*	nucleotide-binding protein, universal stress protein UspA family	-1.06	3.48E-03
lp_2384	*pmi*	mannose-6-phosphate isomerase	-1.45	2.82E-04
lp_2515	*lp_2515*	hypothetical protein	-1.47	7.08E-03
lp_2601	*lp_2601*	hydrolase, HAD superfamily, Cof family	-1.12	3.53E-03
lp_2620	*lp_2620*	cell surface hydrolase, membrane-bound (putative)	-1.25	8.26E-04
lp_2629	*pox3*	pyruvate oxidase	-1.02	8.05E-04
lp_2747	*gtcA2*	teichoic acid glycosylation protein	-1.30	2.69E-03
lp_2755	*lp_2755*	integral membrane protein (putative)	-1.49	4.16E-03
lp_2777	*pbg4*	6-phospho-beta-glucosidase	-3.64	3.02E-05
lp_2778	*pbg5*	6-phospho-beta-glucosidase	-2.13	3.79E-08
lp_2780	*pts20A*	cellobiose PTS, EIIA	-3.81	9.51E-04
lp_2781	*pts20B*	cellobiose PTS, EIIB	-2.60	3.84E-04
lp_2787	*lp_2787*	hydrolase, HAD superfamily, Cof family	-1.18	6.97E-05
lp_2797	*lp_2797*	hypothetical protein	-1.30	1.79E-02
lp_2813	*lp_2813*	hypothetical protein	-1.03	4.78E-03
lp_2814	*lp_2814*	unknown	-1.07	2.23E-02
lp_2923	*lp_2923*	lipase/esterase (putative)	-1.03	1.05E-04
lp_2998	*lp_2998*	integral membrane protein	-1.11	2.70E-03
lp_3009	*pts23B*	cellobiose PTS, EIIB	-2.52	7.12E-04
lp_3010	*pts23C*	cellobiose PTS, EIIC	-2.19	2.73E-04
lp_3011	*pbg6*	6-phospho-beta-glucosidase	-2.60	2.31E-05
lp_3078	*lp_3078*	hydrolase, HAD superfamily	-1.49	9.91E-04
lp_3092	*gabD*	succinate-semialdehyde dehydrogenase (NAD(P)+)	-1.24	2.88E-03
lp_3100	*lp_3100*	oxidoreductase	-1.57	1.17E-03
lp_3124	*lp_3124*	transcription regulator	-1.46	3.70E-11
lp_3234	*lp_3234*	transcription regulator	-1.53	4.92E-02
lp_3236	*lp_3236*	oxidoreductase (putative)	-1.48	2.14E-03
lp_3314	*pflA2*	formate acetyltransferase activating enzyme	-1.37	5.13E-03
lp_3449	*nox5*	NADH oxidase	-1.12	1.49E-05
lp_3476	*lp_3476*	transcription regulator	-2.00	9.47E-04
lp_3484	*lacM*	beta-galactosidase, small subunit	-1.12	3.48E-03
lp_3487	*galM3*	aldose 1-epimerase	-1.76	1.36E-04
lp_3503	*lp_3503*	transport protein	-1.33	1.74E-03
lp_3504	*lp_3504*	integral membrane protein (putative)	-1.30	1.23E-02
lp_3526	*pbg10*	6-phospho-beta-glucosidase	-1.58	8.22E-05
lp_3529	*bglG5*	transcription antiterminator	-1.29	7.80E-04
lp_3606	*iolG2*	myo-inositol 2-dehydrogenase	-1.84	2.53E-04
lp_3607	*iolE*	inositol catabolism protein IolE	-1.06	3.56E-09
lp_3608	*iolG3*	myo-inositol 2-dehydrogenase	-1.07	4.50E-07
lp_3612	*iolG4*	myo-inositol 2-dehydrogenase	-1.93	3.14E-04
lp_3613	*lp_3613*	hypothetical protein	-1.80	3.90E-04
lp_3632	*lp_3632*	unknown	-1.20	5.40E-05
lp_3634	*lp_3634*	alpha-1,2-mannosidase (putative)	-1.41	2.23E-05
lp_3644	*lp_3644*	sugar ABC transporter, permease protein	-1.09	1.10E-05
lp_3658	*rbsU*	ribose transport protein	-1.49	7.24E-05
lp_3659	*rbsD*	ribose transport protein, membrane-associated protein	-1.79	9.94E-03
lp_3660	*rbsK3*	ribokinase	-1.04	1.57E-02

Table 2A: Genes significantly up regulated in cells grown on glucose compared to cells grown on sucrose. Ratio's are in ^{2}log scale.

ORF	*Name*	*Product*	*R*	*p-Value*
lp_0074	*lp_0074*	hypothetical protein	1.35	2.99E-02
lp_0109	*lp_0109*	hypothetical protein	1.22	6.43E-03
lp_0151	*lp_0151*	hypothetical protein	1.38	2.95E-03
lp_0203	*serA1*	phosphoglycerate dehydrogenase	1.76	3.61E-02
lp_0228	*pepD1*	dipeptidase	1.11	2.07E-02
lp_0281	*lp_0281*	transcription regulator	1.01	6.35E-03
lp_0306	*lp_0306*	hypothetical protein	1.10	3.54E-02
lp_0307	*lp_0307*	hypothetical protein	1.09	3.89E-03
lp_0325	*lp_0325*	transcription regulator	1.19	1.04E-02
lp_0350	*hicD1*	L-2-hydroxyisocaproate dehydrogenase	1.41	3.73E-02
lp_0492	*lp_0492*	transport protein	1.20	2.95E-02
lp_0530	*argB*	acetylglutamate kinase	1.37	6.38E-03
lp_0575	*pts9AB*	mannose PTS, EIIAB	3.30	3.96E-06
lp_0576	*pts9C*	mannose PTS, EIIC	3.30	9.82E-05
lp_0577	*pts9D*	mannose PTS, EIID	3.72	1.42E-03
lp_0630	*lp_0630*	prophage P1 protein 7	1.09	3.83E-02
lp_0713	*phnE2*	phosphonates ABC transporter, permease protein	1.34	1.57E-02
lp_0796	*lp_0796*	carboxylesterase	1.05	6.94E-04
lp_1041	*rplP*	ribosomal protein L16	1.42	3.16E-03
lp_1043	*rpmC*	ribosomal protein L29	1.07	1.66E-02
lp_1098	*lp_1098*	hypothetical protein	1.01	2.35E-02
lp_1197	*cps2A*	exopolysaccharide biosynthesis protein	2.91	3.89E-05
lp_1200	*galE2*	UDP-glucose 4-epimerase	1.08	2.50E-03
lp_1201	*cps2E*	priming glycosyltransferase	1.32	1.72E-02
lp_1375	*metE*	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase	1.22	2.86E-02
lp_1734	*mvaD*	diphosphomevalonate decarboxylase	1.53	9.25E-03
lp_1782	*pyrR2*	pyrimidine operon regulator	1.71	6.00E-05
lp_2104	*cps4E*	priming glycosyltransferase	1.04	1.42E-02
lp_2190	*lp_2190*	cell division protein (putative)	1.06	1.20E-02
lp_2463	*lp_2463*	prophage P2b protein 18	2.03	2.85E-02
lp_2659	*xpk1*	phosphoketolase	1.60	5.03E-03
lp_2696	*lp_2696*	integral membrane protein	1.06	4.81E-06
lp_2697	*pyrE*	orotate phosphoribosyltransferase	1.74	4.38E-03
lp_2698	*pyrF*	orotidine-5'-phosphate decarboxylase	1.96	6.23E-04
lp_2699	*pyrD*	dihydroorotate oxidase	2.15	2.68E-03
lp_2701	pyrAA	*carbamoyl-phosphate synthase, pyrimidine-specific, small chain*	*2.23*	*2.79E-02*
lp_2702	*pyrC*	dihydroorotase	2.04	1.20E-02
lp_2710	*lp_2710*	purine transport protein	1.29	1.90E-03
lp_2768	*lp_2768*	transport protein	1.11	1.05E-02
lp_3127	*lp_3127*	cell surface protein precursor, GY family	1.10	3.57E-02

Table 2B: Genes significantly down regulated in cells grown on glucose compared to cells grown on sucrose. Ratio's are in ^{2}log scale.

