The Molecular Response of *Lactobacillus plantarum* to Intestinal Passage and Conditions

Peter Allard Bron

Promotor:	Prof. Dr. W.M. de Vos Hoogleraar in de Microbiologie
Co-promotor:	Dr. M. Kleerebezem Projectleider Wageningen Centre for Food Sciences
Promotiecommissie:	Prof. Dr. M.R. Müller, Wageningen Universiteit, Wageningen Dr. A. Mercenier, Nestlé Research Center, Lausanne, Switzerland Prof. Dr. C.M.J.E. Vandenbroucke-Grauls, Vrije Universiteit, Amsterdam Dr. H.E. Smith, Wageningen Universiteit, Lelystad

Dit onderzoek is uitgevoerd binnen de onderzoekschool VLAG

Peter Allard Bron

The Molecular Response of *Lactobacillus plantarum* to Intestinal Passage and Conditions

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr. Ir. L. Speelman
in het openbaar te verdedigen
op vrijdag 28 mei 2004
des namiddags te half twee in de aula

Bron, P.A.
The Molecular Response of *Lactobacillus plantarum* to Intestinal Passage and Conditions

Thesis Wageningen University, the Netherlands, 2004
With references - With summary in Dutch

ISBN 90-8504-012-4

Keywords: lactic acid bacteria, *Lactobacillus plantarum*, persistence, gastrointestinal tract, bile, *in vivo* expression technology, R-IVET

Contents

Abstract

Lactobacillus plantarum is a lactic acid bacterium (LAB) that is encountered in many environmental niches, including dairy, meat and a variety of vegetable fermentations. Next to the occurrence of *L. plantarum* in our diets, this microbe is frequently encountered as a natural inhabitant of the gastrointestinal tract (GI-tract). This thesis describes the identification and functional analysis of *L. plantarum* promoters and genes that play a role during intestinal passage of this LAB. During these studies the mouse GI-tract and *in vitro* studies were utilized as models for the human situation. Three main strategies that allow the identification of conditionally active genes have been implemented in *L. plantarum*, namely an *in vitro* screen to trap bile-inducible promoters, DNA micro-array technology to identify mRNAs that are up-regulated in the presence of bile, and a resolvase-based *in vivo* expression technology (R-IVET) screen to select promoters that are induced in the GI-tract of a mouse model system. Matching of the genes identified with these three strategies revealed two genes, encoding an integral membrane protein and an argininosuccinate synthase, which appeared to be induced by bile *in vitro* as well as *in vivo* in the mouse GI-tract. As the duodenum is the intestinal site of bile release, expression of these two genes at this specific location in the host's GI-tract was investigated using quantitative reverse transcription PCR. The results demonstrated that the expression levels of these two genes were dramatically increased in *L. plantarum* cells isolated from the mouse duodenum relative to cells grown on standard laboratory medium. Furthermore, gene disruption mutants were constructed in 12 of the *in vivo* induced (*ivi*) genes identified by the R-IVET screen. The effect of the mutations on the survival and persistence of *L. plantarum* were investigated using quantitative PCR to monitor the relative population dynamics of the group of mutants in faecal samples after passage through the GI-tract of mice. These data indicate that the products of three of the disrupted genes (an orphan IIC transport component of a cellobiose PTS system, an extracellular protein that contains an LPQTNE motif involved in anchoring of this protein to the bacterial cell wall, and a copper transporting ATPase) play a key-role in the survival and/or persistence of *L. plantarum* during passage of the GI-tract. Overall, the results described in this thesis represent a first step towards molecular elucidation of the behavior of *L. plantarum* in a highly complex and inaccessible niche like the GI-tract. These results contribute to the development of eventual molecular models that describe GI-tract behavior of LAB, which are fundamental for the explanation of probiotic effects associated with these microbes and related species at the molecular level.

Chapter 1

Introduction and Outline of the Thesis

A modified version of this chapter has been accepted for publication as a chapter in: Gastrointestinal Microbiology (edited by Arthur Ouwehand and Elaine Vaughan, published by Marcel Dekker); Molecular analysis of host-microbe interactions in the gastrointestinal tract, Peter A. Bron, Willem M. de Vos, and Michiel Kleerebezem. Moreover, this chapter has contributed to: Curr. Opin. Biotechnol. (in press); Post-genomics of lactic acid and other bacteria to discover gut functionality, Willem M. de Vos, Peter A. Bron, and Michiel Kleerebezem.

Introduction

From birth to death, the human gastrointestinal tract (GI-tract) is colonized by a vast, and complex consortium of mainly bacterial cells that outnumbers our somatic and germ cells (56). The microbes in this niche are estimated to be composed of at least 500 different species. However, this number is likely to represent a large underestimate, since it has been based on culturing studies that are known to be selective and notably underestimate the large number of gram-positive intestinal bacteria (69). Molecular approaches, such as broad-range sequencing of 16S rRNA genes have been used to monitor the composition of the dominant GI-tract microbiota in different individuals at different time points in their lives. These approaches revealed a relatively stable composition in individual adults, but appeared to be considerably variable when different individuals were compared (69, 71). Moreover, host development (16, 30), host genotype (68), and environmental factors (61) influence the composition of the GI-tract microbes, emphasizing how challenging it is to define and compare bacterial communities within and between specified intestinal niches of a given individual at a particular time point in his or her life. The fact that we have not yet been able to culture the majority of the members of this bacterial community further complicates studies on the activity of individual members of the GI-tract consortium. An important development in this respect are the sophisticated enrichment strategies that have led to the isolation of new bacterial species from faecal samples (25, 70).

Several biological barriers are met by bacteria during residence in and travel through the different parts of the host's GI-tract, such as the gastric acidity encountered in the stomach, the presence of bile salts in the duodenum and stress conditions associated with oxygen gradients that are steep at the mucosal surface, while the colon is virtually anoxic (10). Moreover, considerable bacterial competition is encountered throughout the entire intestinal tract and notably in the colon where cell numbers are highest (56). There are many functions that can be ascribed to the bacterial GI-tract communities, including the processing of undigested food, the stimulation of the host's immune system, and providing colonization resistance to pathogens (28). However, it seems that we are only beginning to understand the dimensions of these interactions. This is evident from the major impact that bacterial colonization seems to have on the host and the presently known response of intestinal bacteria that are reviewed below.

Bacterial responses to the host - *in vitro* approaches

Due to the complex nature of host-specific and chemical stress conditions that are met by bacteria in the GI-tract many studies describe the *in vitro* response of intestinal bacteria to a simplified model that mimics (a component of) the stress encountered in the host's GI-tract. Historically, these studies have been performed in pathogens,

including studies describing the response towards acid stress in enteropathogenic bacteria such as *Salmonella* and *Escherichia coli*, which revealed that RpoS, Fur, PhoP and OmpR are important regulators involved in pH-response (4). More recent studies describe bacteria with proposed GI-tract functionality and their tolerance to acid stress. These studies have focused mainly on physiological aspects such as determination of levels of acid-tolerance (9, 32). Changes in protein synthesis during acid adaptation have been studied in a strain of *Propionibacterium freudenreichii*, an organism which potentially has functionality in the GI-tract, using 2D-gel electrophoresis, indicating an important role in the early acid tolerance response for a biotin carboxyl carrier protein and enzymes involved in DNA synthesis and repair, as well as a role in the late response for the universal chaperones GroEL and GroES (34).

Several studies describe the defense mechanisms of gram-negative enteric bacteria towards bile acids, which include the synthesis of porins, transport proteins, efflux pumps and lipopolysaccharides (24). In addition, a few genome-wide approaches aiming at the identification of proteins important for bile salt resistance in gram-positive bacteria have been described. In *Propionibacterium freudenreichii, Listeria monocytogenes* and *Enterococcus faecalis* differential proteome analysis using 2D-gel electrophoresis led to the identification of several proteins that were expressed at a higher level in the presence of bile salts relative to control conditions lacking bile salts (17, 43, 51). Only in *Propionibacterium freudenreichii* these bile-induced proteins were further analyzed by N-terminal sequencing and peptide-mass fingerprinting, leading to the identification of 11 proteins important in bile stress response. The induced proteins include general stress proteins such as ClpB and the chaperons DnaK and Hsp20 (43). The fact that these general stress proteins are induced by bile is in agreement with the cross-protection against bile stress after thermal or detergent pre-treatment that has been observed in several bacteria, including *Enterococcus faecalis*, *Listeria monocytogenes* and *Bifidobacterium adolescentis* (6, 17, 58). Moreover, proteins involved in signal sensing and transduction, and an alternative sigma factor appeared to be bile-inducible (43). Two other bile-induced proteins in *Propionibacterium freudenreichii* are the superoxide dismutase and cysteine synthase, which could be involved in the protection against the oxidative stress imposed on *Propionibacterium freudenreichii* by bile. In addition, other studies describe the oxidative stress response of GI-tract organisms, including *Campylobacter coli*, *Escherichia coli* and several *Shigella* species (36, 40, 53). A deletion mutant in the gene encoding superoxide dismutase in *Campylobacter coli* displayed poor survival and colonization during infection of an animal model (53). Next to these proteomic approaches, random gene disruption strategies have been applied to *Listeria monocytogenes* and *Enterococcus faecalis*, resulting in strains that are more susceptible to bile salts than the wild-type strains. Subsequent genetic

analysis of the mutants revealed that the disrupted genes encode diverse functions, including an efflux pump homologue (6) and genes involved in oxidative stress response, and cell wall and fatty acid biosynthesis (8). These findings are in agreement with several physiological studies in bacteria with GI-tract functionality such as *Propionibacterium freudenreichii* and *Lactobacillus reuteri* that demonstrated that bile salts induce severe changes in the morphology of the cell membrane and/or cell wall of these organisms (43, 62). Overall, the aforementioned *in vitro* experiments have provided insight in the response of specific bacteria towards components of the complex mixture of stress conditions that is met by these bacteria during residence in or transit through the GI-tract of their hosts. Although these approaches have helped to unravel the response of specific micro-organisms towards certain GI-tract conditions, they will not suffice to describe their behavior in the GI-tract. The full response repertoire will only be triggered *in vivo*, where all physicochemical conditions are combined with specific host-microbe and microbe-microbe interactions. Therefore, more sophisticated approaches have aimed at the development of tools that allow the *in vivo* identification of genes that are important in the GI-tract.

Bacterial responses to the host - *in vivo* strategies

Three main strategies have been developed for the identification of genes that are either highly expressed, differentially expressed or specifically required *in vivo* (Fig. 1). These strategies have mainly been applied for the identification of genes from pathogens which are important during infection of their animal host. Signature tagged mutagenesis (STM) utilizes a negative selection strategy in which an animal host is infected with a pool of sequence-tagged insertion mutants. Mutated genes represented in the initial inoculum but not recovered from the host are essential for growth in the host (47, 66). A major advantage of STM is that this type of screen provides direct proof for the importance of the mutated genes in the relevant niche. Unfortunately, only limited numbers of mutants can be screened per animal model. Therefore, large scale animal experiments are required for genome-wide mutant screens and for this reason STM screens are labor-intensive. Mutants that are slow-growing, contain mutations in genes encoding redundant functions, or that can be complemented in a mixed population remain undetected or are at least underrepresented (45). Moreover, mutants for genes that are essential in the laboratory can never be obtained and, therefore, their importance for persistence *in vivo* cannot be investigated using this technique. Nevertheless, the STM strategy has been applied successfully to identify genes important in GI-tract colonization by at least 6 enteric pathogens, including *Klebsiella pneumoniae*, *Vibrio cholerae*, and *Escherichia coli* (66).