ORF	*Name*	*Product*	*R*	*p-Value*
lp_0063	*lp_0063*	hypothetical protein	-1.24	2.14E-02
lp_0096	*lp_0096*	hypothetical protein	-1.63	6.84E-04
lp_0098	*lp_0098*	adenylyl transferase (putative)	-1.01	1.64E-03
lp_0154	*lp_0154*	hypothetical protein	-2.78	5.96E-04
lp_0155	*lp_0155*	hypothetical protein	-1.92	6.40E-06
lp_0156	*lp_0156*	hypothetical protein	-2.46	1.71E-03
lp_0184	*sacK1*	fructokinase	-4.71	6.48E-04
lp_0185	*pts1BCA*	Sucrose PTS EIIBCA	-6.21	4.51E-06
lp_0187	*sacA*	beta-fructofuranosidase	-6.22	2.31E-06
lp_0188	*sacR*	sucrose operon repressor	-3.97	8.08E-04
lp_0189	*agl2*	alpha-glucosidase	-5.05	7.07E-05
lp_0233	*mtlD*	mannitol-1-phosphate 5-dehydrogenase	-2.26	2.43E-02
lp_0285	*lp_0285*	transcription regulator	-1.22	3.54E-02
lp_0286	*pts6C*	cellobiose PTS, EIIC	-4.73	2.07E-08
lp_0310	*ack2*	acetate kinase	-2.69	5.22E-04
lp_0355	*sufI*	cell division protein SufI	-1.15	2.46E-03
lp_0394	*lp_0394*	transport protein	-1.51	3.16E-02
lp_0395	*lp_0395*	hydrolase, HAD superfamily, Cof family	-1.56	1.47E-02
lp_0396	*lp_0396*	transcription regulator	-1.70	1.03E-04
lp_0436	*pts7C*	cellobiose PTS, EIIC	-1.02	4.68E-02
lp_0472	*lp_0472*	integral membrane protein	-1.05	1.87E-02
lp_0754	*hprK*	bifunctional protein: HPr kinase; P-ser-HPr phosphatase	-1.20	5.26E-05
lp_0852	*pox2*	pyruvate oxidase	-1.42	2.05E-02
lp_0899	*lp_0899*	hypothetical protein	-1.53	1.20E-03
lp_0906	*pbg2*	6-phospho-beta-glucosidase	-1.05	3.54E-02
lp_1069	*ndh2*	NADH dehydrogenase	-1.64	1.15E-04
lp_1070	*lp_1070*	lipoprotein precursor	-1.27	5.52E-06
lp_1101	*ldhL2*	L-lactate dehydrogenase	-1.07	3.34E-06
lp_1102	*lp_1102*	cation transport protein	-1.42	4.87E-03
lp_1168	*lp_1168*	hypothetical protein	-1.84	1.64E-06
lp_1353	*lp_1353*	hydrolase, HAD superfamily, Cof family	-1.95	2.48E-03
lp_1457	*lp_1457*	hypothetical protein	-1.21	4.20E-02
lp_1539	*lp_1539*	lipoprotein precursor	-1.00	4.46E-03
lp_1546	*lp_1546*	prenyltransferase	-1.03	4.24E-04
lp_1566	*lp_1566*	hypothetical protein	-1.57	2.61E-02
lp_1763	*lp_1763*	glycosyltransferase	-2.27	3.90E-03
lp_1872	*lp_1872*	hypothetical protein	-1.01	2.07E-03
lp_2095	*fruR*	transcription regulator of fructose operon	-1.75	6.59E-06
lp_2096	*fruK*	1-phosphofructokinase	-1.35	1.60E-04
lp_2097	*pts16*	fructose PTS, EIIABC	-1.88	1.85E-03
lp_2151	*pdhD*	pyruvate dehydrogenase complex, E3 component; dihydrolipoamide dehydrogenase	-2.12	2.19E-05
lp_2152	*pdhC*	pyruvate dehydrogenase complex, E2 component; dihydrolipoamide S-acetyltransferase	-1.84	3.97E-03
lp_2154	*pdhA*	pyruvate dehydrogenase complex, E1 component, alpha subunit	-4.51	5.75E-05
lp_2340	*lp_2340*	nucleotide-binding protein, universal stress protein UspA family	-1.10	8.32E-03
lp_2601	*lp_2601*	hydrolase, HAD superfamily, Cof family	-1.14	3.65E-03
lp_2629	*pox3*	pyruvate oxidase	-1.05	3.02E-03
lp_2633	*trxH*	thioredoxin H-type	-1.13	2.15E-02
lp_2635	*lp_2635*	integral membrane protein	-1.29	4.49E-02
lp_2647	*pts19A*	N-acetylglucosamine/galactosamine PTS, EIIA	-1.08	5.35E-04
lp_2668	*hsp2*	small heat shock protein	-1.14	7.39E-03
lp_2733	*lp_2733*	oxidoreductase	-1.58	1.94E-02
lp_2747	*gtcA2*	teichoic acid glycosylation protein	-1.69	3.41E-04
lp_2777	*pbg4*	6-phospho-beta-glucosidase	-2.75	1.10E-03
lp_2778	*pbg5*	6-phospho-beta-glucosidase	-2.13	4.26E-07
lp_2780	*pts20A*	cellobiose PTS, EIIA	-3.69	9.58E-07
lp_2781	*pts20B*	cellobiose PTS, EIIB	-3.08	5.69E-05
lp_2787	*lp_2787*	hydrolase, HAD superfamily, Cof family	-1.62	2.38E-03
lp_2813	*lp_2813*	hypothetical protein	-1.34	8.85E-05
lp_2858	*lp_2858*	ABC transporter, ATP-binding protein, N-terminal fragment	-1.31	1.90E-02
lp_2861	*thgA3*	galactoside O-acetyltransferase	-1.57	4.69E-02
lp_2923	*lp_2923*	lipase/esterase (putative)	-1.25	5.66E-03
lp_2975	*lp_2975*	extracellular protein	-1.36	4.90E-02
lp_3009	*pts23B*	cellobiose PTS, EIIB	-2.12	1.81E-05
lp_3010	*pts23C*	cellobiose PTS, EIIC	-1.10	1.94E-02
lp_3011	*pbg6*	6-phospho-beta-glucosidase	-1.11	4.37E-05
lp_3078	*lp_3078*	hydrolase, HAD superfamily	-2.28	8.83E-05
lp_3092	*gabD*	succinate-semialdehyde dehydrogenase (NAD(P)+)	-1.15	1.99E-10

lp_3124	*lp_3124*	transcription regulator	-1.21	9.55E-03
lp_3136	*lp_3136*	transport protein	-1.23	1.31E-04
lp_3232	*lp_3232*	hypothetical protein	-1.42	1.94E-03
lp_3234	*lp_3234*	transcription regulator	-1.63	1.31E-04
lp_3244	*lp_3244*	oxidoreductase	-1.10	1.00E-05
lp_3314	*pflA2*	formate acetyltransferase activating enzyme	-1.23	3.83E-03
lp_3418	*pck*	phosphoenolpyruvate carboxykinase (ATP)	-1.23	1.14E-03
lp_3449	*nox5*	NADH oxidase	-1.42	1.74E-03
lp_3483	*lacL*	beta-galactosidase, large subunit	-1.32	5.62E-05
lp_3484	*lacM*	beta-galactosidase, small subunit	-2.48	1.79E-02
lp_3486	*lacS2*	lactose transport protein	-1.02	1.73E-02
lp_3487	*galM3*	aldose 1-epimerase	-1.83	1.13E-02
lp_3489	*lp_3489*	oxidoreductase	-1.12	3.37E-05
lp_3525	*pbg9*	6-phospho-beta-glucosidase	-1.73	4.85E-05
lp_3526	*pbg10*	6-phospho-beta-glucosidase	-2.16	1.08E-04
lp_3529	*bglG5*	transcription antiterminator	-1.80	4.29E-06
lp_3605	*iolG1*	myo-inositol 2-dehydrogenase	-1.12	7.70E-05
lp_3606	*iolG2*	myo-inositol 2-dehydrogenase	-2.07	1.54E-04
lp_3607	*iolE*	inositol catabolism protein IolE	-1.46	3.57E-09
lp_3608	*iolG3*	myo-inositol 2-dehydrogenase	-1.49	9.10E-05
lp_3611	*lp_3611*	sugar transport protein	-1.47	1.24E-03
lp_3612	*iolG4*	myo-inositol 2-dehydrogenase	-2.46	9.56E-04
lp_3613	*lp_3613*	hypothetical protein	-2.23	3.73E-03
lp_3634	*lp_3634*	alpha-1,2-mannosidase (putative)	-1.40	5.67E-07
lp_3644	*lp_3644*	sugar ABC transporter, permease protein	-1.07	6.26E-03

Table 3A: Genes significantly up regulated in cells grown on glucose compared to cells grown on lactose. Ratio's are in ^{2}log scale.

ORF	*Name*	*Product*	*R*	*p-Value*
lp_0018	*lp_0018*	lipoprotein precursor, peptide binding protein OppA homolog	1.15	4.86E-08
lp_0201	*lp_0201*	lipoprotein precursor, peptide binding protein OppA homolog	1.96	5.66E-05
lp_0202	*lp_0202*	acetyltransferase (putative)	1.98	3.36E-04
lp_0203	*serA1*	phosphoglycerate dehydrogenase	1.74	8.12E-08
lp_0204	*serC*	phosphoserine aminotransferase	1.32	4.44E-07
lp_0255	*metC1*	cystathionine beta-lyase	1.19	6.46E-03
lp_0339	*lp_0339*	N-succinyldiaminopimelate aminotransferase	1.18	1.45E-05
lp_0349	*amtB*	ammonium transport protein	1.10	2.59E-06
lp_0350	*hicD1*	L-2-hydroxyisocaproate dehydrogenase	1.85	1.20E-06
lp_0351	*lp_0351*	integral membrane protein	1.39	2.04E-02
lp_0433	*lp_0433*	hypothetical protein	1.02	2.44E-04
lp_0512	*rpmE*	ribosomal protein L31	1.19	2.54E-05
lp_0575	*pts9AB*	mannose PTS, EIIAB	1.32	2.33E-04
lp_0576	*pts9C*	mannose PTS, EIIC	1.30	9.28E-04
lp_0577	*pts9D*	mannose PTS, EIID	1.32	3.66E-05
lp_1012	*serS2*	serine--tRNA ligase	1.02	3.00E-05
lp_1082	*lp_1082*	malate / lactate dehydrogenase	1.22	1.29E-04
lp_1083	*tkt2*	transketolase	1.08	4.35E-04
lp_1084	*aroD1*	shikimate 5-dehydrogenase	2.04	3.41E-08
lp_1085	*aroA*	phospho-2-dehydro-3-deoxyheptonate aldolase / chorismate mutase	1.13	6.59E-06
lp_1086	*aroB*	3-dehydroquinate synthase	1.32	1.30E-08
lp_1087	*lp_1087*	cation transport protein	1.24	7.41E-04
lp_1089	*ttdB*	L(+)-tartrate dehydratase, subunit B	1.86	1.80E-05
lp_1722	*lp_1722*	amino acid transport protein	1.04	7.95E-03
lp_1765	*lp_1765*	hypothetical protein	1.07	3.35E-02
lp_2033	*aroI*	shikimate kinase	1.97	6.99E-09
lp_2034	*tyrA*	prephenate dehydrogenase	2.23	1.39E-07
lp_2035	*aroE*	3-phosphoshikimate 1-carboxyvinyltransferase	1.62	2.48E-07
lp_2038	*lp_2038*	transport protein	1.77	4.69E-06
lp_2061	*lp_2061*	hypothetical protein	1.32	1.62E-08
lp_2371	*pyrP*	uracil transport protein	1.57	4.61E-06
lp_2697	*pyrE*	orotate phosphoribosyltransferase	1.78	5.46E-10
lp_2698	*pyrF*	orotidine-5'-phosphate decarboxylase	2.11	3.43E-06

lp_2701	*pyrAA*	carbamoyl-phosphate synthase, pyrimidine-specific, small chain	3.21	1.48E-06
lp_2702	*pyrC*	dihydroorotase	1.76	1.15E-09
lp_2703	*pyrB*	aspartate carbamoyltransferase	3.59	1.61E-04
lp_2704	*pyrR1*	pyrimidine operon regulator	2.44	5.21E-06
lp_2710	*lp_2710*	purine transport protein	1.72	2.17E-05
lp_2714	*lp_2714*	protein containing diguanylate cyclase/phosphodiesterase domain 2 (EAL)	1.10	2.29E-02
lp_2788	*panE2*	2-dehydropantoate 2-reductase	2.16	5.54E-07
lp_2789	*lp_2789*	transport protein	1.03	2.91E-07
lp_2825	*lp_2825*	purine nucleosidase	2.02	7.87E-06

Table 3b: Genes significantly down regulated in cells grown on glucose compared to cells grown on lactose. Ratio's are in ^{2}log scale.

ORF	*Name*	*Product*	*R*	*p-Value*
lp_0108	*glpF1*	glycerol uptake facilitator protein	-1.01	4.48E-03
lp_0154	*lp_0154*	hypothetical protein	-1.03	5.68E-07
lp_0155	*lp_0155*	hypothetical protein	-1.19	8.59E-03
lp_0156	*lp_0156*	hypothetical protein	-1.03	2.20E-04
lp_0175	*malE*	maltose/maltodextrin ABC transporter, substrate binding protein	-2.38	2.39E-04
lp_0176	*malF*	maltose/maltodextrin ABC transporter, permease protein	-2.45	6.58E-06
lp_0178	*malA*	maltose/maltodextrin ABC transporter subunit (putatie)	-1.72	1.08E-02
lp_0244	*lp_0244*	oxidoreductase	-1.01	4.99E-03
lp_0266	*lp_0266*	hypothetical protein	-1.07	7.21E-04
lp_0286	*pts6C*	cellobiose PTS, EIIC	-2.74	4.02E-08
lp_0310	*ack2*	acetate kinase	-1.00	4.51E-05
lp_0361	*lp_0361*	hypothetical protein	-2.02	1.55E-04
lp_0472	*lp_0472*	integral membrane protein	-1.11	1.03E-06
lp_0872	*gph1*	phosphoglycolate phosphatase (putative)	-1.20	7.92E-03
lp_0899	*lp_0899*	hypothetical protein	-1.06	8.62E-03
lp_1070	*lp_1070*	lipoprotein precursor	-1.15	4.74E-05
lp_1120	*lp_1120*	amino acid transport protein	-1.02	6.26E-07
lp_1169	*gdh*	glutamate dehydrogenase (NAD(P)+)	-1.33	2.28E-06
lp_1698	*lp_1698*	hypothetical protein	-1.89	2.25E-02
lp_1836	*msrA3*	protein-methionine-S-oxide reductase	-2.31	4.83E-03
lp_2151	*pdhD*	pyruvate dehydrogenase complex, E3 component; dihydrolipoamide dehydrogenase	-1.02	5.83E-05
lp_2152	*pdhC*	pyruvate dehydrogenase complex, E2 component; dihydrolipoamide S-acetyltransferase	-1.17	3.16E-04
lp_2154	*pdhA*	pyruvate dehydrogenase complex, E1 component, alpha subunit	-1.88	2.71E-04
lp_2544	*npr2*	NADH peroxidase	-1.21	1.66E-04
lp_2629	*pox3*	pyruvate oxidase	-1.86	8.85E-08
lp_2685	*dapA2*	dihydrodipicolinate synthase	-1.05	4.47E-04
lp_2777	*pbg4*	6-phospho-beta-glucosidase	-1.15	1.06E-03
lp_2778	*pbg5*	6-phospho-beta-glucosidase	-1.22	4.26E-04
lp_2780	*pts20A*	cellobiose PTS, EIIA	-1.82	1.87E-05

lp_2781	*pts20B*	cellobiose PTS, EIIB	-1.71	4.64E-05
lp_2799	*lp_2799*	amino acid transport protein	-1.24	4.31E-05
lp_2872	*lp_2872*	oxidoreductase	-1.16	7.04E-06
lp_2873	*adh2*	alcohol dehydrogenase	-1.26	4.71E-04
lp_2874	*lp_2874*	short-chain dehydrogenase/oxidoreductase	-1.28	3.15E-05
lp_2969	*pts22CBA*	N-acetylglucosamine PTS, EIICBA	-1.60	6.63E-07
lp_3092	*gabD*	succinate-semialdehyde dehydrogenase (NAD(P)+)	-1.17	8.88E-06
lp_3124	*lp_3124*	transcription regulator	-1.06	4.02E-04
lp_3278	*lp_3278*	amino acid transport protein	-1.57	1.70E-07
lp_3356	*lp_3356*	acetyltransferase (putative)	-1.62	4.61E-03
lp_3449	*nox5*	NADH oxidase	-1.89	1.53E-06
lp_3468	*lacS1*	lactose transport protein	-3.42	6.44E-10
lp_3469	*lacA*	beta-galactosidase I	-4.73	2.81E-05
lp_3471	*ram1*	alpha-L-rhamnosidase (putative)	-1.18	1.06E-04
lp_3472	*lp_3472*	sugar transport protein	-1.47	2.89E-04
lp_3478	*lp_3478*	transcription regulator	-1.54	2.88E-05
lp_3480	*galT*	UTP--hexose-1-phosphate uridylyltransferase	-4.20	1.72E-08
lp_3481	*galE4*	UDP-glucose 4-epimerase	-4.33	2.75E-08
lp_3482	*galK*	galactokinase	-4.93	1.83E-08
lp_3483	*lacL*	beta-galactosidase, large subunit	-3.80	1.44E-05
lp_3484	*lacM*	beta-galactosidase, small subunit	-3.14	4.66E-07
lp_3485	*melA*	alpha-galactosidase	-2.65	1.55E-05
lp_3486	*lacS2*	lactose transport protein	-3.00	2.25E-06
lp_3487	*galM3*	aldose 1-epimerase	-3.38	7.27E-05
lp_3489	*lp_3489*	oxidoreductase	-1.61	1.21E-05
lp_3589	*pox5*	pyruvate oxidase	-1.99	5.06E-04
lp_3619	*pts37BC*	sorbitol PTS, EIIBC	-1.52	3.20E-03

Table 4A: Genes significantly up regulated in cells grown on glucose compared to cells grown on fructose. Ratio's are in ^{2}log scale.

ORF	*Name*	*Product*	*R*	*p-Value*
lp_0201	*lp_0201*	lipoprotein precursor, peptide binding protein OppA homolog	2.53	3.82E-02
lp_0317	*potB*	spermidine/putrescine ABC transporter, permease protein	1.25	1.59E-03
lp_0329	*acdH*	acetaldehyde dehydrogenase	1.64	1.70E-05
lp_0339	*lp_0339*	N-succinyldiaminopimelate aminotransferase	1.07	2.33E-03
lp_0349	*amtB*	ammonium transport protein	1.68	4.94E-02
lp_0367	*choS*	glycine betaine/carnitine/choline ABC transporter, substrate binding and permease protein	1.33	1.62E-05
lp_0512	*rpmE*	ribosomal protein L31	1.48	3.04E-03
lp_0575	*pts9AB*	mannose PTS, EIIAB	3.32	1.58E-06
lp_0576	*pts9C*	mannose PTS, EIIC	4.03	1.06E-07
lp_0577	*pts9D*	mannose PTS, EIID	4.28	3.89E-10
lp_0625	*lp_0625*	prophage P1 protein 2	1.13	1.55E-04
lp_0628	*lp_0628*	prophage P1 protein 5	1.73	9.43E-04
lp_0642	*lp_0642*	prophage P1 protein 19	1.38	3.06E-03
lp_0658	*lp_0658*	prophage P1 protein 35	1.06	5.04E-04
lp_0790	*pgk*	phosphoglycerate kinase	1.16	2.50E-10
lp_0802	*lp_0802*	glutamine ABC transporter, substrate binding and permease protein	1.01	1.59E-04
lp_0803	*glnQ1*	glutamine ABC transporter, ATP-binding protein	1.03	1.32E-04
lp_0874	*lp_0874*	bifunctional protein: amino acid aminotransferase; 2-hydroxyacid dehydrogenase	1.06	1.45E-02
lp_0915	*lp_0915*	integral membrane protein	1.20	1.32E-04
lp_0938	*hsdR*	type I site-specific deoxyribonuclease, HsdR subunit	1.06	5.77E-06
lp_0997	*cspC*	cold shock protein CspC	1.49	7.46E-03
lp_1032	*rpsJ*	ribosomal protein S10	1.19	2.81E-05
lp_1033	*rplC*	ribosomal protein L3	1.19	3.28E-04
lp_1034	*rplD*	ribosomal protein L4	1.29	8.62E-06
lp_1035	*rplW*	ribosomal protein L23	1.75	2.22E-05
lp_1036	*rplB*	ribosomal protein L2	1.51	6.84E-05
lp_1038	*rpsS*	ribosomal protein S19	1.61	4.76E-05
lp_1039	*rplV*	ribosomal protein L22	1.44	4.24E-06

lp_1040	*rpsC*	ribosomal protein S3	1.47	1.41E-04
lp_1041	*rplP*	ribosomal protein L16	1.55	1.79E-06
lp_1044	*rpsQ*	ribosomal protein S17	1.75	1.38E-04
lp_1045	*rplN*	ribosomal protein L14	1.49	2.42E-06
lp_1046	*rplX*	ribosomal protein L24	1.36	5.39E-06
lp_1047	*rplE*	ribosomal protein L5	1.61	5.55E-08
lp_1050	*rpsH*	ribosomal protein S8	1.51	5.56E-06
lp_1051	*rplF*	ribosomal protein L6	1.49	8.21E-06
lp_1052	*rplR*	ribosomal protein L18	1.46	2.01E-05
lp_1053	*rpsE*	ribosomal protein S5	1.19	3.62E-05
lp_1055	*rplO*	ribosomal protein L15	1.53	4.15E-04
lp_1056	*secY*	preprotein translocase, SecY subunit	1.48	8.43E-04
lp_1058	*adk*	adenylate kinase	1.28	3.69E-05
lp_1059	*infA*	translation initiation factor IF-1	1.15	2.38E-04
lp_1059a	*rpmJ*	ribosomal protein L36	1.13	1.39E-03
lp_1060	*rpsM*	ribosomal protein S13	1.09	3.88E-03
lp_1061	*rpsK*	ribosomal protein S11	1.06	2.71E-03
lp_1062	*rpoA*	DNA-directed RNA polymerase, alpha subunit	1.10	7.29E-03
lp_1063	*rplQ*	ribosomal protein L17	1.14	7.18E-03
lp_1082	*lp_1082*	malate / lactate dehydrogenase	1.31	6.86E-03
lp_1083	*tkt2*	transketolase	1.83	2.38E-02
lp_1084	*aroD1*	shikimate 5-dehydrogenase	2.51	6.73E-08
lp_1085	*aroA*	phospho-2-dehydro-3-deoxyheptonate aldolase / chorismate mutase	1.56	3.16E-03
lp_1086	*aroB*	3-dehydroquinate synthase	1.84	1.15E-07
lp_1087	*lp_1087*	cation transport protein	1.65	4.69E-04
lp_1088	*lp_1088*	hypothetical protein	1.43	3.87E-03
lp_1089	*ttdB*	L(+)-tartrate dehydratase, subunit B	1.31	2.98E-03
lp_1090	*ttdA*	L(+)-tartrate dehydratase, subunit A	1.31	2.62E-02
lp_1102	*lp_1102*	cation transport protein	2.24	1.30E-02
lp_1105	*mae*	malic enzyme, NAD-dependent	1.11	1.76E-02
lp_1107	*citD*	citrate lyase, acyl carrier protein	1.07	6.62E-07
lp_1110	*lp_1110*	unknown	1.01	1.31E-03
lp_1112	*fum*	fumarate hydratase	3.27	2.89E-02
lp_1151	*lp_1151*	RNA methyltransferase	1.22	3.24E-02
lp_1160	*cspP*	cold shock protein CspP	1.13	1.61E-03
lp_1169	*gdh*	glutamate dehydrogenase (NAD(P)+)	1.47	3.72E-02
lp_1176	*glf1*	UDP-galactopyranose mutase	1.04	6.25E-06
lp_1177	*cps1A*	polysaccharide biosynthesis protein	1.10	2.24E-04
lp_1179	*cps1C*	repeat unit transporter	1.14	1.94E-05
lp_1180	*cps1D*	glycosyltransferase	1.16	1.05E-05
lp_1181	*cps1E*	acyltransferase/acetyltransferase	1.24	4.98E-03
lp_1182	*cps1F*	exopolysaccharide biosynthesis protein	1.24	1.60E-05
lp_1184	*cps1H*	glycosyltransferase (rhamnosyltransferase)	1.16	2.55E-05
lp_1185	*cps1I*	polysaccharide polymerase	1.07	4.79E-05
lp_1186	*rfbA*	glucose-1-phosphate thymidylyltransferase	1.07	3.68E-07
lp_1187	*lp_1187*	unknown	1.16	1.70E-03
lp_1188	*rfbC*	dTDP-4-dehydrorhamnose 3,5-epimerase	1.00	1.60E-03
lp_1190	*rfbD*	dTDP-4-dehydrorhamnose reductase	1.11	3.52E-03
lp_1245	*hicD2*	L-2-hydroxyisocaproate dehydrogenase	1.18	5.23E-07
lp_1261	*oppA*	oligopeptide ABC transporter, substrate binding protein	1.06	5.02E-05
lp_1330	*mscL*	large conductance mechanosensitive channel	1.08	4.27E-03
lp_1336	*lp_1336*	ABC transporter, ATP-binding protein	1.00	1.76E-03
lp_1357	*lp_1357*	extracellular protein, membrane-anchored (putative)	2.94	8.33E-04
lp_1368	*lp_1368*	hypothetical protein	1.02	1.58E-06
lp_1375	*metE*	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase	1.18	7.91E-04
lp_1391	*argS*	arginine--tRNA ligase	1.12	1.15E-02
lp_1640	*rplS*	ribosomal protein L19	1.05	1.82E-02
lp_1744	*lp_1744*	amino acid ABC transporter, ATP-binding protein	1.65	3.68E-02
lp_1765	*lp_1765*	hypothetical protein	1.21	6.84E-03
lp_1766	*lp_1766*	hypothetical protein	1.27	1.28E-02
lp_1767	*lp_1767*	lysin	2.29	3.63E-02
lp_1782	*pyrR2*	pyrimidine operon regulator	1.44	4.90E-07
lp_1783	*pyrAA2*	carbamoyl-phosphate synthase (glutamine-hydrolysing), small chain	1.67	3.10E-06
lp_1784	*pyrAB2*	carbamoyl-phosphate synthase (glutamine-hydrolysing), large chain, truncated	1.52	1.60E-06
lp_1785	*pgm5*	phosphoglycerate mutase (putative)	1.09	3.06E-04
lp_1965	*glyQ*	glycine--tRNA ligase, alpha chain	1.26	1.16E-06
lp_1981	*hisS*	histidine--tRNA ligase	1.15	6.83E-07
lp_2019	*dltA*	D-alanine activating enzyme DltA	1.09	1.83E-03
lp_2021	*pbpX2*	serine-type D-Ala-D-Ala carboxypeptidase	1.03	1.92E-04
lp_2033	*aroI*	shikimate kinase	2.07	1.07E-05