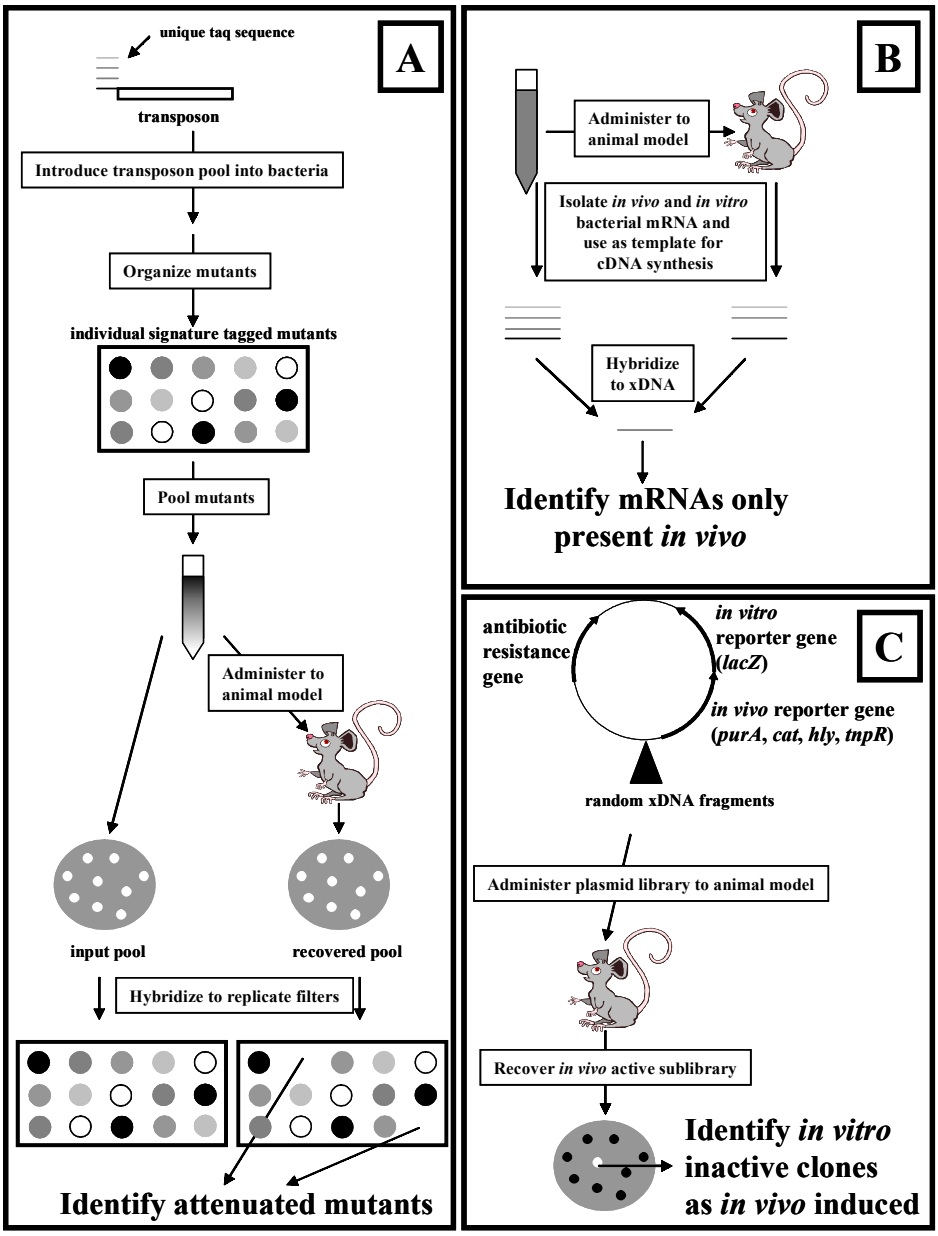


Fig. 1: Schematic representation of the basic principles of STM (A), SCOTS (B) and (R)-IVET (C). See text for details (xDNA is chromosomal DNA).

Lipopolysaccharides have been recognized as an important factor in GI-tract persistence and colonization of several gram-negative bacteria, as they have emerged as a common theme in the STM-based studies. In addition, the importance of the global regulator of anaerobic metabolism Fnr was highlighted by several STM screens, which is not surprising considering the low oxygen tension in the colon. Moreover, the alternate sigma factor RpoN was found in several of the STM screens and is likely to associate with RNA polymerase to promote the transcription of genes that are specifically required in the GI-tract niche. Finally, STM studies revealed the importance of specific adhesins, including the type IV pili of *Vibrio cholerae* and *Citrobacter rodentium* (66).

A second strategy that has been applied for the identification of *in vivo* transcribed genes is selective capture of transcribed sequences (SCOTS). This approach allows selective capture of bacterial cDNAs from total cDNA, prepared from infected cells or tissues. cDNA mixtures obtained are then enriched for sequences that are transcribed preferentially during growth in the host, using additional hybridizations to bacterial genomic DNA in the presence of cDNA similarly prepared from bacteria grown *in vitro*. This strategy is very effective for the identification of mRNAs that are highly abundant *in situ* and are also expressed to a lower level in the laboratory. In contrast to the STM strategy, genes that are essential in the laboratory can be investigated for their importance in GI-tract colonization. Nevertheless, major disadvantages of SCOTS are the instability of bacterial mRNA for the construction of cDNA libraries, the low abundance of mRNA from transiently or lowly expressed genes, and the technical difficulty in isolation of sufficient high-quality mRNA from small populations of bacteria *in vivo* (45). SCOTS has only been applied in a limited number of studies and the majority of these screens was performed to identify bacterial genes expressed within macrophages (11, 22, 31, 48). Recently, the first SCOTS strategy utilizing an animal model to identify genes important during infection was performed (13). This approach resulted in the identification of *Escherichia coli* genes of which the expression is either relatively abundant or induced *in vivo*. Similar to the STM approaches described above, this SCOTS approach revealed the induction of expression of genes involved in pilus formation and lipopolysaccharides biosynthesis. Other genes identified included iron-responsive, and plasmid- and phage-encoded genes.

The third strategy that has been used to identify genes that are specifically induced or required during infection is *in vivo* expression technology (IVET). Similar to SCOTS, the IVET strategy is capable of identifying genes that are non-essential or redundant, while in an STM approach genes are only identified that are essential *in vivo*. An important difference between IVET and SCOTS lies in the fact that SCOTS is capable of identifying genes that are active in the laboratory, but, nevertheless, are induced in the host, while IVET only identifies *in vivo* induced genes that are very lowly or not expressed in the laboratory. Moreover, the SCOTS strategy directly identifies mRNA, while IVET is a promoter trapping system. Hence, the IVET approach relies on the generation of transcriptional fusions of genomic sequences to a reporter gene encoding an enzymatic activity. Nowadays, four variations of IVET utilizing different reporter genes have evolved. The original technology involves a tandem set of two promoterless reporter genes, namely *purA* and *lacZ*, which were used to identify promoters that are specifically switched on in *Salmonella typhimurium* during infection (46). Purine auxotroph mutants (Δ*purA*) of *Salmonella typhimurium* were only able to survive in a mouse model system when complemented *in trans* with a plasmid encoding and expressing *purA*. The promoterless *purA* gene

was thereby utilized as a reporter for the identification of chromosomal fragments that are capable to complement the mutants, thereby strongly selecting for chromosomal fragments which harbor promoter elements that are active in the mouse model system. Subsequently, the *in vivo* active promoters are tested for the absence of promoter activity *in vitro* utilizing the second reporter gene (*lacZ*). The second variation of IVET is based on selection of an antibiotic resistance gene as selectable marker. One obvious disadvantage of this second variation of IVET is that the antibiotic must be administered to the host animal and this is highly likely to disturb the natural occurring microbes in the GI-tract. Therefore, the screening conditions assessed with this variant of IVET significantly differ from the native, *in vivo* situation. On the other hand, the addition of different levels of the selective antibiotic allows for selection of *in vivo* induced genes in a wider range of promoter activities. The third type of IVET selection uses a single gene as a dual reporter. The first example of such a dual reporter was *hly*, encoding the pore-forming haemolysin listeriolysin O (LLO) of *Listeria monocytogenes* (19). LLO mediates lysis of the phagosomal membrane in macrophages following infection. This reporter provides an *in vivo* selection for active fusions that allow for escape from the phagosomal compartment and subsequent multiplication. Moreover, a convenient screen on blood agar plates can be performed to identify inactive fusions *in vitro*, since clones harboring such fusions do not display haemolysis on these plates. The major drawback of the three aforementioned IVET variations is that the experimental set-up is designed in such a way that gene activity is required throughout the residence of the bacteria in the host. Hence, genes that are weakly expressed in the laboratory or transiently expressed only in a specific compartment of the host's GI-tract slip through the selection procedure without being noticed. The fourth IVET variation circumvents this disadvantage by using the irreversible enzymatic activity of resolvases as reporter gene. Recombination-based IVET (R-IVET) is the only IVET approach that functions as a genetic screen. An antibiotic resistance marker flanked by two resolvase-recognition sites is integrated into the chromosome of the bacterium of interest. Subsequently, a promoterless copy of a resolvase-encoding gene, typically the *tnpR* gene from Tnγδ, is introduced on a plasmid and used to trap transcriptional activation by monitoring changes in the antibiotic resistance phenotype. Importantly, this approach does not rely on selective pressure during the animal experiments, as promoter activations are irreversibly trapped by the excision of the antibiotic resistance marker and can be identified after recovery of the bacterium under investigation from the host.

In the first decade, (R)-IVET was extensively utilized for the identification of genes important during infection of at least 15 different pathogens, including *Klebsiella pneumoniae*, *Salmonella enterica* and *Listeria monocytogenes* (3, 45). Thereby, (R)-IVET is the most extensively applied screen for the identification of *in vivo* induced genes during infection of animal models. The number of genes that is identified with an individual (R)-IVET screen varies strongly and ranges from 1 to approximately 100 genes (3). Several of these screens identified genes that were already known to be involved in virulence and this observation was considered an intrinsic validation of these (R)-IVET screens (45). An exemplary finding along these lines is the identification of *agrA* using R-IVET in *Staphylococcus aureus* (44). This gene encodes a quorum-sensing transcriptional activator and *agrA* mutants constructed in this organism prior to the R-IVET screen had already been shown to display a virulence defective phenotype (20). In general, regulators are one of the predominant classes of genes identified with (R)-IVET (45). Another frequently encountered class of *in vivo* induced genes in pathogenic bacteria are involved in the uptake of divalent cations, including many examples of Fe^{2+} transporters (45). The harsh conditions these pathogens encounter when they transit from rich laboratory media to the host's GI-tract apparently results in the induction of this group of genes. This suggestion is further supported by the observation that several *in vivo* induced genes were demonstrated to be similarly regulated under low Fe^{2+} concentrations *in vitro* (35, 42, 59). Other genes that frequently arise from (R)-IVET screens have functions in a variety of generally recognized functional categories, including cell metabolism, DNA repair and general stress response.

Recently, the first report appeared that describes the utilization of IVET in a food-grade organism in order to determine the specific induction of gene expression in *L. reuteri* after introduction in the GI-tract of animal models. This approach was based on *in vivo* selection of an antibiotic resistant phenotype (the second variation of IVET) and led to the identification of 3 genes important for this organism during colonization of the GI-tract of Lactobacillus-free mice (65). One of these genes encodes a peptide methionine sulfoxide reductase which has previously been identified using IVET in the non-food-associated *Streptococcus gordonii* during endocarditis (37). Although not noticed by the authors at that time, this was an important clue suggesting an overlap in the genetic response triggered in the pathogenic and non-pathogenic world following contact with the host.

Insights from genomics

Nowadays more and more bacteria are undergoing genome sequencing and as a result over 130 completed bacterial genomes have become available in the public domain (http://wit.integratedgenomics.com/GOLD/). Following the first example of *Haemophilus influenzae* in 1995 (18) the major focus of these efforts has initially

been on pathogenic bacteria and includes the completion of several genome sequences of food-borne pathogens, including *Bacillus cereus* (33), *Salmonella typhimurium* (49), and *Listeria monocytogenes* (21). Over the last years sequencing of the genomes of food-associated, non-pathogenic bacteria has received considerable attention, including the elucidation of the complete genome sequence of *Bacillus subtilis* (41) and *Lactococcus lactis* (7). To date, only a single high-fidelity genome sequence of a *Lactobacillus* species, *L. plantarum* strain WCFS1, has been published (39). Additional genomes of other lactic acid bacteria (LAB) are nearing completion and draft genome information has become available in the public domain in 2002 with the publication and appearance of genome sequences for LAB provided by the Joint Genome Institute (http://www.jgi.doe.gov/) in collaboration with the lactic acid bacteria genomics consortium (12, 38). These developments will provide insight in diversity, evolutionary relationship and functional differences between these bacteria and might eventually shed light on the behavior of these food-associated microbes during residence in the human GI-tract. Next to this large amount of sequence data from LAB, additional efforts have led to determination of the (complete) genome sequences of members of our normal colonic microbiota, in particular *Bacteroides thetaiotaomicron* (67) and *Bifidobacterium longum* (57) (Fig. 2).

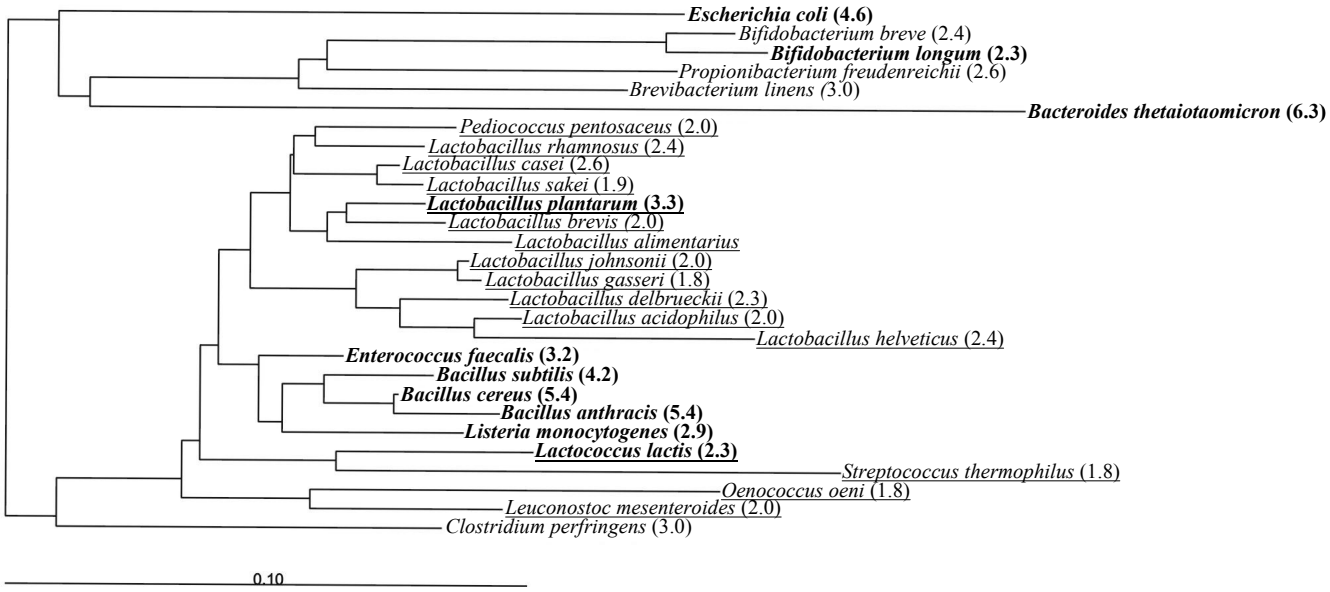


Fig. 2: Phylogenetic relationship based upon the neighbor-joining method of partial 16S rDNA sequences *(Escherichia coli* positions 107 to 1434). It should be noted that for some species the genome sequence has (partially) been determined for multiple strains. LAB genomes are underscored, and published, complete genomes are shown in bold. The estimated genome sizes are indicated between brackets. (The authors gratefully acknowledge Sergey Konstantinov for the construction of this figure).