lp_2034	*tyrA*	prephenate dehydrogenase	2.18	1.38E-04
lp_2035	*aroE*	3-phosphoshikimate 1-carboxyvinyltransferase	1.57	2.95E-06
lp_2038	*lp_2038*	transport protein	1.47	8.29E-06
lp_2125	*rpsO*	ribosomal protein S15	1.29	4.31E-03
lp_2312	*glnH2*	glutamine ABC transporter, substrate binding protein	1.46	2.55E-02
lp_2313	*glnQ4*	glutamine ABC transporter, ATP-binding protein	1.54	3.32E-02
lp_2371	*pyrP*	uracil transport protein	3.02	5.70E-05
lp_2449	*lp_2449*	prophage P2a protein 8	1.44	7.73E-03
lp_2450	*lp_2450*	prophage P2a protein 7	1.28	5.01E-05
lp_2451	*lp_2451*	prophage P2a protein 6	1.65	1.13E-06
lp_2453	*lp_2453*	prophage P2a protein 4	1.25	3.56E-06
lp_2537	*metA*	homoserine O-succinyltransferase	1.17	3.84E-02
lp_2552	*hisE*	phosphoribosyl-ATP pyrophosphatase	1.49	6.76E-03
lp_2553	*hisI*	phosphoribosyl-AMP cyclohydrolase	1.29	5.05E-03
lp_2556	*hisA*	phosphoribosylformimino-5-aminoimidazole carboxamideribotide isomerase	1.23	2.76E-02
lp_2557	*hisH*	amidotransferase	1.41	8.76E-03
lp_2560	*hisG*	ATP phosphoribosyltransferase	1.19	2.05E-02
lp_2561	*hisX*	histidine--tRNA ligase (putative)	1.34	2.10E-03
lp_2659	*xpk1*	phosphoketolase	1.72	2.90E-05
lp_2697	*pyrE*	orotate phosphoribosyltransferase	2.19	1.01E-04
lp_2698	*pyrF*	orotidine-5'-phosphate decarboxylase	2.64	3.51E-05
lp_2699	*pyrD*	dihydroorotate oxidase	4.97	8.50E-05
lp_2701	*pyrAA*	carbamoyl-phosphate synthase, pyrimidine-specific, small chain	5.16	3.36E-04
lp_2702	*pyrC*	dihydroorotase	2.61	1.89E-03
lp_2703	*pyrB*	aspartate carbamoyltransferase	4.96	1.08E-04
lp_2704	*pyrR1*	pyrimidine operon regulator	3.80	1.94E-06
lp_2708	*pucR*	purine transport regulator	1.13	3.42E-06
lp_2710	*lp_2710*	purine transport protein	1.60	2.05E-03
lp_2714	*lp_2714*	protein containing diguanylate cyclase/phosphodiesterase domain 2 (EAL)	1.00	3.44E-04
lp_2759	*lp_2759*	hydrolase, HAD superfamily, Cof family	1.19	2.27E-04
lp_2760	*lp_2760*	hypothetical protein	1.06	1.37E-05
lp_2788	*panE2*	2-dehydropantoate 2-reductase	1.77	6.52E-05
lp_2825	*lp_2825*	purine nucleosidase	3.23	7.13E-04
lp_2958	*wapA*	cell surface protein precursor	1.04	3.36E-05
lp_2984	*livB*	branched-chain amino acid ABC transporter, permease protein	1.09	1.73E-02
lp_3020	*tag2*	DNA-3-methyladenine glycosylase I	1.03	4.78E-02
lp_3091	*lp_3091*	hypothetical protein	1.50	3.66E-02
lp_3150	*lp_3150*	malate dehydrogenase (putative)	1.27	4.01E-07
lp_3151	*acm3-N*	muramidase, N-terminal fragment	1.39	1.49E-07
lp_3154	*acm3-C*	muramidase, C-terminal fragment	1.60	1.70E-02
lp_3169	*lp_3169*	hypothetical protein	1.33	1.05E-03
lp_3193	*prtM2*	peptidylprolyl isomerase	1.46	9.21E-07
lp_3207	*lp_3207*	aminotransferase	2.42	2.20E-08
lp_3217	*lp_3217*	integral membrane protein (putative)	1.14	3.05E-02
lp_3278	*lp_3278*	amino acid transport protein	1.04	7.33E-03
lp_3283	*lp_3283*	hypothetical protein	1.15	4.22E-07
lp_3338	*nha2*	Na(+)/H(+) antiporter	1.20	2.02E-03
lp_3488	*galR2*	galactose operon repressor	1.17	1.05E-05
lp_3491	*lp_3491*	fumarate reductase, flavoprotein subunit precursor	1.23	2.84E-02
lp_3495	*lp_3495*	transcription regulator	1.02	4.47E-07
lp_3502	*lp_3502*	transcription regulator	1.09	1.39E-09
lp_3508	*lp_3508*	glucokinase regulatory protein	1.19	7.15E-03
lp_3579	*spx5*	negative regulator of proteolysis	1.04	1.30E-05
lp_3607	*iolE*	inositol catabolism protein IolE	1.26	2.51E-02
lp_3672	*lp_3672*	transcription regulator	1.32	5.38E-09
lp_3673	*pepC2*	cysteine aminopeptidase	1.01	2.22E-03

Table 4B: Genes significantly down regulated in cells grown on glucose compared to cells grown on fructose. Ratio's are in ^{2}log scale.

ORF	*Name*	*Product*	*Ratio*	*p-Value*
lp_0219	*lp_0219*	ABC transporter subunit (putative)	-1.28	5.21E-03
lp_0248	*lp_0248*	hypothetical protein	-1.22	1.12E-03
lp_0286	*pts6C*	cellobiose PTS, EIIC	-1.44	2.85E-03
lp_0302	*lp_0302*	extracellular protein	-1.69	1.19E-06
lp_0473	*lp_0473*	lipoprotein precursor (putative)	-1.01	7.29E-06
lp_0538	*pth*	aminoacyl-tRNA hydrolase	-1.08	1.03E-02
lp_0554	*lp_0554*	hypothetical protein	-1.13	1.46E-03
lp_0584	*lp_0584*	transport protein (putative)	-1.07	1.20E-02
lp_0761	*trxB1*	thioredoxin reductase (NADPH)	-1.03	1.07E-06
lp_0783	*lp_0783*	lipoprotein precursor, peptide binding protein OppA homolog	-1.38	6.74E-05
lp_0822	*glmS1*	glutamine-fructose-6-phosphate transaminase (isomerizing)	-1.36	2.57E-04
lp_0852	*pox2*	pyruvate oxidase	-1.13	3.31E-04
lp_0865	*lp_0865*	hypothetical protein	-1.74	6.96E-03
lp_0872	*gph1*	phosphoglycolate phosphatase (putative)	-1.09	1.41E-02
lp_0875	*lp_0875*	hypothetical protein	-1.94	1.43E-04
lp_0889	*lp_0889*	transcription regulator	-1.01	2.41E-03
lp_0935	*lp_0935*	hypothetical protein	-1.07	7.35E-04
lp_0990	*lp_0990*	hypothetical protein	-1.21	1.51E-02
lp_0992	*lp_0992*	hypothetical protein	-1.36	1.81E-04
lp_1171	*pbpX1*	serine-type D-Ala-D-Ala carboxypeptidase	-1.10	2.01E-02
lp_1239	*lp_1239*	hypothetical protein	-1.01	2.82E-02
lp_1362	*lp_1362*	hypothetical protein	-1.58	6.91E-06
lp_1403	*lp_1403*	cell surface protein, ErfK family	-1.20	1.30E-02
lp_1435	*lp_1435*	integral membrane protein	-1.35	1.31E-06
lp_1552	*lp_1552*	integral membrane protein	-1.05	2.07E-03
lp_1611	*lp_1611*	hypothetical protein	-1.47	2.01E-04
lp_1670	*fabZ1*	(3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase	-1.40	1.45E-07
lp_1672	*acpA2*	acyl carrier protein	-1.42	9.29E-07
lp_1678	*accC2*	acetyl-CoA carboxylase, biotin carboxylase subunit	-1.09	1.69E-04
lp_1682	*lp_1682*	phosphopantetheinyltransferase	-1.50	6.47E-06
lp_1684	*lp_1684*	integral membrane protein	-1.31	2.01E-05
lp_1685	*lp_1685*	transcription regulator	-1.18	8.19E-12
lp_1686	*lp_1686*	acyl-CoA thioester hydrolase (putative)	-1.19	9.66E-07
lp_1687	*lp_1687*	GTPase	-1.08	1.16E-04
lp_1763	*lp_1763*	glycosyltransferase	-1.41	1.77E-02
lp_1812	*lp_1812*	lipoprotein precursor (putative)	-1.02	1.81E-03
lp_1908	*lp_1908*	integral membrane protein	-1.74	6.33E-04
lp_1913	*lp_1913*	hypothetical protein	-1.10	4.23E-02
lp_2056	*lp_2056*	hydrolase, HAD superfamily	-1.24	3.92E-08
lp_2076	*lp_2076*	nitrate ABC transporter, ATP-binding protein	-2.12	1.77E-02
lp_2078	*rodA1*	rod-shape determining protein	-1.16	2.35E-02
lp_2095	*fruR*	transcription regulator of fructose operon	-2.18	2.18E-05
lp_2096	*fruK*	1-phosphofructokinase	-2.17	4.67E-07
lp_2097	*pts16*	fructose PTS, EIIABC	-1.55	7.61E-06
lp_2099	*cps4J*	repeat unit transporter	-1.68	2.80E-03
lp_2100	*cps4I*	glycosyltransferase	-1.47	2.14E-03
lp_2403	*lp_2403*	prophage P2a protein 54	-1.28	3.39E-03
lp_2629	*pox3*	pyruvate oxidase	-1.34	5.93E-04
lp_2668	*hsp2*	small heat shock protein	-1.73	8.81E-05
lp_2669	*lp_2669*	hypothetical protein	-1.47	3.83E-04
lp_2770	*lp_2770*	hypothetical protein	-1.40	7.63E-08
lp_2771	*natC2*	nicotinate phosphoribosyltransferase	-3.08	2.89E-05
lp_2774	*lp_2774*	ABC transporter, ATP-binding protein	-1.23	1.73E-03
lp_2780	*pts20A*	cellobiose PTS, EIIA	-1.34	2.49E-07
lp_2781	*pts20B*	cellobiose PTS, EIIB	-1.20	3.11E-06
lp_2827	*napA3*	Na(+)/H(+) antiporter	-1.22	6.10E-03
lp_2909	*lp_2909*	acetyltransferase (putative)	-1.14	5.71E-07
lp_3014	*lp_3014*	extracellular protein	-2.07	2.98E-05
lp_3043	*zmp4*	extracellular zinc metalloproteinase	-1.09	4.20E-02
lp_3077	*lp_3077*	extracellular protein (putative)	-1.61	1.23E-07
lp_3165	*lp_3165*	transposase, fragment	-1.96	4.59E-03
lp_3172	*xylR*	xylose operon regulator	-1.01	2.52E-05
lp_3256	*lp_3256*	DegV family protein	-1.54	9.10E-07
lp_3279	*kup2*	potassium uptake protein	-1.75	1.71E-06
lp_3280	*lp_3280*	cation efflux protein	-1.26	1.64E-07
lp_3351	*lp_3351*	hypothetical protein	-1.04	4.43E-06
lp_3356	*lp_3356*	acetyltransferase (putative)	-1.88	1.93E-08
lp_3463	*glpF6*	glycerol uptake facilitator protein	-1.00	1.03E-05
lp_3589	*pox5*	pyruvate oxidase	-1.39	1.88E-04
lp_3676	*lp_3676*	extracellular protein	-1.46	4.71E-07
lp_3678	*lp_3678*	cell surface protein precursor	-1.61	3.81E-05
lp_3679	*lp_3679*	extracellular protein	-2.46	7.37E-05

Chapter 7

Summary, Concluding Remarks and Future Perspectives

The transcriptional response of bacteria to changing environmental conditions is an important mechanism for maintaining the bacterial viability (20). The research in this thesis describes the transcriptional response of *Lactobacillus plantarum* to a selected set of specific stress and other environmental conditions, and to targeted mutation of genes encoding global regulatory functions. These studies expanded our understanding of transcriptional mechanisms underlying sugar transport, sugar utilization, oxidative stress response and growth. Below, a summary is provided of main experimental approaches and their results that are placed in a general context and discussed with specific attention for their impact and future directions

Developments in microarray techniques

At the start of this PhD-project in 2002, transcriptome analysis was a relative new technology as elaborated in Chapter 1 and only a few papers applying the technique were available addressing the transcriptome responses in low G+C Gram-positive bacteria. The complete genome sequence of *L. plantarum* WCFS1 was available, but not yet published, and a cloned-based microarray had been constructed for this organism. Clone-based arrays do not require complete annotated genome and therefore can be produced at an early stage in (functional) genomic research. The lactate and bile salt stress response of *L. plantarum* as well as its response to a new quorum sensing peptide were elucidated using this array (4, 7, 17, 21). In addition, a DNA-DNA comparison of *L. plantarum* strains was performed to identify unique and/or variable regions in the genomes of these strains relative to the WCFS1 genome (13). However, this type of array has some drawback as it remains uncertain whether all genes are present on the array, until the entire genome is known. In addition, the gene identity is not known beforehand, and one spotted fragment may represent more than a single gene (16). The availability of a complete and annotated sequence of *L. plantarum* WCFS1 enabled the production of a full genome amplicon-based microarray that ideally contains spotted PCR-amplicons of each predicted gene. The advantage of this array design is that only one gene per spot is present and that the coverage of the array is known in advance. However, consistent production of high quality arrays of this type has proven to be a technical challenge. At first, amplification of all desired amplicons was not possible using a standard PCR-mixture. However, as amplicons were obtained for 97% of the genes, the coverage of the array was satisfactory. More severe problems occurred during the production of the array itself: the concentration and impurity of amplicons in a PCR-reaction mixture led to low signal-to-noise ratio and irregular spot shapes. Only purification and tenfold concentration of the amplicons led to satisfactory and consistent results and effectively applicable arrays.

As the technique of *in situ* oligonucleotide synthesis advanced, custom arrays became more readily accessible, and an *in situ* synthesised oligonucleotide array of *L. plantarum* is nowadays available and used in many experimental approaches in our institute (18, 19). The advantages of this array relative to the spotted amplicon-based array are mainly the higher signal-to-background ratio and the higher density of spots per square mm, allowing a higher number of probes on a slide.

Technical development of arrays and their production is still going on. An example is the tiling array which can cover a whole bacterial genome sequence, and not just the predicted open reading frames, which allows identification of point-mutations in a mutated strain by DNA-DNA hybridizations of the strain versus its wild type. This full genome coverage also allows one to obtain a highly detailed view of transcription, as not only data of predicted ORFs but also of the intergenic regions will be generated. Such an array will not only provide knowledge about up and down regulation of genes but also about transcription starts and termination, which might lead to identification of promoters, *cis*-acting elements, alternative transcription starts, non-coding RNAs, and anti-termination events. This will definitely generate a lot of useful data and lead to better biological understanding of transcriptional regulation.

The transcription of genes to RNA is just the first step to the functional unit of the protein. Proteomics is a technique with still some limitations, due to the different chemical properties and localization of proteins, which makes a complete isolation and separation with one standard method impossible. Proteome studies of *L. plantarum* led to identification 200 genes on a 2DE gels, which is an impressive number, but still less then 10% of the total number of genes (5). The availability of transcriptome as well as proteome data sets of the same samples from a wild-type and *ccpA*-mutant allowed us to compare the results of both ~omics approaches (**Chapter 5**). These comparative analyses revealed that while 80% of the proteins identified displayed the same direction of regulation as the corresponding transcript, approximately 30% appeared to be affected to a quantitatively similar extend at the level of transcript and protein abundance. The limited amount of identifiable proteins is a major drawback in proteome-transcriptome comparisons**.** An antibody microarray which allows high-throughput qualitative and quantitative identification of proteins on a glass slide is an emerging technology (3) and if this technology develops as its “older brother” the DNA-microarray has done over the past 12 years, a combined full transcriptomic-proteomic approaches will give more insight into the biology of the cell.

Microarray data interpretation

In the first years of transcriptomic research technical difficulties were a draw-back in performing analyses but it has now developed into a common tool in many areas of research. Nowadays, in the post-genomic era understanding transcriptome data and integrating them with data obtained from other ~omics approaches is the next challenge for biologists. Systems biology approaches that integrate functional genomics data by mathematical modelling techniques have huge potential in the field of data integration and might ultimately lead to prediction of adaptive behaviour of an organism (22). The metabolic model that was developed for *L. plantarum* (23) was used in **Chapter 4** as a mapping tool of array data. This data mapping revealed a remarkable activation of CO_2-producing pathways in aerobically grown mid-logarithmic cells as compared to early logarithmic cells, suggesting low CO_2-concentration in the medium led to a temporary reduction of the growth rate and to increased expression of CO_2-producing enzymes. This hypothesis could be confirmed by supplementation of the aerobic growth conditions by providing higher CO_2 gas pressure during these growth conditions, which was shown to eliminate the observed growth delay.

However, interpreting and understanding a relative small set of array data is already difficult, despite the presence of analyses tools such as data mapping and cluster analyses. The increasing amount of transcriptome data will also lead to an increasing amount of noise and maybe even false data. This makes the interpretation of data not only difficult, it might even obstruct the building of models that go beyond a straightforward interpretation of changes during limited changes of circumstances. It is therefore that initiatives like MGED (Microarray Gene Expression Data)-society have agreed on a number relevant data standards for microarray experiments. The main components are Minimum Information About a Microarray Experiment (MIAME), which concerns the minimal amount of information that should be reported about an array experiment and MAGE (MicroArray Gene Expression), which defines sets of common terms and annotation rules for microarray experiments (1). These standards prevent ambiguous interpretation of microarray data and will enable correct modelling, integration and interpretation of microarray data.

Transcriptome data

The original goal of the IOP-project, in which this project was embedded, was to perform transcriptional analyses in four Gram-positive bacteria (*Bacillus subtilis, Lactococcus lactis, L. plantarum*, and *B. cereus*) under similar conditions and additionally compare and predict the transcriptional responses in the four bacteria. Within the work package stress, lactate

stress was of high interest as the acid inhibits bacterial growth and is used as a food preservative. However, lactic acid bacteria are generally more resistant to lactate than other microorganisms. This is illustrated by the lactate shock response of *L. plantarum* compared to that of the food-pathogen *B. cereus*. The experiments were performed with identical L-lactate concentrations and pH (100 mM, pH 5.0). These conditions led to a significant change in the transcriptome of *B. cereus* (W. van Schaik, M. van der Voort, personal communication), while the response of *L. plantarum* to these conditions was highly limited (M. Stevens, unpublished data). It appeared that the chosen stress conditions were not harsh enough to trigger a stress response in *L. plantarum*, which is in agreement with previous observations (17). Only 10 genes were affected in all samples taken after 15, 30, and 60 minutes of stress exposure (Table 1).