L. plantarum is a versatile and flexible organism that is able to grow on a wide variety of sugar sources. This phenotypic trait is reflected in the genome sequence of *L. plantarum*, which harbors a remarkably high number of 25 complete PTS enzyme II complexes as well as several incomplete complexes. This high number of PTS systems is far more than that found in other complete bacterial genomes, and similar only to *Listeria monocytogenes* (21) and *Enterococcus faecalis* (50). In addition to these PTS systems, the *L. plantarum* genome encodes 30 transporters that are predicted to be involved in the transport of carbon sources. This high sugar uptake flexibility has also been observed in the (incomplete) genomes of other LAB, such as *L. johnsonii* and *L. acidophilus* (2, 52). Moreover, a remarkably high percentage of regulatory genes (8.5%) is encoded in the *L. plantarum* genome. Similar percentages were found in *Listeria monocytogenes*, in which 7.3% of all the encoded genes is involved in regulatory functions. This could be a reflection of the many different environmental conditions that these bacteria face. Moreover, during residence of these bacteria in the proximal parts of the GI-tract these sophisticated regulatory systems might enable these organisms to adapt quickly to changes in the nutrient (including sugar) availability caused by the host's diet (Fig. 3).

The genomes of *B. thetaiotaomicron* and *Bifidobacterium longum* encode an elaborate apparatus for acquiring and hydrolyzing otherwise indigestible dietary polysaccharides (57, 67). In *B. thetaiotaomicron* this colonic substrate dependence is associated with an environment-sensing system consisting of a large repertoire of extracytoplasmic function sigma factors and one- and two-component signal transduction systems (67). In contrast, genes involved in sugar transport and hydrolysis in *Bifidobacterium longum* are organized in operons which are predominantly regulated by LacI-type, sugar responsive repressors (57). The tight regulation of sugar utilization observed in these bacteria allows a stringent response to environmental changes and is in accordance with the fact that *Bifidobacterium longum* and *B. thetaiotaomicron* need to adapt to wide fluctuations in substrate availability in the colon (57, 67). It is speculated that the mode of regulation via repression of genes could allow a more rapid response in *Bifidobacterium longum* (57). In analogy, an operon in *L. acidophilus* involved in utilization of the prebiotic compound fructooligosaccharide contains a LacI-type repressor. Moreover, the expression of this operon is subject to global catabolite repression in the presence of readily fermentable sugars (5). Another interesting finding in the genome of *B. thetaiotaomicron* is that it encodes the capacity to use a variety of host-derived glycoproteins and glycoconjugates. Sixty-one percent of its glycosylhydrolases are predicted to be located in the periplasm, outer membrane, or extracellularly. This suggests that these enzymes are not only important for fulfilling the needs of *B. thetaiotaomicron* but may also help shape the metabolic milieu of the intestinal ecosystem, thereby maintaining a microbiota that supplies the host with 10 to 15% of our daily calories as

fermentation products of dietary polysaccharides (67) (Fig. 3). Similarly, the genome sequence of *Bifidobacterium longum* revealed insights into the interaction of bifidobacteria with their host, as genes encoding polypeptides with homology to glycoprotein-binding fimbriae are present in the genome. Moreover, a eukaryotic-type serine protease inhibitor is encoded in the genome and could be involved in the reported immunomodulatory activity of bifidobacteria (57).

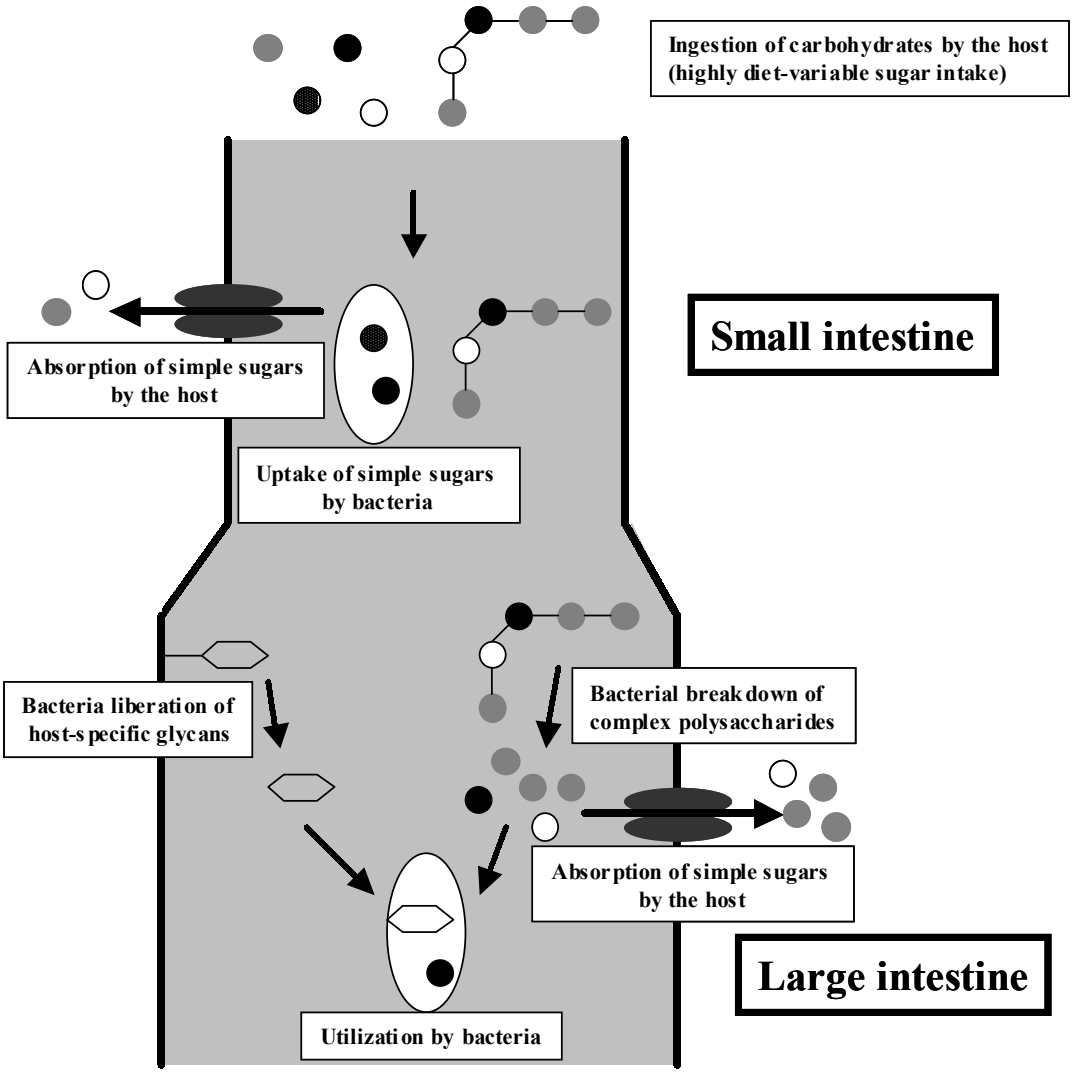


Fig. 3: Molecular model of bacterial sugar utilization in the GI-tract. In the small intestine mono- and disaccharides are rapidly consumed by the host. Typically, bacteria that live in this niche display highly flexible sugar utilization capacities, allowing them to quickly adapt to changes in the carbon source availability that is determined by the host's diet. This high sugar flexibility is required to compete with the host for carbon acquisition. In the large intestine more complex oligo-and polysaccharides are the only available C-source. Therefore, bacteria in this niche are usually able to hydrolyze complex dietary polysaccharides and host-derived glycoproteins and glycoconjugates. Subsequently, the released, simpler sugars are utilized as C-source by the host and the bacteria residing in the colon (26).

Overall, the availability of the genome sequences together with their annotations provides valuable clues towards the survival strategy of these bacteria during their residence in the human GI-tract. Additionally, these complete genome sequences are powerful tools for the convenient and effective interpretation of the data generated by the *in vitro* and *in vivo* screening procedures described above.

in situ profiling of transcription in the GI-tract

As soon as sequence data is available for a few genes in a bacterium of interest, one could think of several sophisticated tools that allow investigation of the *in situ* expression levels of specific genes. One example of such an approach is the implementation of quantitative reverse transcriptase PCR (qRT-PCR) in the gram-negative bacterium *Helicobacter pylori* (54). This study describes the assessment of gene expression in this pathogen within the mouse and human gastric mucosae. Three genes, encoding urease, catalase and a putative adhesin specific for adherence to human gastric mucosa, were selected for this study, as their role during host residence was already established. Using minute quantities of mRNA isolated from human and mouse infected mucosae, the *in situ* expression of these three genes could be established. Moreover, the results of this study indicate that the relative abundance of transcripts was the same in the human and mouse model system. Hence, this study demonstrates that qRT-PCR is a powerful tool for the detection and quantification of bacterial gene expression in the GI-tract (54).

Studies in germ-free mice have indicated that there is specific signaling between the commensal bacterium *B. thetaiotaomicron* and its host. Synthesis of host epithelial glycans is elicited by a *B. thetaiotaomicron* signal whose expression is regulated by a fucose binding bacterial transcription factor. This factor senses environmental levels of fucose and coordinates the decision to generate a signal for production of host fucosylated glycans when environmental fucose is limited or to induce expression of the bacteria's fucose utilization operon when fucose is abundant (29). Additional studies have evaluated the global intestinal response to colonization of gnotobiotic mice with *B. thetaiotaomicron*. This colonization dramatically affected the host's gene expression, including several important intestinal functions such as nutrient absorption, mucosal barrier fortification, and postnatal intestinal maturation (28). Interestingly, comparison of the changes in global host gene expression after colonization with *B. thetaiotaomicron*, *Bifidobacterium infantis* or *Escherichia coli* revealed that part of this host response was only induced by colonization with *B. thetaiotaomicron* (28). Moreover, from these *in situ* global transcription profiles and follow-up experiments it could be established that the production of a previously uncharacterized angiogenin is induced when gnotobiotic mice are colonized with *B. thetaiotaomicron*, revealing a mechanism whereby intestinal commensal bacteria influence GI-tract bacterial ecology and shape innate immunity (27). The cellular

origin of the angiogenin response was investigated in different intestinal cell types that were separated by laser-capture microdissection and analyzed by qRT-PCR, revealing that angiogenin-3 mRNA is specifically induced only in crypt epithelial cells. Hence, these experiments strongly suggest an intestinal tissue specific response of the host during colonization (28). Although the aforementioned studies on *B. thetaiotaomicron* colonization of gnotobiotic mice have provided valuable information on the influence of one particular member of the microbiota on the host, it remains to be investigated whether this response can also be established in a conventional, colonized animal model. Moreover, a future challenge lies within the translation of these mice host response analyses to the human system.

Concluding remarks

Historically, research on the bacterial flora of the GI-tract has concentrated on the inhabitants that have negative effects on their hosts. More recently, research has expanded from these pathogenic to non-pathogenic bacteria, including symbionts and commensals. One obvious reason for this is the accumulating evidence that certain bacteria, especially strains from the genus *Lactobacillus* and *Bifidobacterium*, may have probiotic effects in man and animals (1). At present molecular tools are available that allow a detailed indexation of the distribution of specific microbes residing in the human GI-tract, including the variations that can occur between different individuals (63, 69, 71). In addition, knowledge on the activity and response of specific species to the conditions encountered when they transit through this complex niche is starting to accumulate. Several *in vitro* studies mimicking specific conditions in the GI-tract have been performed, which allowed the identification of the repertoire of genes and their corresponding proteins that respond to the condition applied. More sophisticated, *in vivo* approaches aiming at the identification of bacterial genes that are induced during passage of the GI-tract have been performed in several pathogens. Unfortunately, only one report describes the *in vivo* response of a food-grade bacterium during passage through a (non-conventionally colonized) host, while such reports are completely lacking for commensal bacteria.

A promising prospect from the increasing availability of complete genome sequences is the construction of DNA micro-arrays in several laboratory working on food-associated microbes. These, genomics-based, global investigations of gene expression in food-grade microbes under various conditions will further detail our future understanding of their behavior. Besides the application of DNA micro-array technology to elucidate the bacterial side of host-microbe interactions in the GI-tract, this technology has been used by Hooper and co-workers in several elegant studies aiming at identification of the response of germ-free mice to colonization with the commensal *B. thetaiotaomicron* (28). However, an important question that still remains to be answered is to what extent the data obtained on host and bacterial gene

expression using gnotobiotic animal models can be extrapolated to the situations in conventionally raised animals and, ultimately, humans. Nevertheless, these kind of studies could eventually lead to the construction of first generation molecular models describing specific host-microbe interactions. Such hypothetical models can subsequently be confirmed or falsified by similar analyses using mutant bacterial or host species in which specific changes have been introduced (overexpression or deletion) in factors predicted to play a role in the interaction. Finally, confirmed mouse-bacterium interaction models might eventually be analyzed in humans using a gene targeted approach.

Outline of this thesis

This thesis primarily aims at the identification and characterization of genes that are important in a LAB during residence in, and travel through the GI-tract. LAB are applied extensively in the production of fermented food products. Via the frequent consumption of dairy, olives, sausages and other fermented food products, large amounts of LAB are ingested by humans on a daily basis. Moreover, LAB have the potential to serve as delivery vehicles of health-promoting or therapeutic compounds to the human GI-tract (23, 60). Among the LAB, *L. plantarum* is encountered in many environmental niches, including dairy, meat and a variety of vegetable fermentations (14, 15, 55). Next to the occurrence of *L. plantarum* in our diets, this microbe is frequently encountered as a natural inhabitant of the GI-tract (1). Hence, *L. plantarum* has been selected as a model organism of which the GI-tract behavior will be studied. Other important criteria for the selection of this lactic acid bacterium are its high genetic accessibility and the fact that the complete genome sequence of *L. plantarum* WCFS1 has been determined (39). Moreover, the *L. plantarum* WCFS1 strain is a single colony isolate of the strain NCIMB8826, which is of human origin and effectively survives passage of the human stomach in an active form, reaches the ileum in high numbers as compared to other strains, and is detectable in the colon (64).

The current chapter **(Chapter 1)** describes the state of the art in the research field on both the host and the microbial side of their interactions in the GI-tract, as well as the lack of knowledge on the *in situ* behavior of food-associated and commensal bacteria in this niche. The next two chapters describe the development of genetic tools in LAB. **Chapter 2** describes the exploitation of the alanine racemase encoding gene (*alr*) as a food-grade selection marker in *Lactococcus lactis* and *L. plantarum*. It is demonstrated that the *alr* gene can be exploited as a dominant and complementation marker. In **Chapter 3** the possibility to use the *alr* gene as a promoter probe for the identification of conditionally active promoters is explored. An exemplary screen is performed and results in the identification of high-salt inducible promoters in *L. plantarum*. In **Chapter 4** the *alr* promoter probe is applied in a

genome-wide screen, resulting in the identification of bile salt inducible genes in *L. plantarum*. Moreover, a qRT-PCR approach focused on two of the identified bile salt inducible genes, which led to the conclusion that the results obtained with this plasmid-based promoter probe can be extrapolated to the single copy situation on the *L. plantarum* chromosome. In addition, the *in situ* induction of expression of these two genes in the duodenum of a mouse model system is demonstrated. **Chapter 5** describes a global transcriptome analysis using DNA micro-array technology, resulting in the identification of *L. plantarum* genes of which the expression level is induced by bile salts. The obtained transcript profiles are compared to the results obtained with the *alr* promoter probe in chapter 4. In **Chapter 6** the implementation of a novel R-IVET system in *L. plantarum* is described. This system is applied in the screening of a chromosomal library in a conventionally colonized mouse model system, resulting in the identification of 72 *L. plantarum* genes of which the expression is induced during passage of the mouse GI-tract as compared to laboratory conditions. **Chapter 7** deals with the construction of 12 gene disruption mutants in a selection of the genes that were identified in chapter 6 as *in vivo* induced in the GI-tract. The growth characteristics of the constructed mutants on laboratory media are described, as well as assessment of the *in vivo* persistence of these mutants in a mouse model system. **Chapter 8** describes a functional analysis of sortase in *L. plantarum*. The results from the R-IVET screen (Chapter 6) suggest that sortase dependent surface exposed proteins could play a role during persistence in the GI-tract. Therefore, a *L. plantarum* sortase mutant was constructed and its *in vitro* agglutination and *in vivo* persistence characteristics were investigated. In **Chapter 9** general conclusions coming from the research described in this thesis are drawn, and the future perspectives in the research field are discussed.

References

1. **Ahrne, S., S. Nobaek, B. Jeppsson, I. Adlerberth, A. E. Wold, and G. Molin.** 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. J. Appl. Microbiol. **85:**88-94.

2. **Altermann, E., R. J. Cano, and T. Klaenhammer.** 2003. Structural and functional genome-comparison between two related *Lactobacillus* probiotic species. Proceedings of the 7th symposium on lactic acid bacteria.

3. **Angelichio, M. J., and A. Camilli.** 2002. *In vivo* expression technology. Infect. Immun. **70:**6518-23.

4. **Audia, J. P., C. C. Webb, and J. W. Foster.** 2001. Breaking through the acid barrier: an orchestrated response to proton stress by enteric bacteria. Int. J. Med. Microbiol. **291:**97-106.

5. **Barrangou, R., E. Altermann, R. Hutkins, R. Cano, and T. R. Klaenhammer.** 2003. Functional and comparative genomic analyses of an operon involved in fructooligosaccharide utilization by *Lactobacillus acidophilus*. Proc. Natl. Acad. Sci. U.S.A. **100:**8957-62.

6. **Begley, M., C. G. Gahan, and C. Hill.** 2002. Bile stress response in *Listeria monocytogenes* LO28: adaptation, cross-protection, and identification of genetic loci involved in bile resistance. Appl. Environ. Microbiol. **68:**6005-12.

7. **Bolotin, A., P. Wincker, S. Mauger, O. Jaillon, K. Malarme, J. Weissenbach, S. D. Ehrlich, and A. Sorokin.** 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. lactis IL1403. Genome Res. **11:**731-753.

8. **Breton, Y. L., A. Maze, A. Hartke, S. Lemarinier, Y. Auffray, and A. Rince.** 2002. Isolation and characterization of bile salts-sensitive mutants of *Enterococcus faecalis*. Curr. Microbiol. **45:**434-9.

9. **Chou, L. S., and B. Weimer.** 1999. Isolation and characterization of acid- and bile-tolerant isolates from strains of *Lactobacillus acidophilus*. J. Dairy Sci. **82:**23-31.

10. **Cummings, J. H., and G. T. Macfarlane.** 1991. The control and consequences of bacterial fermentation in the human colon. J. Appl. Bacteriol. **70:**443-59.

11. **Daigle, F., J. E. Graham, and R. Curtiss, 3rd.** 2001. Identification of *Salmonella typhi* genes expressed within macrophages by selective capture of transcribed sequences (SCOTS). Mol. Microbiol. **41:**1211-22.

12. **de Vos, W. M.** 2001. Advances in genomics for microbial food fermentations and safety. Curr. Opin. Biotechnol. **12:**493-8.

13. **Dozois, C. M., F. Daigle, and R. Curtiss, 3rd.** 2003. Identification of pathogen-specific and conserved genes expressed *in vivo* by an avian pathogenic *Escherichia coli* strain. Proc. Natl. Acad. Sci. U.S.A. **100:**247-52.

14. **Duran Quintana, M. C., P. Garcia Garcia, and A. Garrido Fernandez.** 1999. Establishment of conditions for green table olive fermentation at low temperature. Int. J. Food Microbiol. **51:**133-43.

15. **Enan, G., A. A. el-Essawy, M. Uyttendaele, and J. Debevere.** 1996. Antibacterial activity of *Lactobacillus plantarum* UG1 isolated from dry sausage: characterization, production and bactericidal action of plantaricin UG1. Int. J. Food Microbiol. **30:**189-215.

16. **Favier, C. F., E. E. Vaughan, W. M. De Vos, and A. D. Akkermans.** 2002. Molecular monitoring of succession of bacterial communities in human neonates. Appl. Environ. Microbiol. **68:**219-26.

17. **Flahaut, S., J. Frere, P. Boutibonnes, and Y. Auffray.** 1996. Comparison of the bile salts and sodium dodecyl sulfate stress responses in *Enterococcus faecalis*. Appl. Environ. Microbiol. **62:**2416-20.

18. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, and et al.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496-512.

19. **Gahan, C. G., and C. Hill.** 2000. The use of listeriolysin to identify *in vivo* induced genes in the gram- positive intracellular pathogen *Listeria monocytogenes*. Mol. Microbiol. **36:**498-507.

20. **Gillaspy, A. F., S. G. Hickmon, R. A. Skinner, J. R. Thomas, C. L. Nelson, and M. S. Smeltzer.** 1995. Role of the accessory gene regulator (*agr*) in pathogenesis of staphylococcal osteomyelitis. Infect. Immun. **63:**3373-80.

21. **Glaser, P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend, F. Baquero, P. Berche, H. Bloecker, P. Brandt, T. Chakraborty, A. Charbit, F. Chetouani, E. Couve, A. de Daruvar, P. Dehoux, E. Domann, G. Dominguez-Bernal, E. Duchaud, L. Durant, O. Dussurget, K. D. Entian, H. Fsihi, F. Garcia-del Portillo, P. Garrido, L. Gautier, W. Goebel, N. Gomez-Lopez, T. Hain, J. Hauf, D. Jackson, L. M. Jones, U. Kaerst, J. Kreft, M. Kuhn, F. Kunst, G. Kurapkat, E. Madueno, A. Maitournam, J. M. Vicente, E. Ng, H. Nedjari, G. Nordsiek, S. Novella, B. de Pablos, J. C. Perez-Diaz, R. Purcell, B. Remmel, M. Rose, T. Schlueter, N. Simoes, A. Tierrez, J. A. Vazquez-Boland, H. Voss, J. Wehland, and P. Cossart.** 2001. Comparative genomics of *Listeria* species. Science **294:**849-52.

22. **Graham, J. E., and J. E. Clark-Curtiss.** 1999. Identification of *Mycobacterium tuberculosis* RNAs synthesized in response to phagocytosis by human macrophages by selective capture of transcribed sequences (SCOTS). Proc. Natl. Acad. Sci. U.S.A. **96:**11554-9.

23. **Grangette, C., H. Muller-Alouf, M. Geoffroy, D. Goudercourt, M. Turneer, and A. Mercenier.** 2002. Protection against tetanus toxin after intragastric administration of two recombinant lactic acid bacteria: impact of strain viability and *in vivo* persistence. Vaccine **20:**3304-9.

24. **Gunn, J. S.** 2000. Mechanisms of bacterial resistance and response to bile. Microbes Infect. **2:**907-13.

25. **Heilig, H. G., E. G. Zoetendal, E. E. Vaughan, P. Marteau, A. D. Akkermans, and W. M. de Vos.** 2002. Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl. Environ. Microbiol. **68:**114-23.

26. **Hooper, L. V., T. Midtvedt, and J. I. Gordon.** 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu. Rev. Nutr. **22:**283-307.

27. **Hooper, L. V., T. S. Stappenbeck, C. V. Hong, and J. I. Gordon.** 2003. Angiogenins: a new class of microbicidal proteins involved in innate immunity. Nat. Immunol. **4:**269-73.

28. **Hooper, L. V., M. H. Wong, A. Thelin, L. Hansson, P. G. Falk, and J. I. Gordon.** 2001. Molecular analysis of commensal host-microbial relationships in the intestine. Science **291:**881-4.

29. **Hooper, L. V., J. Xu, P. G. Falk, T. Midtvedt, and J. I. Gordon.** 1999. A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem. Proc. Natl. Acad. Sci. U.S.A. **96:**9833-8.

30. **Hopkins, M. J., R. Sharp, and G. T. Macfarlane.** 2001. Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles. Gut **48:**198-205.

31. **Hou, J. Y., J. E. Graham, and J. E. Clark-Curtiss.** 2002. *Mycobacterium avium* genes expressed during growth in human macrophages detected by selective capture of transcribed sequences (SCOTS). Infect. Immun. **70:**3714-26.

32. **Hyronimus, B., C. Le Marrec, A. H. Sassi, and A. Deschamps.** 2000. Acid and bile tolerance of spore-forming lactic acid bacteria. Int. J. Food Microbiol. **61:**193-7.

33. **Ivanova, N., A. Sorokin, I. Anderson, N. Galleron, B. Candelon, V. Kapatral, A. Bhattacharyya, G. Reznik, N. Mikhailova, A. Lapidus, L. Chu, M. Mazur, E. Goltsman, N. Larsen, M. D'Souza, T. Walunas, Y. Grechkin, G. Pusch, R. Haselkorn, M. Fonstein, S. D. Ehrlich, R. Overbeek, and N. Kyrpides.** 2003. Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. Nature **423:**87-91.

34. **Jan, G., P. Leverrier, V. Pichereau, and P. Boyaval.** 2001. Changes in protein synthesis and morphology during acid adaptation of *Propionibacterium freudenreichii*. Appl. Environ. Microbiol. **67:**2029-36.