Table 1: [2]Log-ratio of genes higher expressed at each of the three samples points during lactate stress conditions in L. plantarum. Samples were taken after 15, 30, and 60 minutes and the applied cut-off was an average 2 times differential expression over 2 experiments.

ORF	*Product*	*15'*	*30'*	*60'*
lp_0100	cobalt ABC transporter, ATP-binding protein (putative)	1.9	1.9	2.1
lp_0101	cobalt transport protein	3.7	3.7	4.0
lp_0102	cobalamin biosynthesis protein	3.4	3.4	3.5
lp_0103	transcription regulator	3.4	3.4	3.4
lp_0104	Unknown	5.6	5.6	5.7
lp_0105	NCAIR mutase, PurE-related protein	5.7	5.7	5.9
lp_0106	Unknown	5.6	5.6	5.4
lp_0107	Unknown	5.0	5.0	5.0
lp_0109	Unknown	5.6	5.6	5.8
lp_0928	Unknown	1.1	1.1	1.8

The highest differential expression (10 to 80-fold induction) was found for a locus known to be involved in lactate-racemization (7). The increased expression of this operon was expected as it is activated by L-lactate (7), and by the fact that responses of other genes putatively involved in lactate utalization were also seen in *L. lactis* and *B. cereus* exposed to L-lactate stress (D. Molenaar, A. Zomer). As the specific lactate stress response reflected the lifestyle of the different bacterial species, interspecies comparisons applying lactate stress were of limited use. As this was not to be expected from comparing the transcriptional response to inactivation of global regulators in different genera, we focused in more detail on the comparison of the response to deletion of the gene encoding the alternative sigma factor σ^{54} (**Chapter 2)** or catabolite control protein A, CcpA (**Chapter 5).** While σ^{54} is present in *L. plantarum* and *Bacillus* spec. but not in *L. lactis* (12), the *ccpA*-gene is present in all four

bacteria. Moreover, the relatively easy *in silico* reconstruction of the regulon based on a genome-based prediction of *cre*-sites and the availability of *ccpA*-mutants transcriptome data sets for each bacterium, enables a detailed comparison of this regulon.

Approximately 60 spotted amplicon-based microarray experiments were performed for the research described in this thesis leading to a massive amount of transcription data. Some of these data could not be discussed within the frameworks of the chapters in this thesis, although they undoubtedly represent interesting biological information.

A detailed look at data of genes encoding ribosomal proteins revealed changes in transcription of these genes under certain circumstances. The observed differential expression of these genes between early stationary phase and exponential phase might indicate a growth rate-dependent regulation (Table 2). However, differences in gene expression of ribosomal proteins between the wild-type and the *ccpA*-mutant during exponential phase were found to be minimal, while the growth rate between these strains differed significantly. This suggests that the expression of these genes does not reflect accurately growth and hence these ribosomal protein genes are not accurate transcript-markers for growth rate. Further supporting this non-growth depending expression of the ribosomal genes derives from their analysis during peroxide stress (**Chapter 3**) and lactate stress (data not shown). Although both stresses led to temporary growth stagnation, a decreased expression of ribosomal genes was not observed. Ribosome formation and ribosomal gene expression in *Escherichia coli* is tuned by the need of the cell for protein synthesis (11). Protein synthesis is one of the fundamental processes in living cells, its regulation is highly conserved (many antibiotics target ribosomes and are therefore effective against a wide range of species (25)). From analyses of the transcriptome data it appears that regulation of ribosomal genes in *L. plantarum* is similar: depending on the need for proteins and potentially independent of growth rate.

Table 2: Regulation of genes encoding ribosomal proteins in comparisons between stationary and exponential growing cells. The cut-off was two times locally weighted differential expression.

Condition		*Growth rate*	*Number of ribosomal genes higher expressed (100% is 60 genes)*
Anaerobic:	Exponential	$0.8h^{-1}$	45 (75%)
	vs		
	Stationary	0	2 (7%)
Aerobic:	Exponential	0.8^{-1}	15 (25%)
	vs		
	Stationary	almost 0	0 (0%)

As mentioned above, many comparisons performed in this thesis deal with samples of cells that were not in the same growth phase or had a different rate of growth at the moment of sampling. Therefore, these datasets might allow the discovery of growth rate dependent genes by comparing samples with different growth rates. For example, a comparison between anaerobic exponential en stationary samples revealed that apart from the ribosomal proteins mentioned above, additional categories were regulated (Fig. 1). An obvious category of genes anticipated to be regulated in correspondence with growth rate alterations is the one containing genes that are predicted to code for cell division proteins, as no growth and cell division occurs in the stationary phase. The category of genes predicted to code for an H^+-ATPase involved in proton motive force maintenance is involved in regulation of the intracellular pH and is apparently most needed in fast acidifying, exponentially growing cells. The expression of tRNAs is similar as ribosomal genes probably depending on the rate of protein synthesis and the cell wall related proteins to synthesis of new cell wall during cell growth. The regulation of amino acid transporters suggests high flux into proteins during exponential phase, but could also be due to exhaustion of amino acids in the medium in the stationary phase. Regulation of sugar utilization genes indicates relief of repression of this category in the stationary phase, probably due to decreased CcpA activity.

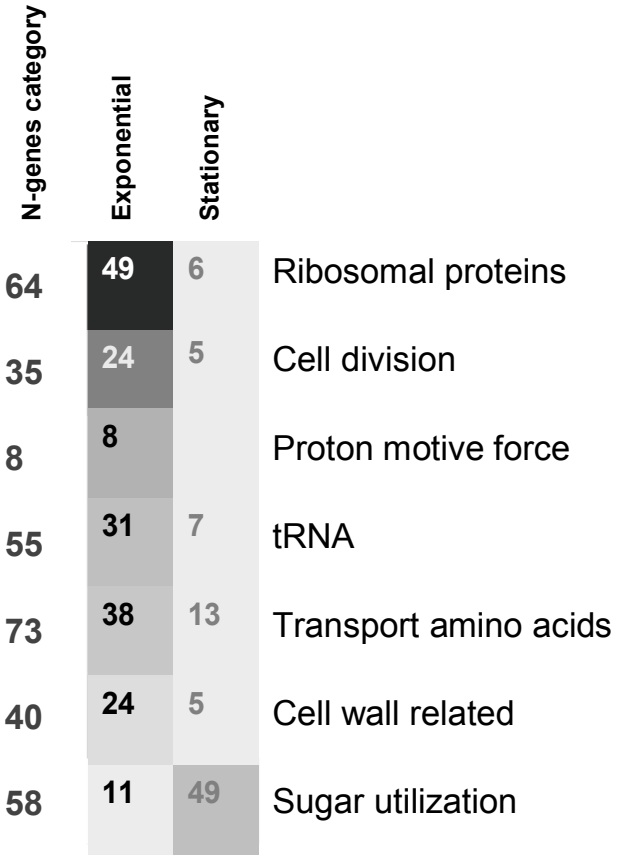

Figure 1: Analyses of functional categories in a exponential vs. stationary phase comparison using FIVA (2). The numbers left to the boxes represent the total number of genes in the functional class, the number in the boxes the number of regulated genes under the condition. The darkness of the box increases with the significance of the regulation of the classes under the condition.

A remarkable regulation was observed of the pyruvate oxidase genes (*pox*) that already had been extensively studied in *L. plantarum* strain Lp80 (8, 10). The *pox*-genes of this strain are highly similar to their counterparts in WCFS1 (>99% identity in nucleotide sequence (8)). However, regulation and functionality appears to be different. PoxB and PoxF are apparently the only functional enzymes in Lp80 (8) and are induced at the end of growth under aerobic conditions. Additional analyses in an available *ccpA*-mutant in strain LM-3 (15) revealed catabolite repression of all *pox*-genes except *poxD*. Of the latter gene no transcript could be detected under the conditions tested (8). Expression of *pox*-genes in WCFS1 was observed in this thesis under a number of conditions described in **Chapters 3, 4, 5** and **6** (Table 3). *PoxB* is the only *pox*-gene affected by peroxide and *poxF* the only affected by aerobic conditions. No differential expression under aerobic conditions was observed for the others, not even in the stationary phase. CcpA-dependent regulation was observed for *poxD* and *poxE*, although the latter was only affected in the early growth phase. Analogous to its CcpA-dependent repression, *poxE* was repressed by glucose compared to other sugars. Similar regulation was observed for *poxF*, but remarkably not for the CcpA-dependent *poxD*. No differential expression was observed for *poxC* under any of the tested conditions. The comparison between expression of *pox*-genes indicates that strain variation may occur on the level of regulation. However, it is more likely that these differences occur due to slightly different experimental conditions. The experiments in this thesis were performed with a substantial amount of acetate in the growth medium (0.5%), and since this compound is known to inhibit Pox activity (8) this could explain the differential behaviour of these genes in the different experiments.

Table 3: Expression of pox genes in WCFS1 and LP80 (8, 10) and their specific regulation during experiments performed in this thesis. ND stands for no-differential expression observed.

ORF	*Gene*	*Oxidative stress (Chapter 2)*	*Aerobic Growth (Chapter 4)*	*CcpA-mutation (Chapter 5)*	*Different sugar (Chapter 6)*
lp_0849	*poxD*	ND	ND	Higher in mutant (entire growth)	ND
lp_0852	*poxE*	ND	ND	Higher in mutant (exponential growth)	fructose, mannose, sucrose
lp_2629	*poxF*	ND	higher under aerobic conditions	ND	fructose, lactose, mannose, sucrose
lp_3587	*poxC*	ND	ND	ND	ND
lp_3589	*poxB*	Higher on peroxide	ND	ND	lactose

These additional analyses show that interpretation of transcriptome data can lead to a range of hypothesis and insights. This kind of data-mining will lead to even more reliable conclusion by using large data sets and sophisticated bio-informatics tools, as was done by Wels (24).

Sugar utilization and control: CcpA is the main player

CcpA is the main player in catabolite control in *L. plantarum*, as already described in two other low G+C Gram-positive organism *L. lactis* and *B. subtilis* (14, 26). Deletion of *ccpA* affects the regulation of approximately 200 genes in the exponential growth phase. CcpA mainly acts as a repressor of transport systems and utilization genes of non-favourable sugars (**Chapter 5).** However, this basal level of global regulation is overruled by specific conditions as is apparent from the observation that during growth on glucose the sucrose PTS is approximately 3-fold induced in a *ccpA*-deletion strain compared to the wild type, whereas differential expression increases up to 70-fold in sucrose-grown cells compared to glucose-grown cells. The sucrose operon is probably regulated by a regulator of the LacI-family (6) and this regulator seems to fine-tune regulation to much higher levels of expression during growth on sucrose.