35. **Janakiraman, A., and J. M. Slauch.** 2000. The putative iron transport system SitABCD encoded on SPI1 is required for full virulence of *Salmonella typhimurium*. Mol. Microbiol. **35:**1146-55.

36. **Khanduja, V., G. Kang, D. P. Rajan, and K. A. Balasubramanian.** 1998. Oxidative stress response in *Shigella* & nonpathogenic gut bacteria. Indian J. Med. Res. **108:**3-7.

37. **Kiliç, A. O., M. C. Herzberg, M. W. Meyer, X. Zhao, and L. Tao.** 1999. Streptococcal reporter gene-fusion vector for identification of *in vivo* expressed genes. Plasmid **42:**67-72.

38. **Klaenhammer, T., E. Altermann, F. Arigoni, A. Bolotin, F. Breidt, J. Broadbent, R. Cano, S. Chaillou, J. Deutscher, M. Gasson, M. van de Guchte, J. Guzzo, A. Hartke, T. Hawkins, P. Hols, R. Hutkins, M. Kleerebezem, J. Kok, O. Kuipers, M. Lubbers, E. Maguin, L. McKay, D. Mills, A. Nauta, R. Overbeek, H. Pel, D. Pridmore, M. Saier, D. van Sinderen, A. Sorokin, J. Steele, D. O'Sullivan, W. de Vos, B. Weimer, M. Zagorec, and R. Siezen.** 2002. Discovering lactic acid bacteria by genomics. Antonie Van Leeuwenhoek **82:**29-58.

39. **Kleerebezem, M., J. Boekhorst, R. Van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. De Vries, B. Ursing, W. M. De Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U.S.A. **100:**1990-1995.

40. **Krogfelt, K. A., M. Hjulgaard, K. Sorensen, P. S. Cohen, and M. Givskov.** 2000. rpoS gene function is a disadvantage for *Escherichia coli* BJ4 during competitive colonization of the mouse large intestine. Infect. Immun. **68:**2518-24.

41. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, and A. Danchin.** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature**:**249-56.

42. **Lai, Y. C., H. L. Peng, and H. Y. Chang.** 2001. Identification of genes induced *in vivo* during *Klebsiella pneumoniae* CG43 infection. Infect. Immun. **69:**7140-5.

43. **Leverrier, P., D. Dimova, V. Pichereau, Y. Auffray, P. Boyaval, and G. Jan.** 2003. Susceptibility and adaptive response to bile salts in *Propionibacterium freudenreichii*: physiological and proteomic analysis. Appl. Environ. Microbiol. **69:**3809-18.

44. **Lowe, A. M., D. T. Beattie, and R. L. Deresiewicz.** 1998. Identification of novel staphylococcal virulence genes by *in vivo* expression technology. Mol. Microbiol. **27:**967-76.

45. **Mahan, M. J., D. M. Heithoff, R. L. Sinsheimer, and D. A. Low.** 2000. Assessment of bacterial pathogenesis by analysis of gene expression in the host. Annu. Rev. Genet. **34:**139-164.

46. **Mahan, M. J., J. M. Slauch, and J. J. Mekalanos.** 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259:**686-8.

47. **Mecsas, J.** 2002. Use of signature-tagged mutagenesis in pathogenesis studies. Curr. Opin. Microbiol. **5:**33-7.

48. **Morrow, B. J., J. E. Graham, and R. Curtiss, 3rd.** 1999. Genomic subtractive hybridization and selective capture of transcribed sequences identify a novel *Salmonella typhimurium* fimbrial operon and putative transcriptional regulator that are absent from the *Salmonella typhi* genome. Infect. Immun. **67:**5106-16.

49. **Parkhill, J., G. Dougan, K. D. James, N. R. Thomson, D. Pickard, J. Wain, C. Churcher, K. L. Mungall, S. D. Bentley, M. T. Holden, M. Sebaihia, S. Baker, D. Basham, K. Brooks, T. Chillingworth, P. Connerton, A. Cronin, P. Davis, R. M. Davies, L. Dowd, N. White, J. Farrar, T. Feltwell, N. Hamlin, A. Haque, T. T. Hien, S. Holroyd, K. Jagels, A. Krogh, T. S. Larsen, S. Leather, S. Moule, P. O'Gaora, C. Parry, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S. Whitehead, and B. G. Barrell.** 2001. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. Nature**:**848-52.

50. **Paulsen, I. T., L. Banerjei, G. S. Myers, K. E. Nelson, R. Seshadri, T. D. Read, D. E. Fouts, J. A. Eisen, S. R. Gill, J. F. Heidelberg, H. Tettelin, R. J. Dodson, L. Umayam, L. Brinkac, M. Beanan, S. Daugherty, R. T. DeBoy, S. Durkin, J. Kolonay, R. Madupu, W. Nelson, J. Vamathevan, B. Tran, J. Upton, T. Hansen, J. Shetty, H. Khouri, T. Utterback, D. Radune, K. A. Ketchum, B. A. Dougherty, and C. M. Fraser.** 2003. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. Science **299:**2071-4.

51. **Phan-Thanh, L., and T. Gormon.** 1997. Stress proteins in *Listeria monocytogenes*. Electrophoresis **18:**1464-71.

52. **Pridmore, R. D.** *et al*. 2003. submitted for publication.

53. **Purdy, D., S. Cawthraw, J. H. Dickinson, D. G. Newell, and S. F. Park.** 1999. Generation of a superoxide dismutase (SOD)-deficient mutant of *Campylobacter coli*: evidence for the significance of SOD in *Campylobacter* survival and colonization. Appl. Environ. Microbiol. **65:**2540-6.

54. **Rokbi, B., D. Seguin, B. Guy, V. Mazarin, E. Vidor, F. Mion, M. Cadoz, and M. J. Quentin-Millet.** 2001. Assessment of *Helicobacter pylori* gene expression within mouse and human gastric mucosae by real-time reverse transcriptase PCR. Infect. Immun. **69:**4759-66.

55. **Ruiz-Barba, J. L., J. C. Piard, and R. Jimenez-Diaz.** 1991. Plasmid profiles and curing of plasmids in *Lactobacillus plantarum* strains isolated from green olive fermentations. J. Appl. Bacteriol. **71:**417-21.

56. **Savage, D. C.** 1977. Microbial ecology of the gastrointestinal tract. Annu. Rev. Microbiol. **31:**107-33.

57. **Schell, M. A., M. Karmirantzou, B. Snel, D. Vilanova, B. Berger, G. Pessi, M. C. Zwahlen, F. Desiere, P. Bork, M. Delley, R. D. Pridmore, and F. Arigoni.** 2002. The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. Proc. Natl. Acad. Sci. U.S.A. **99:**14422-7.

58. **Schmidt, G., and R. Zink.** 2000. Basic features of the stress response in three species of bifidobacteria: *B. longum*, *B. adolescentis*, and *B. breve*. Int. J. Food Microbiol. **55:**41-5.

59. **Smith, H. E., H. Buijs, R. R. de Vries, H. J. Wisselink, N. Stockhofe-Zurwieden, and M. A. Smits.** 2001. Environmentally regulated genes of *Streptococcus suis*: identification by the use of iron-restricted conditions in vitro and by experimental infection of piglets. Microbiology **147:**271-80.

60. **Steidler, L., W. Hans, L. Schotte, S. Neirynck, F. Obermeier, W. Falk, W. Fiers, and E. Remaut.** 2000. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin- 10. Science **289:**1352-1355.

61. **Sullivan, A., C. Edlund, and C. E. Nord.** 2001. Effect of antimicrobial agents on the ecological balance of human microflora. Lancet Infect. Dis. **1:**101-14.

62. **Taranto, M. P., M. L. Fernandez Murga, G. Lorca, and G. F. de Valdez.** 2003. Bile salts and cholesterol induce changes in the lipid cell membrane of *Lactobacillus reuteri*. J. Appl. Microbiol. **95:**86-91.

63. **Vaughan, E. E., M. C. de Vries, E. G. Zoetendal, K. Ben-Amor, A. D. Akkermans, and W. M. de Vos.** 2002. The intestinal LABs. Antonie Van Leeuwenhoek **82:**341-52.

64. **Vesa, T., P. Pochart, and P. Marteau.** 2000. Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. Aliment. Pharmacol. Ther. **14:**823-8.

65. **Walter, J., N. C. Heng, W. P. Hammes, D. M. Loach, G. W. Tannock, and C. Hertel.** 2003. Identification of *Lactobacillus reuteri* Genes Specifically Induced in the Mouse Gastrointestinal Tract. Appl. Environ. Microbiol. **69:**2044-51.

66. **West, N. P., P. J. Sansonetti, G. Frankel, and C. M. Tang.** 2003. Finding your niche: what has been learnt from STM studies on GI colonization. Trends Microbiol. **11:**338-44.

67. **Xu, J., M. K. Bjursell, J. Himrod, S. Deng, L. K. Carmichael, H. C. Chiang, L. V. Hooper, and J. I. Gordon.** 2003. A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. Science**:**2074-6.

68. **Zoetendal, E. G., A. D. Akkermans, W. M. Akkermans-van Vliet, J. A. G. M. de Visser, and W. M. de Vos.** 2001. The host genotype affects the bacterial community in the human gastrointestinal tract. Microbial Ecol. Health Dis. **13:**129-134.

69. **Zoetendal, E. G., A. D. Akkermans, and W. M. De Vos.** 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol. **64:**3854-9.

70. **Zoetendal, E. G., C. M. Plugge, A. D. Akkermans, and W. M. de Vos.** 2003. *Victivallis vadensis* gen. nov., sp. nov., a sugar-fermenting anaerobe from human faeces. Int. J. Syst. Evol. Microbiol. **53:**211-5.

71. **Zoetendal, E. G., A. von Wright, T. Vilpponen-Salmela, K. Ben-Amor, A. D. Akkermans, and W. M. de Vos.** 2002. Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. Appl. Environ. Microbiol. **68:**3401-7.

Chapter 2

Use of the *alr* Gene as Food-Grade Selection Marker in Lactic Acid Bacteria

PETER A. BRON,[1] MARCOS G. BENCHIMOL,[2] JOLANDA LAMBERT,[1] EMMANUELLE PALUMBO,[2] MARIE DEGHORAIN,[2] JEAN DELCOUR,[2] WILLEM M. DE VOS,[1] MICHIEL KLEEREBEZEM,[1] AND PASCAL HOLS.[2]

[1] Wageningen Centre for Food Sciences, P.O. Box 557, 6700 AN Wageningen, The Netherlands. [2] Université catholique de Louvain, Unité de Génétique, Place Croix du sud 5, 1348 Louvain-la-Neuve, Belgium.

Published in Appl. Environ. Microbiol. (2002) 68: 5663-5670.

Abstract

Both *Lactococcus lactis* and *Lactobacillus plantarum* contain a single *alr* gene, encoding an alanine racemase (EC 5.1.1.1), which catalyses the interconversion of D-alanine and L-alanine. The *alr* genes of these lactic acid bacteria were investigated for their application as food-grade selection markers in a heterologous complementation approach. Since isogenic mutants of both species carrying an *alr* deletion (Δ*alr*) showed auxotrophy for D-alanine, plasmids carrying a heterologous *alr* were constructed and could be selected, as they complemented D-alanine auxotrophy in the *L. plantarumΔalr* and *Lactococcus lactisΔalr* strains. Selection was found to be highly stringent and plasmids were stably maintained over 200 generations of culturing. Moreover, the plasmids carrying the heterologous *alr* genes could be stably maintained in wild-type strains of *L. plantarum* and *Lactococcus lactis* by selection for resistance to D-cycloserine, a competitive inhibitor of Alr (600 and 200 μg/ml, respectively). In addition, a plasmid carrying the *L. plantarum alr* gene under control of the regulated *nisA* promoter was constructed to demonstrate that D-cycloserine resistance of *Lactococcus lactis* is linearly correlated to the *alr* expression level. Finally, the *Lactococcus lactis alr* gene controlled by the *nisA* promoter, together with the nisin regulatory genes *nisRK*, were integrated into the chromosome of *L. plantarumΔalr*. The resulting strain could grow in the absence of D-alanine only when expression of the *alr* gene was induced with nisin.

Introduction

Alanine racemases are pyridoxal 5'-phosphate dependent enzymes involved in the interconversion of D-alanine (D-ala) and L-alanine (L-ala). Most well-studied alanine racemases originate from bacteria, including *Escherichia coli* (52), *Pseudomonas putida* (41), *Salmonella typhimurium* (51) and several *Bacillus* species (21, 30, 39, 55). More recently, eukaryotic alanine racemases were investigated in more detail, e.g. in the fungus *Tolypocladium niveum* where this enzyme is involved in the biosynthesis of cyclosporin A (27). Since in many bacteria, D-ala is involved in the cross-linking of cell wall peptidoglycan layers, this component is essential for their growth.

In *E. coli*, the *dadX* gene is involved in L-ala catabolism. It encodes an alanine racemase and is situated in an operon together with the *dadA* gene, encoding a D-alanine dehydrogenase. The transcription of *dadX* and *dadA* was found to be repressed by glucose, while it was induced by the presence of L-ala (53). The *dadX* gene is responsible for 85% of the total alanine racemase activity in *E. coli* and a second gene named *alr* is present, which is constitutively expressed (52, 53). Only the *alr dadX* double mutant is auxotroph for D-ala (53).