Another mode of regulation of sugar utilization is mediated via the mannose PTS (**Chapter 2**). It was shown that the σ^{54}-regulated mannose PTS is an important glucose transporter in *L. plantarum* and that presence of the mannose PTS is essential for growth initiation on galactose. Furthermore, the mannose PTS appeared to drain PEP-pools, thereby regulating the capability of the cell to start growth on a non-PTS sugar. However, little is known concerning the mannose PTS activation. It is strictly regulated by σ^{54}, a class II sigma factor that needs an additional activator to initiate transcription (**Chapter 2**), but the mechanism that leads to transcriptional activation is unknown. The σ^{54}-activator designated ManR contains a Man-IIA domain (NCBI-conserved domain search) that can potentially be phosphorylated at the expense of HPr-(His15-P). This suggests an HPr-dependent regulatory mechanism that leads to high expression of the mannose PTS during excess of glucose. It even suggests a self-inducing mechanism as the IIA-domain seems to have high affinity for HPr-(His15-P).

Stress response

Chapter 3 of this thesis is the only chapter describing experiments in which a direct stress treatment was given to the cell and the transcriptional response of the cell to that stress was analyzed. Peroxide stress survival experiments with *L. plantarum* strains lacking expression of a functional mannose PTS showed that a strain without a functional mannose PTS is more

sensitive to hydrogen peroxide treatment as compared with the wild-type strain. However, in the circumstances described in the other chapters the cell frequently had to deal with stress: acid stress at the end of fermentation and oxidative stress during aerobic growth. Oxidative stress was in fact studied twice, once in the comparison anaerobic versus aerobic growth and once in the hydrogen peroxide stress. This resulted in the identification of the transcriptional regulator, later annotated OxyR, a regulator involved in redox sensing (9), which was highly expressed under both aerobic and hydrogen peroxide stress conditions. At first glance, the responses between the aerobic growth and peroxide treatment looked different: only 4 genes were induced during both aerobic condition and peroxide stress, amounting to only 5% of the total response. However, when focusing on the annotations of genes it was observed that a number of functions, 10 in total, were similarly expressed under both circumstances (Table 4). It is therefore plausible that the expression of different genes but with assumed identical functions is due to a specific regulation.

Table 4: Functions significantly differential expressed ($p < 0.05$) during both aerobic and hydrogen peroxide conditions.

Putative function	*Aerobic conditions*	*Peroxide Stress*
- beta-galactosidase	lp_3483	lp_3469
- thioredoxin	lp_2633	lp_3437, lp_2270
- short-chain dehydrogenase/oxidoreductase	lp_3033	lp_3096
- transcription regulator OxyR	lp_0889	lp_0889
- pyruvate oxidase	lp_2629	lp_3589
- glutathione reductase	lp_1253	lp_1253
- fumarate reductase, flavoprotein subunit precursor	lp_0055	lp_1113
- glycerol-3-phosphate ABC transporter	lp_1327	lp_1327
- cell surface hydrolase, membrane-bound (putative)	lp_0461	lp_1935
- protein containing diguanylate cyclase/phosphodiesterase domain 2 (EAL)	lp_0823	lp_0823

Conclusion

The investigations of the transcriptional response of *L. plantarum* as performed in this thesis have given further insights in the biology of this organism. From the literature and the knowledge generated in this thesis, a number of conclusions can be drawn (Fig. 2). Glucose is taken up by the σ^{54}-regulated by mannose PTS, concomitantly phosphorylated to glucose-6-phosphate and via the glycolysis converted to pyruvate and subsequently to lactate. Another, hydrogen peroxide sensitive glucose transporter is postulated. Further, hydrogen peroxide has a global impact on gene expression. Glycolysis influences CcpA activity via Hpr/HprK-

regulation and CcpA impacts expression of approximately 200 genes. Within this group of CcpA regulated genes are genes involved in the pyruvate metabolism and utilization of non-favourable sugars (not shown). Environmental factors (e.g. oxygen) affect the expression of genes. Additionally, the expression of genes and the fermentation products have an influence at the environment itself, contributing to flavour, perception, and quality of food products and to the probiotic activity of the bacterium itself.

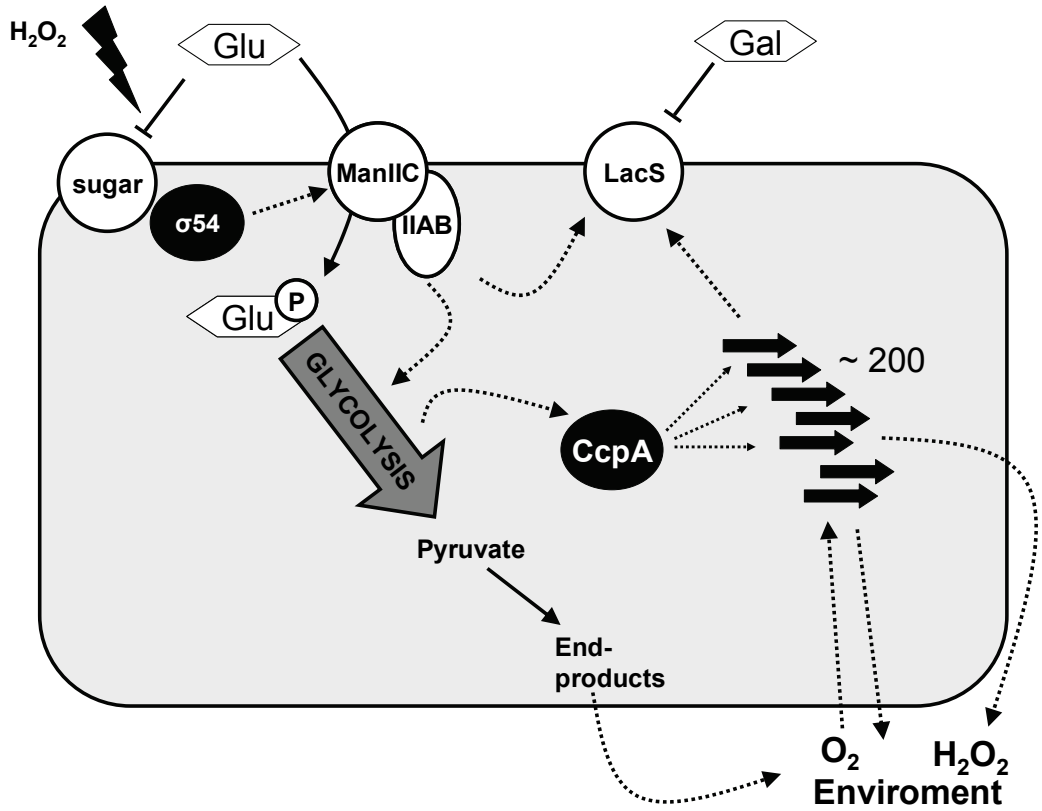

Figure 2: Sugar utilization en transcriptional regulation in L. plantarum as concluded from the work in this thesis. Regulation is displayed by dotted lines, reactions by solid lines.H_2O_2-stress target as a flash.

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Nederlandse Samenvatting

Melkzuurbacteriën zijn een groep van bacteriën die suikers relatief snel omzetten in melkzuur en dit melkzuur veroorzaakt een verlaging van de zuurgraad waardoor andere micro-organismen niet meer kunnen groeien. Door deze eigenschap worden melkzuurbacteriën vaak gebruikt in voedselfermenaties zoals bijvoorbeeld zuurkool, yoghurt en worst. De bacterie *Lactobacillus plantarum* behoort tot de groep van de melkzuurbacteriën en wordt naast voedselfermenaties ook aangetroffen in het humane maagdarmstelsel, waar het een positieve invloed op de gezondheid kan hebben, het zogenaamde pro-biotische effect. De complete genoomsequentie van *L. plantarum* stamWCFS1 is bekend en deze informatie én de set van beschikbare genetische "tools" maakt *L. plantarum* een geschikt organisme voor genetisch onderzoek.

Eind 20ste eeuw werd een methode ontwikkeld om de relatieve expressiepatronen van veel verschillende genen in één experiment te kunnen analyseren: de transcriptomic techniek. Gebruikmakend van deze techniek kon het verschil in genexpressie, veroorzaakt door gendeletie, door stress of door omgevingsfactoren worden bestudeerd. Dit onderzoek heeft geresulteerd in dit proefschrift.

Een steeds veranderende factor in de omgeving van bacteriën is de beschikbaarheid van suikers. Onder optimale groeiomstandigheden zet *L. plantarum* glucose geheel om naar lactaat, wat leidt tot een snelle verzuring van het medium. De aanwezigheid van glucose in het groeimedium controleert de expressie van genen in een proces dat bekend staat als "catabolite control". De belangrijkste regulator van catabolite controle in de groep van Gram-positieve bacteriën, waartoe *L. plantarum* behoord, is het "catabolite control protein A" (CcpA). Deletie van het *ccpA* gen leidde tot verlies van "catabolite control" in de bacterie resulterend in een differentiële expressie in het wild type vergeleken met de deletiestam van ongeveer 200 genen (Hoofdstuk 5). Naast deze veranderingen in genexpressie leidde de deletie verder nog tot een langzamere groei van de bacterie en tot een gemixte lactaat-acetaat fermentatie op glucose. De gemixte fermentatie resulteerde in een langzamere verzuring van het medium waardoor de gemuteerde bacterie langer kon doorgroeien. De conversie van glucose naar acetaat i.p.v. naar lactaat levert de bacterie meer energie op (en dus meer biomassa), maar is waarschijnlijk ook de oorzaak van de lagere groeisnelheid.

Verdere analyse van het suikerverbruik door *L. plantarum* werd gedaan in hoofdstuk 6, waar het expressiepatroon tijdens groei op verschillende suikers werd vergeleken. Dit resulteerde

de identificatie van suikerspecifieke genen zoals transporters en genen betrokken bij het omzetten van deze suikers. De expressie van zulke genen op alleen de specifieke suiker duidt op lokale en globale expressiecontrole. De globale controle uitgevoerd door CcpA en de lokale door regulators geactiveerd door de suiker zelf.