In *Bacillus subtilis* the alanine racemase gene (*dal*) is involved in alanine conversion and a *dal* deletion mutant was dependent on D-ala supplementation when grown in rich media. However, in minimal medium, growth of the *dal* mutant was only affected after addition of L-ala. Hence, it was suggested that *B. subtilis* possesses a second, L-ala repressible alanine racemase (21). The complete genome sequence of *B. subtilis* confirms the existence of a second gene (*yncD*) that shows high homology to alanine racemases (35). Furthermore, alanine racemases from *Bacillus* species are postulated to be involved in spore formation, since racemase activity is generally higher in spores as compared to vegetative cells (39, 40, 46). Spore alanine racemase converts the germinant L-ala into its competitive inhibitor D-ala and may regulate spore germination (54). The most extensively studied alanine racemase originates from *B. stearothermophilus* and includes sequence analysis of the gene and protein, determination of the catalytic site residues, and characterization of the biochemical properties of the protein (30, 44, 47). Moreover, the 3D-structure of the alanine racemase of this organism was determined by X-ray crystallography (43).

Several allosteric inhibitors of alanine racemases have been described, including D-cycloserine, hydroxylamine and β-chloroalanine (1, 32, 36, 50). Inactivation of *alrA*, encoding alanine racemase, resulted in a 30-fold lower MIC value for D-cycloserine in *Mycobacterium smegmatis* (9). Furthermore, a D-cycloserine resistant mutant of *M. smegmatis* was shown to display elevated alanine racemase activity, caused by a promoter up mutation. Similarly, increasing the *alrA* gene dosage by cloning on a multicopy plasmid resulted in increased D-cycloserine resistance in *M. intracellulare* and *M. bovis* (6).

In the lactic acid bacteria (LAB) *Lactobacillus plantarum* and *Lactococcus* alanine racemase activity is encoded by homologous *alr* genes. Disruption of *alr* in both LAB resulted in auxotrophy for D-ala on rich media (28, 29). Additionally, no growth of the *L. plantarum* deletion mutant was observed on minimal medium with or without L-ala, indicating that L-ala does not suppress another putative alanine racemase. In contrast with *B. subtilis*, *alr* appears to be the sole gene coding for alanine racemase activity in *L. plantarum* (28). LAB are important organisms for the production of fermented foods and feeds. Moreover, LAB are used as probiotics and have great potential to serve as delivery vehicles of health-promoting compounds to the human gastro-intestinal tract (23, 24, 45). To further optimize these microbes for industrial exploitation, genetic modification approaches have been used (14). However, the application of genetically modified microorganisms in food products requires safe and sustainable genetic tools. Therefore, sophisticated food-grade marker systems are being developed that circumvent the addition of undesirable components like antibiotics to industrial fermentation processes. Several food-grade marker systems have been developed for the selection of a plasmid in LAB, both of the dominant and complementation type (14). Recently, a third type of food-grade selection markers was described, using a two-plasmid system for food-grade selection in Lactococci (17).

D-ala is not a regular constituent of industrial fermentation media, suggesting that the alanine racemase encoding gene of LAB could be exploited as a food-grade complementation marker. Previously, a similar approach in *B. subtilis* showed that the *dal* gene, encoding alanine racemase, could be used as a functional and stringent complementation marker in rich media (21). Here, we describe the use of the *alr* gene as a heterologous food-grade selection marker in both *L. plantarum* and *Lactococcus lactis*. Plasmids expressing *alr* were introduced into *alr* mutants of both organisms and into their wild-type parent strains, using D-cycloserine resistance for selection of transformants in the latter strains. Selection appeared highly stringent, and plasmids were stably maintained during culturing. Moreover, *alr* expression levels in *Lactococcus lactis* could be correlated with D-cycloserine resistance levels and a nisin dependent *alr* mutant strain of *L. plantarum* was constructed that showed a conditional lethal phenotype.

Material and Methods

Bacterial strains, plasmids and primers

Lactococcus lactis MG1363 (22) and *L. plantarum* NCIMB8826 (26) were used in this study, and their derivatives, the used plasmids and primers are listed in Table 1. *E. coli* MC1061 (8) was used as intermediate cloning host and was handled as described by Sambrook *et al* (42). *L. plantarum* was grown at 30 ^{o}C in MRS broth (Difco, Surrey, U.K.) without aeration. *Lactococcus lactis* was grown at 30 ^{o}C in M17

broth (Merck Darmstadt, Germany), supplemented with 0.5% (wt/vol) glucose, without aeration. D-ala (200 μg/ml) was added to these media when indicated. Where appropriate, antibiotics were added to the different media; for *E. coli*, ampicillin (50 μg/ml), erythromycin (250 μg/ml), and chloramphenicol (20 μg/ml); for *Lactococcus lactis,* chloramphenicol (10 μg/ml); for *L. plantarum*, erythromycin (5 μg/ml) plus lincomycin (10 μg/ml), and chloramphenicol (10 μg/ml).

DNA manipulations

Plasmid DNA was isolated from *E. coli* on a small scale using the alkaline lysis method (2, 42). Large scale plasmid DNA isolations were performed using Jetstar columns according to the manufacturer's instructions (Genomed GmbH, Bad Oberhausen, Germany). DNA isolation and transformation in *Lactococcus lactis* and *L. plantarum* were performed as described previously (15, 19, 31). Standard procedures were applied for DNA manipulations in *E. coli* (42). Restriction endonucleases, *Taq* and *Pwo* polymerase, T4 DNA ligase and Klenow polymerase were used following the recommendations of the manufacturer (Promega Biotech, Roosendaal, the Netherlands and Boehringer Mannheim, Germany). Primers were purchased from Pharmacia Biotech (Roosendaal, the Netherlands) or Genset Oligos (Paris, France).

Plasmid constructions

Lactococcus lactis MG1363 genomic DNA (22) was used as a template to amplify the *alr* gene, using the primers LLALR-5 and LLALR-11 that were designed on the basis of the *Lactococcus lactis* IL1403 genome sequence (3). Sequence analysis of the obtained amplicons revealed 98% identity between the *alr* genes of *Lactococcus lactis* IL1403 and *Lactococcus lactis* MG1363. The 1.3-kb PCR product was digested with *Bam*HI and *Sal*I (restriction sites introduced by the primers) and inserted into similarly digested pJDC9, resulting in pGIP009. To drive expression of the *Lactococcus lactis alr* gene by the *ldhL* promoter of *L. plantarum* (18), pGIP009 was digested with *Hin*dIII and *Sma*I, followed by ligation to pGIT032, previously digested with *Hin*dIII and *Hpa*I. The resulting plasmid was designated pGIP011 and was introduced into *L. plantarum* NCIMB8826 and MD007. In analogy, to place *alr* expression under control of the *nisA* promoter of *Lactococcus lactis* (11), pGIP009 was digested with *Hind*III and *Bam*HI and, subsequently, ligated to similarly digested pNZ2650, yielding pGIP012, which was introduced into *L. plantarum* MD007Int6 carrying the *Lactococcus lactis nisRK* genes on the chromosome.

Table 1: Strains, plasmids and primers used in this study and their relevant characteristics and references. Underscored base pairs in primers indicate introduced restriction sites.

Material	Relevant Features[b]	Source of reference
Strains		
L. plantarum[a]		
MD007	*L. plantarum* NCIMB8826*Δalr*	(M. Deghorain, unpublished data)
MD007Int6	Em^R, *nisRK*, pMEC10 chromosomal integration in MD007 at $tRNA^{ser}$	this work
MD007::pGIP014	Em^R, *nisRK*, pGIP014 chromosomal integration in MD007 at $tRNA^{ser}$	this work
Lactococcus lactis		
NZ3900	MG1363 with a chromosomal insertion of *nisRK*	(12)
PH3960	NZ3900Δ*alr*, *nisRK*	(29)
Plasmids		
pJDC9	Em^R	(10)
pGIP009	Em^R, pJDC9 derivative containing *Lactococcus lactis alr* gene	this work
pGIT032	Em^R, Cm^R, P_{ldhL},	(18)
pNZ2650	Cm^R, P_{nisA}	(29)
pGIP011	Em^R, Cm^R, pGIT032 derivative containing *Lactococcus lactis alr* downstream of P_{ldhL}	this work
pGIP012	Cm^R, pNZ2650 derivative containing *Lactococcus lactis alr* downstream of P_{nisA}	this work
pNG8048-Cre	Cm^R	(7)
pUC-*Nco*I	Ap^R, pUC18 derivative containing *Nco*I and RBS of *prtP*	(20)
pNZ7110	Ap^R, pUC derivative containing *cre* and T_{pepN}	this work
pNZ7115	Ap^R, pNZ7110 with *cre* replaced by *L.plantarum alr* PCR product	this work
PNZ8020	Cm^R	(11)
pGIP013	pNZ8020 derivative containing *L.plantarum alr* from pNZ7115	this work
pGEMT-easy	Ap^R	Promega
pGIP010	Ap^R, pGEMT-easy derivative containing *Lactococcus lactis alr* PCR product	this work
pMEC10	Em^R, *nisRK*	(38)
pGIP014	Em^R, *nisRK*, pMEC10 derivative containing *alr* fragment of pGIP010	this work
Primers		
LLALR-5	5'-CGAGGATCCGCATAGTAATTTAGAAGCTGTTGC-3'	this work
LLALR-11	5'-ACGCGTCGACATTTGTAAAGGCTTTTATGAGAT-3'	this work
LLALR-12	5'-GGGGTACCATTTGTAAAGGCTTTTATGAGAT-3'	this work
LLALR-13	5'-CGGAATTCATTACAGCTCCAAGACTAGTC-3'	this work
TRNA	5'-GCGAACCGGCTAATACCGGC-3'	this work
409301L1	5'-AACAGAAGGTGGGACAGTAG-3'	this work
LplAlr_F	5'-AGGCAATTTGCCATGGTTGTAATTGG –3'	this work
LplAlr_R	5'-ACGTTCTAGATTAATCTATATAAACTCTCGG-3'	this work

[a] Strain NCIMB8826 was obtained from the National Collections of Industrial, Food and Marine Bacteria, Aberdeen, Scotland, UK

[b] Ap^R, ampicillin resistant, Cm^R, chloramphenicol resistant, Em^R, erythromycin resistant

The strategy described above was also used to construct plasmids expressing the *L. plantarum alr* gene for transformation and selection in *Lactococcus lactis*. To remove the *Hind*III site from pNG8048-cre (7), this plasmid was digested with *Hin*dIII, blunt-ended with Klenow DNA polymerase, and backligated. The *cre*-T_{pepN} fragment in this vector was removed with *Nco*I and *Xho*I, and ligated to pUC-*Nco*I (20) digested with *Nco*I and *Sal*I. Finally, digestion of this vector with *Eco*RI and *Hin*dIII, followed by Klenow DNA polymerase treatment, yielded a fragment containing RBS-*cre*-T_{pepN} , which was inserted into a pUC18 derivative previously digested with *Sma*I. The resulting plasmid was designated pNZ7110. The *alr* gene was obtained by a PCR reaction, using primers LplAlr_F and LplAlr_R, and *L. plantarum* NCIMB8826 chromosomal DNA as template. The 1.1-kb PCR product was digested with *Nco*I and *Xba*I and ligated to pNZ7110 digested with *Nco*I and *Nhe*I, in order to exchange *cre* for *alr*. The resulting plasmid was designated pNZ7115. Finally, RBS-*alr*-T_{pepN} was digested from pNZ7115 as a *Bam*HI-*Eco*RI fragment, and cloned downstream of the *nisA* promoter in similarly digested pNZ8020, yielding pGIP013. This plasmid was used for transformation of NZ3900 and strain PH3960.

The *alr* mutant strains of *L. plantarum* NCIMB8826 and *Lactococcus lactis* NZ3900, designated MD007 and PH3960 (Table 1), respectively, carry a stable deletion of an internal fragment of 100 and 30 bp in the *alr* gene, resulting in auxotrophy for D-ala in both organisms (28, 29). Since a large part of the *alr* genes in MD007 and PH3960 remain present in the chromosome, a heterologous complementation strategy was applied here, in order to avoid integration of the plasmid into the chromosome. Therefore, pGIP011 and pGIP012, containing the lactococcal *alr* gene under control of the *ldhL* and *nisA* promoter were used for complementation of the *L. plantarumΔalr* strains MD007 and MD007Int6, respectively. Similarly, pGIP013, containing the *L. plantarum alr* gene under control of the *nisA* promoter, was used for complementation of *Lactococcus lactisΔalr* strain PH3960.

To obtain a conditional *alr* mutant using the NICE system (12), a plasmid was designed for integration of the *Lactococcus lactis alr* gene into the chromosome of *L. plantarum* MD007. pGIP012 was used as template to amplify the *nisA* promoter fused to the *alr* gene, using primers LLALR-12 and LLALR-13 for the PCR reaction. The 1.6-kb PCR product was cloned in pGEMT-easy (Promega Biotech, Roosendaal, The Netherlands), resulting in a vector designated pGIP010. This vector was digested with *Sac*I and *Kpn*I and the resulting fragment containing P_{nisA} and *alr* was subcloned into similarly digested pMEC10 (38), yielding vector pGIP014. This vector was used for integration into the genome of *L. plantarum* MD007.

Alanine racemase assay

Lactococcus lactis strain PH3960 harboring pGIP013 was cultured overnight at 30 ^{o}C in GM17 with chloramphenicol. The culture was used to inoculate fresh medium (starting OD_{600}= 0.25) at 30 ^{o}C. Growth was continued to an OD_{600} of 0.6. Nisin was then added to the cultures at concentrations of 0, 0.001, 0.01, 0.1 or 1.0 ng/ml, and growth was continued for 2.5 h. Cultures were harvested by centrifugation and the pellets were washed with 2 ml of distilled water and recentrifuged. Subsequently, the cells were resuspended in distilled water to an OD_{600} of 20 and 1 gram of zirconium beads (48) was added to the cell suspension and the cells were mechanically disrupted. After centrifugation the cell extract was transferred to fresh tubes and stored at 4 ^{o}C. Alanine racemase activity was measured by determining the D-ala conversion into L-ala by following spectrophotometrically the production of NADH in a coupled reaction, as the addition of L-alanine dehydrogenase (L-ADH) from *B. sphaericus* converts L-ala into pyruvate and ammonia. L-ADH was obtained from *Lactococcus lactis* NZ3900 harboring plasmid pNZ2650 (29). After induction with 1 ng/ml nisin, a cell-free extract was prepared and used as source of L-ADH. The assay used here contained 0.001 to 0.015 units of alanine racemase, depending on nisin induction level, 0.025 units of L-ADH, 100 mM sodium phosphate buffer pH 8.0, 10 mM NAD^{+}, and 50 mM D-alanine, in a total reaction volume of 1 ml. After addition of the sample containing Alr activity, the OD_{340} at 25 ^{o}C was followed for at least 5 minutes and enzyme activities were correlated to total protein content of the cell extracts as described previously (4).

Measurement of D-cycloserine resistance in cultures

PH3960 harboring pGIP013 was grown overnight in GM17 containing chloramphenicol and D-ala. Cultures were diluted 1:20 and grown in the same medium to an OD_{600} of 1.3. Afterwards 1:8 dilutions were cultured for 1 hour in the same medium with nisin concentrations ranging from 0 to 1 ng/ml. Subsequently, cultures were diluted 1:10 in the same medium with D-cycloserine ranging from 0 to 1 mg/ml. OD_{600} values were measured, growth was continued for 2 h and OD_{600} values were measured again. The OD increase of the cultures grown in the presence of D-cycloserine were compared to that observed for cultures grown without added D-cycloserine.

Nucleotide sequence accession numbers

The nucleotide sequences of the *alr* genes of *L. plantarum* NCIMB8826 and *Lactococcus lactis* MG1363 have been deposited in the GenBank database under accession numbers Y08941 and Y18148, respectively.

Results

Heterologous complementation of MD007 and PH3960

To evaluate the potential of the alanine racemase encoding gene *alr* as a heterologous complementation marker, pGIP011 (encoding the *Lactococcus lactis alr*) was introduced into the *alr* deletion variant of *L. plantarum* NCIMB8826 (MD007). After introduction of pGIP011 into MD007, cells were plated on MRS or MRS containing erythromycin, lincomycin and D-ala. After approximately 16 h of incubation, the MRS plates contained full-grown colonies selected by alanine racemase production, whilst after approximately 40 h similar amounts of colonies were obtained on the antibiotic resistance selection plate. In addition, colonies selected by *alr* expression appeared more homogenous in size. These results indicate that *in trans* complementation by the *Lactococcus lactis alr* gene under control of the *ldhL* promoter results in regeneration of growth of the *Δalr* strain of *L. plantarum* without D-ala supplementation. Similar results were obtained using pGIP012 for transformation into the *L. plantarumΔalr* strain MD007int6. Colonies appeared faster and were more homogenous in size when grown on MRS plates as compared to MRS plates containing chloramphenicol and D-ala. Similar observations were made when pGIP013 was introduced into the *Lactococcus lactisΔalr* strain PH3960 and subsequently plated on GM17 or GM17 containing chloramphenicol and D-ala. Although tightly regulated, the high-copy NICE vector containing *alr* apparently provides sufficient *alr* transcription to fully complement the *alr* phenotype in both hosts, even without addition of nisin, suggesting that only relatively low expression levels are required for growth.

Plasmid integrity was investigated in pGIP011 and pGIP013 transformants of *L. plantarum* MD007 and *Lactococcus lactis* PH3960, respectively. Twenty of these *L. plantarum* and *Lactococcus lactis* colonies were inoculated in medium containing erythromycin or chloramphenicol, respectively. All cultures were fully-grown overnight. Following isolation of plasmids pGIP011 and pGIP013, restriction analyses revealed that the plasmids were apparently intact (data not shown), indicating that selection of the transformants is equally stringent on basis of *alr*, as compared to the antibiotic markers.

To evaluate the stability of these food-grade selectable plasmids, MD007Int6 colonies harboring pGIP012 and PH3960 colonies harboring pGIP013 were pre-grown for 200 generations in MRS or GM17 medium, with or without D-ala, respectively. After four successive cycles of growth for fifty generations, cells were plated on medium containing D-ala, and the presence of the plasmid in the resulting colonies was assessed through the Alr phenotype by replica plating to plates with or without D-ala (Table 2). After 200 generations of culturing in the presence of D-ala, plasmid pGIP012 was undetectable in MD007Int6. However, all cells that were cultured without D-ala still contained pGIP012 after 200 generations. This indicates

that the *alr* marker strongly contributes to the stability of the plasmid in *L. plantarum*. In contrast, all tested cells of strain PH3960 contain plasmid pGIP013 after 200 generations, independent of the presence or absence of selective pressure. Plasmid DNA isolation of pGIP012 from MD007Int6 and pGIP013 from PH3960 suggests that the copy number of the lactococcal plasmid is 5-10 times higher (data not shown), possibly explaining the higher stability of pGIP013 compared with pGIP012. Fermentation media do not usually contain D-ala, making *alr* a stable, food-grade complementation marker under industrial conditions for both *Lactococcus lactis* and *L. plantarum*.

Table 2: Stability of *alr* encoding plasmids pGIP012 and pGIP013 in *L. plantarum* MD007Int6 and *Lactococcus lactis* PH3960, respectively. Cells were pre-grown with or without D-ala for multiples of 50 generations and the presence of the plasmids was judged by the ability of the cells to grow without D-ala. After culturing under selection pressure (without D-ala), both *L. plantarum* and *Lactococcus lactis* have stably maintained their plasmids in all tested colonies. The results for the cultures grown without selection pressure (with D-ala) are presented in the table below.

generations with D-ala	MD007Int6 Alr^+ (%)	PH3960 Alr^+ (%)
50	100	100
100	34	100
150	2	100
200	2	100

Selection of pGIP011 and pGIP013 in wild type strains

Several competitive inhibitors for Alr have previously been described (1, 32, 36, 50). In *M. smegmatis*, resistance to D-cycloserine could be correlated to the expression level of *alr* (6). This suggests that D-cycloserine resistance can be used for the dominant selection of transformants harboring a plasmid expressing *alr*. To evaluate this possibility in LAB, pGIP011 and pGIP013 were used for selection in the wild-type *L. plantarum* NCIMB8826 and *Lactococcus lactis* NZ3900. Plasmid pGIP011 was introduced in *L. plantarum* NCIMB8826 and cells were plated on MRS with increasing concentrations of D-cycloserine. After approximately 96 h of growth at 30 ^oC, colonies appeared on the plates with D-cycloserine concentrations up to 600 μg/ml (column 'cfu' in Table 3). The presence of pGIP011 in these colonies was confirmed by replica plating to MRS plates containing erythromycin and lincomycin (Column ' antibiotic resistance' in Table 3). The vast majority (96%) of the colonies originating from the MRS plate containing 600 μg/ml D-cycloserine plate harbored pGIP011. Similarly, the possibility of selecting pGIP013 transformants of *Lactococcus lactis* strain NZ3900 was evaluated. Since pGIP013 contains the *L. plantarum alr* gene under control of the *nisA* promoter, dominant D-cycloserine-based selection was evaluated on plates containing 1 ng/ml nisin. Addition of increasing

concentrations of D-cycloserine resulted in a decrease of cfu appearing after approximately 96 h. Replica plating proved that all tested colonies from the plate containing 200 μg/ml D-cycloserine, possessed the Cm^R phenotype, indicating that pGIP013 is present (Table 3). These results indicate that *alr* can be used as a dominant selection marker in wild-type variants of these LAB.

Table 3: D-cycloserine-based dominant selection of plasmids pGIP011 and pGIP013 in the wild-type strains of *L. plantarum* and *Lactococcus lactis*, respectively. Numbers of primary transformants are indicated (cfu). Selection stringency depends on the concentration of D-cycloserine present in the media, as judged by replica plating in order to determine the antibiotic resistance phenotype of the colonies growing at different D-cycloserine concentrations (percentage antibiotic resistance). Control plates without D-cycloserine contain the appropriate antibiotic. Data are representative for three independent experiments.

Organism	[D-cycloserine] (μg/ml)	cfu	antibiotic resistance (%)
L. plantarum	0 (+Ery)	746	100
	450	442	11
	500	264	43
	550	139	75
	600	109	96
Lactococcus lactis	0 (+Cm)	326	100
	100	>1000	25
	150	769	96
	200	418	100

Nisin inducible D-cycloserine resistance in PH3960

The correlation between D-cycloserine resistance and *alr* expression in *Lactococcus lactis* was further investigated. Firstly, strain PH3960 harboring pGIP013 was used in a nisin induction experiment. Cell free extracts were prepared from cultures grown in the presence of different concentrations of nisin and used for determination of the specific alanine racemase activity. Results from this experiment proved that induction with nisin led to overexpression of the alanine racemase enzyme, as indicated by the higher levels of alanine racemase activity (Table 4). Subsequently, strain PH3960 harboring pGIP013 was used for determination of D-cycloserine resistance levels at different nisin induction levels. The data clearly indicated that at higher nisin induction levels, the elevating Alr levels resulted in higher D-cycloserine resistance levels, as could be judged from culture growth (Fig. 1). Subsequently, an analysis was performed to verify if this correlation between nisin and D-cycloserine resistance could be detected on plates. Cells were grown to an OD_{600} of 0.5 and plated on GM17 containing chloramphenicol and varying concentrations of nisin and D-cycloserine. After growth at 30 ^oC colonies appeared on plates with D-cycloserine concentration up to 300 μg/ml. A similar correlation between nisin and D-cycloserine resistance was

found, as judged by the higher number of colonies formed after higher levels of nisin induction (data not shown). This experiment demonstrated that stepwise increase of *alr* expression led to a stepwise increase of the D-cycloserine resistance level in *Lactococcus lactis*.

Table 4: Nisin induction experiment for the overexpression of *L. plantarum alr* in *Lactococcus lactis*. Alr activity was measured in PH3960 harboring pGIP013 after induction with nisin concentrations ranging from 0-1 ng/ml.

nisin (ng/ml)	specific activity (U/g protein)
0	3.4
0.001	5.6
0.01	10.7
0.1	36.8
1	70.7

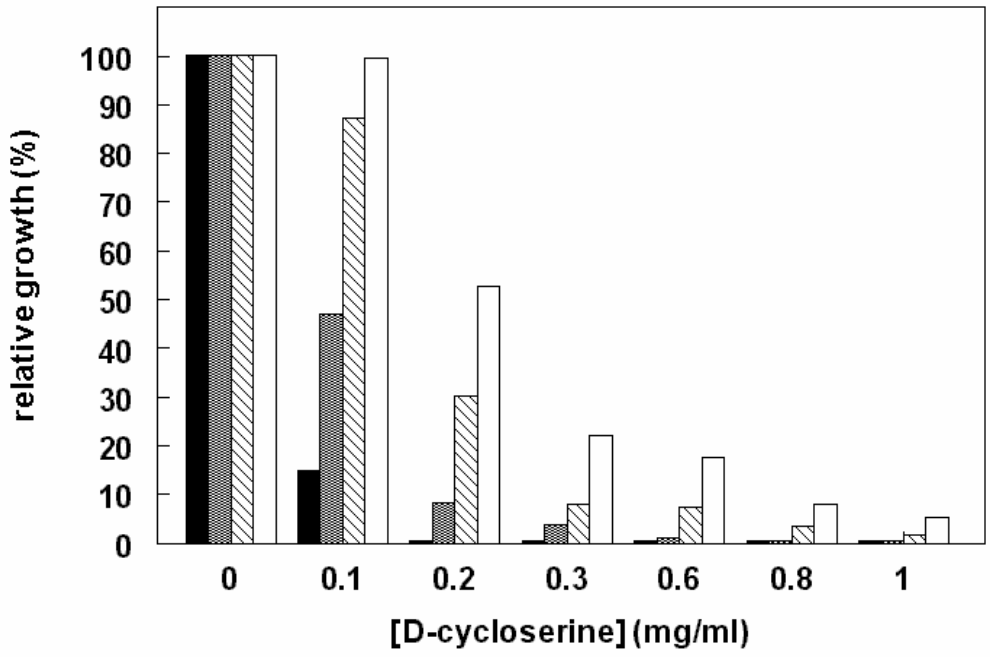


Fig. 1: Correlation between level of Alr and level of D-cycloserine resistance in *Lactococcus lactis*. PH3960 harboring pGIP013 was cultured with different concentrations of nisin and D-cycloserine. After 2 h growth rate was determined by OD_{600} measurement. Cultures were induced with 0, 0.01, 0.1 or 1 ng/ml nisin (black, gray, lined or white bars, respectively). Note that growth without D-cycloserine was similar for all nisin concentrations and taken as 100%.