De regulatie van een andere potentiële globale regulator, de sigma factor 54 (σ^{54}), wordt beschreven in hoofdstuk 2 en 3. Een voorspelling van het regulon van σ^{54} met behulp van bio-informatica tools leverde een aantal kandidaat-genen op, waaronder de genen van het mannose phosphotranferase systeem (PTS). De genetische koppeling van deze genen aan een σ^{54}-activator-gen maakt regulatie van het mannose PTS door σ^{54} aannemelijk. Transcriptome-proeven met een mutant in het *rpoN* gen (coderend voor σ^{54}) bevestigden de voorspelde regulatie van het mannose PTS terwijl geen andere σ^{54} gereguleerde genen konden worden geïdentificeerd (hoofdstuk 2). Een gedetailleerde analyse van het mannose PTS identificeerde het transport systeem als een belangrijke gluocse-transporter van het PTS, zoals ook beschreven in andere lactobacilli. Verder bleek het mannose PTS als glucose transporter direct betrokken bij "catabolite control". In tegenstelling tot het wild type konden cellen die geen mannose PTS meer tot expressie brengen, groei initiëren op galactose. Verder was de concentratie van het glycolytische tussenproduct fosfo-enolpyruvaat (PEP) veel hoger in rustende mannose PTS deletie stammen vergeleken met het wild type, een indicatie dat de "catabolite control" waarschijnlijk wordt gereguleerd door de concentratie van glycolytische tussenproducten.

De *rpoN* deletiestam bleek gevoeliger voor waterstofperoxide (hoofdstuk 3), een fenomeen dat kon worden verklaard door de voorspelde regulatie door σ^{54} van een glutathion peroxidase (*gpo*), een gen coderend voor een enzym dat waarschijnlijk betroken is bij de bescherming tegen waterstofperoxide. Een transcriptome analyse van de deletiestam tijdens een waterperoxide-behandeling toonde echter aan dat er geen verschil is in de levels waarop *gpo* tot expressie komt, wat regulatie van *gpo* door σ^{54} onwaarschijnlijk maakt. Verder bleken de mannose PTS deletiemutanten ook gevoeliger voor waterstofperoxide te zijn, hetgeen duidt op een directe relatie tussen het mannose PTS en waterstofgevoeligheid. Een verklaring hiervoor is de robuustheid van het mannose PTS t.o.v. peroxide in vergelijking tot andere PTSen. Tijdens peroxide stress worden de andere systemen beschadigd en glucoseopname zal via het mannose PTS moeten plaatsvinden. In de deletiestammen is dit niet meer mogelijk en dat leidt tot een lage glucose opname en daardoor tot energietekorten in de cel. Omdat de stress response tegen peroxiden veel energie vergt, zullen de deletiestammen sneller sterven.

De natuurlijk niche van *L. plantarum* is anaëroob. Desalniettemin codeert het genoom van *L. plantarum* WCFS1 een groot aantal genen betrokken in de bescherming tegen de bij oxidatieve processen in de aanwezigheid van zuurstof vrijkomende radicale zuurstof species (ROS). Om de reactie van *L. plantarum* op de aanwezigheid van zuurstof te onderzoeken, werd op globaal niveau naar de verschillende genexpressie van een anaërobe t.o.v. een aërobe cultuur gekeken (hoofdstuk 4). Uit deze analyse bleek dat een groot aantal genen betrokken bij de protectie tegen ROS hoger tot expressie kwamen tijdens aërobe groei, duidend op een verbruik van zuurstof. Verder bleek ook dat de groei van de bacterie tijdelijk stagneerde in de aërobe cultuur, een stagnatie die gerelateerd bleek aan de hoeveelheid bacteriën in het inoculum. Transcriptome analyse tussen cellen voor en na de stagnatie toonde aan dat veel CO_2-producerende reacties hoger tot expressie kwamen na de groeistagnatie. Dit kan erop duiden dat de oorzaak van de groeistagnatie een CO_2-tekort is en dat de cel daarvoor compenseert door CO_2-producerende reacties aan te schakelen. *L. plantarum* (en vele andere organismen) heeft CO_2 nodig om te kunnen groeien omdat het essentieel is voor de productie van purines en pyrimidines. Een vernieuwde aërobe fermentatie in 1% CO_2 verrijkte lucht, liet inderdaad zien dat de groeistagnatie werd veroorzaakt door CO_2-tekort. Dit experiment geeft mooi aan hoe transcriptome analyse en verregaande data interpretatie tot een betere fermenatatietechniek kunnen leiden.

Het werk beschreven in dit proefschrift geeft nieuwe inzichten in de transcriptionele respons van *L. plantarum* tijdens stress, bij mutaties en in verschillende suiker achtergronden. Deze inzichten kunnen leidden tot verbeterde fermentatie toepassingen in de industrie, terwijl de gebruikte technieken en de data-analysen kunnen worden gebruikt en verder ontwikkeld in toekomstig *L. plantarum*-onderzoek.

Nawoord:

Het leven bestaat uit de interacties die je hebt met individuen om je heen. Sommigen van die individuen hebben mij gevormd en gesteund en daardoor bijgedragen aan de totstandkoming van dit proefschrift. Daarvoor wil ik ze graag bedanken.

Ten eerste mijn begeleiders: Michiel en Douwe. Het zit erop voor mij, Ik heb veel van jullie geleerd, bedankt voor alle hulp! Hetzelfde geldt voor Willem, verder weg, maar wel altijd bereid om te helpen, ook jij bedankt! Veel succes verder met alles en we zullen elkaar nog wel eens zien.

De mensen op het NIZO, allemaal bedankt! Het zijn er te veel om allemaal op te noemen, maar enkele wil ik toch bij naam noemen. Eerst Iris (die lief is): de dagen dat jij niet werkte, waren maar half zo leuk. De mensen in de denktank: Mariela, zie Iris, en succes met jouw boekje! Arno, tja wat rijmt er op Arno? En nog Jolalalalanda, ook succes met je boekje. Peter (mijn dank grenst aan…), Wilbert, en helemaal in het begin Ingeborg en Patrick, tanx!

Verder nog Eddy (Eddy Merckx!), Anne (je schrijft Anne, maar je zegt Onne), Marke, Roger (nu moet je zelf RNA isoleren), Bert (Straks ben jij aan de beurt), Patrick en Filipe: thank you darling!

Het is altijd plezierig als andere mensen werk voor jou doen en dus wil ik bij deze mijn stagiare Vivian bedanken: Vivian, het gaat je goed in Japan!

Verder ook collega's van het WCFS bedankt, speciaal de mensen van het project C-008. We hebben altijd vruchtbare discussies kunnen voeren over mijn werk waar ik mijn voordeel mee kon doen.

De mensen van Microbiologie in Wageningen (Eline, Mirjana en de ander mensen van de trip naar Japan), leuk om jullie te kennen. David, bedankt voor de samenwerking op CcpA gebied, en Elaine ook jij bedankt.

Mensen in Groningen, met name Oscar en Anne, bedankt dat ik bij jullie veel heb kunnen leren over transcriptomics en ook steeds hulp kreeg als dat nodig was.
Dan nog mensen die het leven de afgelopen jaren zoveel leuker maakten. Vrienden in Valkeburg: Henny, Angele, Marc, Bianca, Robbie en vele anderen, bedankt! Mijn lieve nichtje Saskia, bedankt! En ook neef Roel en neef Roedie. Karin, bedankt! BGA-ers: Daan en de anderen, het was prettig om als eenzame Limburger bij jullie te kunnen wonen, dat heeft er zeker voor gezorgt dat ik me thuisvoelde in Wageningen. Dus jullie ook bedankt! Freunden in Duitsland, (Maarten), Danke en Glück Auf! Verder alle mensen die ik vergeten ben te noemen, ook bedankt hoor!
En dan als laatste mijn familie: Papa en Mama: bedankt voor het Papa en Mama zijn. Beter kan ik het niet weergeven wat jullie doen voor mij, Danke! En de rest van de clan: Servi (boef, grote broer, paranimf), Antoinette (vooral voor het lekkere eten) en Carolijn en Natan (voor het geven van levensvreugde). Bedankt voor alle steun en support.

MS

Curriculum Vitea

Marc Stevens werd geboren op 24 mei 1976 in Valkenburg-Houthem. Na de lagere school in Valkenburg, volgde hij middelbaar onderwijs aan de scholengemeenschap Stella Maris te Meerssen. Al tijdens zijn middelbare schooltijd onstond zijn interesse voor de moleculaire biologie en na het behalen van zijn VWO-diploma in 1995, begon hij aan een studie biologie aan de technische universiteit Aken (RWTH Aachen). Hij deed een afstudeeronderzoek bij het instituut voor microbiologie van de universiteit onder begeleiding van Prof. Dr. K. Wolf en Dr. M. Zimmermann, waarna hij in 2001 afstudeerde in de richting van de moleculaire microbiologie. In 2002 begon hij als AIO voor het Wageningen Centre for Food Sciences met als werklocatie NIZO Food Reserach, onder begeleiding van Prof. Dr. Michiel Kleerebezem, Dr. Douwe Molenaar en Prof. Dr. Willem de Vos. Hij deed onderzoek naar het transcriptionele netwerk van de melkzuurbacterie *Lactobacillus plantarum* wat resulteerde in dit proefschrift.

list of publications

M. J. A Stevens, Douwe Molenaar, Oscar P. Kuiper, Willem M. De Vos, Eddy J. Smid, Anne Wiersma and Michiel Kleerebezem.
Genome wide analysis of *Lactobacillus plantarum* adapted to aerobic growth.
Manuscript in preparation

M. J. A Stevens, Vivian Kong Tse Lam. Douwe Molenaar, Willem M. De Vos, and Michiel Kleerebezem.
Analyses of carbohydrate utilization in *Lactobacillus plantarum* WCFS1.
Manuscript in preparation

M. J. A Stevens, Douwe Molenaar, Anne de Jong, Willem M. De Vos, and Michiel Kleerebezem.
Sigma 54 mediated control of the mannose PTS in *Lactobacillus plantarum* impacts on carbohydrate metabolism
Manuscript in preparation

M. J. A Stevens, Douwe Molenaar, Anne de Jong, Willem M. De Vos, and Michiel Kleerebezem.
Global Transcriptional Analysis of the Peroxide Response in *Lactobacillus plantarum* and Involvement of the Mannose PTS in Survival
Manuscript in preparation

M. J. A Stevens, David P.A Cohen, Douwe Molenaar, Oscar P. Kuiper, Willem M. De Vos, Elaine E. Vaughan and Michiel Kleerebezem.
Growth Phase dependent Comparative Transcriptome and Proteome Analyses of the CcpA-regulon of *Lactobacillus plantarum*.
Manuscript in preparation

Activities in the frame of the VLAG Graduate School

- Radiation Expert 5B, Larenstein, Velp, 2002
- Bioinformation Technology I, VLAG, 2003
- Genetics and Physiology of food-related microorganisms, VLAG, 2005
- Statistics for Microarray, University Utrecht, 2004
- Scientific Writing, Wageningen UR, 2003
- Discussion Techniques, Wageningen UR, 2005
- WCFS-Food Summit, 2004.
- International Conference on Lactic Acid Bacteria, 2005, Egmond aan Zee, NL
- International Conference Grampositive Genomics, 2005, San Diego, USA
- Nederlands Biotechnologie Congres, 2006, Ede, NL
- ALW Genetics Conferences 2002-2005
- Genomic momentum Conference 2003
- VLAG PhD-Trip Microbiology 2004

Lay out en Cover Design: Marc Stevens

Printed by: Ponsen & Looijen BV, Wageningen