Conditional *alr* mutant of *L. plantarum*

In addition to the use of the plasmid pGIP013 for food-grade selection in *Lactococcus lactis*, the possibility to use *alr* as a single copy chromosomal marker was investigated. For this purpose pGIP014 was constructed and integrated into the chromosome of *L. plantarum* MD007. This vector contains the lactococcal *alr* under control of the *nisA* promoter. Other important features of the construct are the *nisRK* genes that are required for functional implementation of the NICE system in *L. plantarum* NCIMB8826. The *alr* fusion was cloned in opposite orientation relative to the $tRNA^{ser}$ to minimize read through from upstream promoters (Fig. 2). Finally, pGIP014 encodes the *int* gene, involved in the integration of the plasmid into the chromosome via site specific recombination between the *attP* and *attB* sites. This plasmid was transformed to the *L. plantarumΔalr* strain MD007 and the anticipated genomic organization after correct integration was verified by PCR (data not shown). A single colony possessing the correct genotype was designated MD007::pGIP014 and growth characteristics were analyzed in correlation to its D-ala requirement under conditions in which the nisin concentration varied. These experiments revealed that without addition of nisin this strain was unable to grow for at least 24 h in the absence of D-ala in the plates. After pre-growth in MRS medium with D-ala, the strain was grown in MRS without D-ala and increasing concentrations of nisin (0.1-20 ng/ml). During 24 h the growth of the cultures was followed by OD_{600} measurements (Fig. 3). These measurements revealed that addition of 20 ng/ml of nisin led to growth very similar to a control culture that was grown in the presence of D-ala. Moreover, gradual induction of *alr* expression using the NICE system leads to gradual increase in growth rate. These experiments show that the *alr* gene can be used for single copy selection on the chromosome as well.

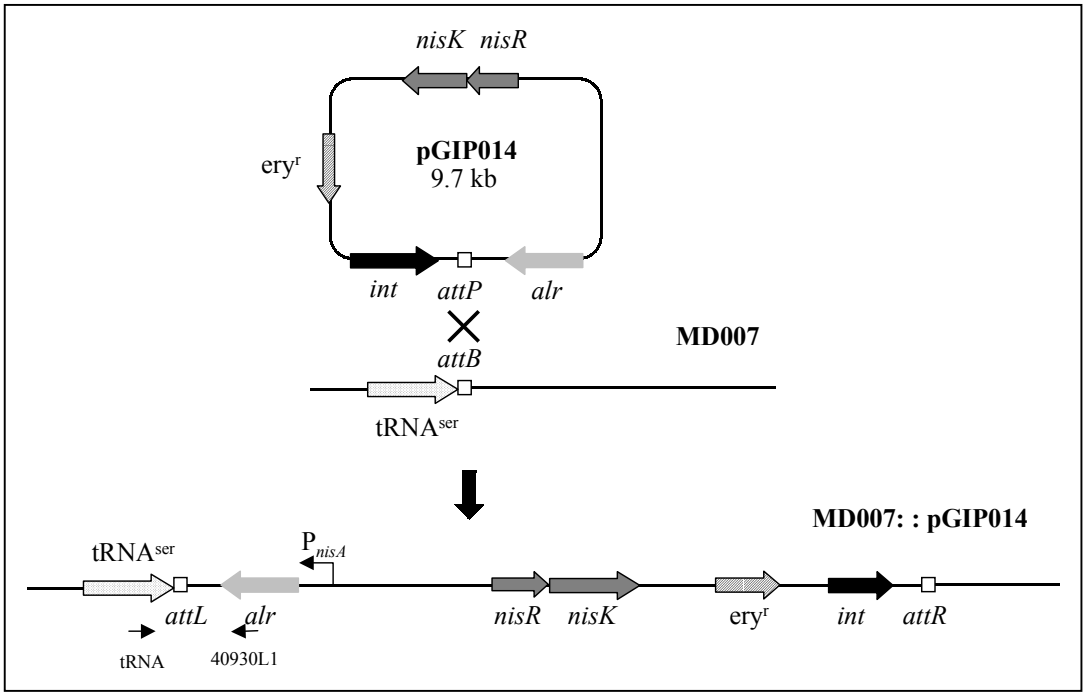


Fig. 2: Integration of pGIP014 into the chromosome of MD007 at the $tRNA^{ser}$ locus by site specific integration. pGIP014 harbors the *attP* site, which is identical to the chromosomally localized *attB* site. The *int* gene product catalyses the recombination event between the *attB* and *attP* sites. Indicated primers tRNA and 40930L1 were used to identify the recombination event. The final strain was designated MD007::pGIP014. In this strain the *alr* gene is orientated opposite to all neighboring genes, preventing read-through expression from surrounding genes.

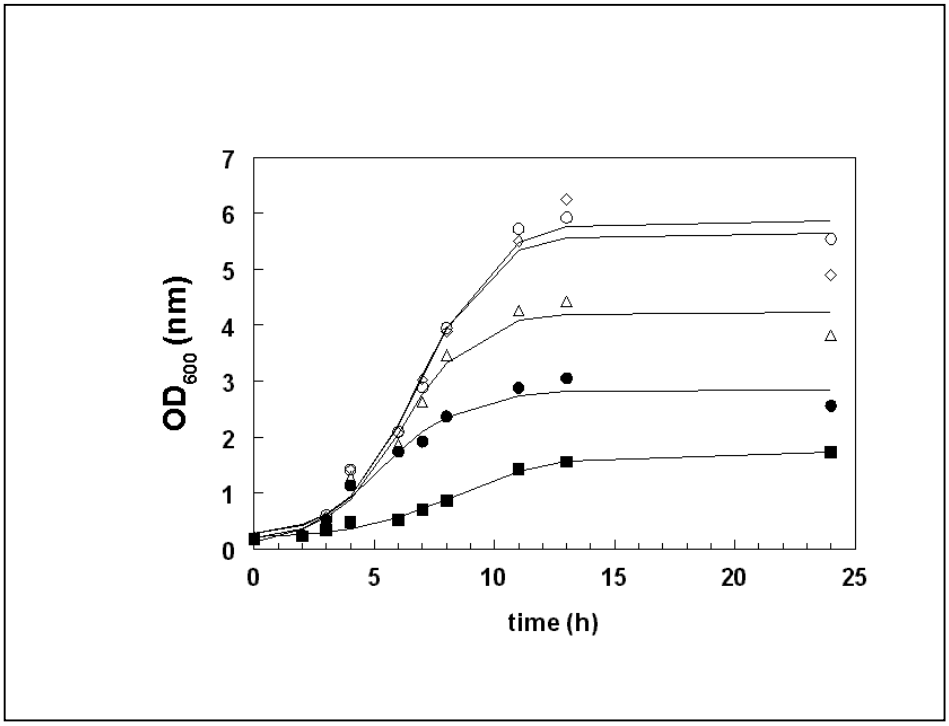


Fig. 3: Growth of strain MD007::pGIP014 depends on nisin addition. An overnight culture of MD007::pGIP014 in MRS containing D-alanine was used to inoculate MRS containing nisin concentrations of 0.1 (closed squares), 1 (closed circles), 3 (triangles) and 20 (diamond) ng/ml. Open circles represents a control culture grown with D-ala. Growth was followed during 24 h by OD_{600} measurement. Data are representative for three independent experiments.

Discussion

In the present study we report the exploitation of the alanine racemase encoding gene as a safe, food-grade complementation marker for *Lactococcus lactis* and *L. plantarum*. Selection based on *alr* expression appeared to be faster and equally stringent when compared to selection based on antibiotic resistance. Several food-grade selection markers, both of the dominant and complementation type, have been described for various bacteria, including the use of *alr* in *B. subtilis* (21). The experiments presented here demonstrate that the strategy in *B. subtilis* could readily be expanded to LAB. The plasmids used here were highly stable during culturing under selective pressure, making *alr* a valuable, sustainable, food-grade marker in these lactic acid bacteria, as D-ala is absent in most industrial fermentation media. Additionally, some LAB are considered suitable candidate microorganisms for the delivery of health-promoting factors to the human GI-tract (23, 24, 45). The *alr* gene thus provides a valuable tool for stable maintenance of plasmids. Historically, both complementation and dominant markers have been reported that use the capacity to ferment a given sugar as the selection criterion (14). This includes for Lactobacilli the use of the *lacLM* genes as a complementation marker in *L. helveticus* (25). However, the latter marker only functions in media containing lactose as the sole carbon source. Hence, to our knowledge the *alr* marker presented here is the first food-grade marker in a *Lactobacillus* species that can be used independently of the carbon source available in the medium. Moreover, this is the first complementation marker described in *L. plantarum*.

D-cycloserine, a competitive inhibitor of alanine racemase activity, could be used for selection of plasmids expressing *alr* in the wild type strains of *L. plantarum* and *Lactococcus lactis*. Essentially, the results prove that *alr* can be used both as a complementation and dominant marker, which to our knowledge is a novel feature among the food-grade marker systems described to date. Since several other compounds inhibiting alanine racemase activity are known (1, 32, 36, 50), substitution of D-cycloserine by any of the other inhibitors seems possible in this dominant selection system. Even though application possibilities with *alr* using dominant selection are limited, it is a functional tool in the laboratory, especially in *L. plantarum* and other LAB, as the number of functional markers in these organisms is limited. Hence, *alr*-based selection could provide a useful expansion of the genetic tools available for these LAB. Application of the *alr* gene as a food-grade complementation marker can probably be expanded to many other LAB, including industrial strains. Construction of genetically modified Alr deficient variants of such strains might not be required, since it seems feasible to select natural *alr* mutants of LAB by screening for D-ala requirement for growth. Despite the fact that this strategy would require a negative screening procedure the high-throughput screening methods that have become available, might allow such an approach.

We have demonstrated how the stepwise overexpression of *alr* with the NICE system, leads to concomitant stepwise increase in the levels of D-cycloserine resistance in *Lactococcus lactis*, both in liquid and solid media. These results show that the activity of the *nisA* promoter and the cognate Alr expression levels in the cell can be correlated to D-cycloserine resistance levels. Tools for screening promoter libraries for conditionally active promoters have been described previously in LAB, e.g. using the promoterless *cat* gene (49). However, correlation of the level of chloramphenicol resistance to promoter strength was only possible in a short range of promoter activities and subtle differences in promoter strength probably remain undetected using this system. Using the NICE system (12), our experiments suggest that an *alr*-based system enables the selection on basis of differences in promoter strength, in a wide dynamic range.

Thus far, the NICE system has been mainly used for overexpression of proteins (29, 37, 38). However, recently the NICE system has also been applied in more tailor made expression strategies (13). Here we describe the introduction of the *alr* gene in the chromosome of *L. plantarum* under control of the NICE system resulting in a nisin-controlled conditional mutant, hence nisin acts as the trigger for the change in growth phenotype. To our knowledge, in a *Lactobacillus* species this is the first report describing a conditional mutant in an essential gene using the NICE system. A similar essential gene disruption strategy could also be applied in the original host *Lactococcus lactis* (34) (Boels, unpublished data). However, NICE can be implemented in many gram-positive hosts (5, 16, 33, 38) generating similar regulatory characteristics, including promoter silence under non-inducing conditions. These findings suggest that a broad application of this system allows to study essential gene mutation phenotypes in a wide range of microorganisms.

Overall, we expanded the set of food-grade markers available in LAB for the expression of heterologous proteins with our *alr* selection system. Furthermore, our system will be helpful in the development of delivery vehicles, since cell lysis can be easily manipulated. Currently we are investigating the potential use of *alr* as a qualitative and quantitative reporter that can be applied in conditional promoter and promoter strength selection procedures.

Acknowledgements

We thank Steve Kotsonis for critically reviewing this manuscript. This work was supported by the EU projects LABVAC (BIO4-CT96-0542) and LABDEL (EU-QLRT-2000-00340).

References

1. **Badet, B., and C. Walsh.** 1985. Purification of an alanine racemase from *Streptococcus faecalis* and analysis of its inactivation by (1-aminoethyl)phosphonic acid enantiomers. Biochemistry **24:**1333-1341.

2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513-1523.

3. **Bolotin, A., P. Wincker, S. Mauger, O. Jaillon, K. Malarme, J. Weissenbach, S. D. Ehrlich, and A. Sorokin.** 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. lactis IL1403. Genome Res. **11:**731-753.

4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248-54.

5. **Bryan, E. M., T. Bae, M. Kleerebezem, and G. M. Dunny.** 2000. Improved vectors for nisin-controlled expression in gram-positive bacteria. Plasmid **44:**183-190.

6. **Caceres, N. E., N. B. Harris, J. F. Wellehan, Z. Feng, V. Kapur, and R. G. Barletta.** 1997. Overexpression of the D-alanine racemase gene confers resistance to D- cycloserine in *Mycobacterium smegmatis.* J. Bacteriol. **179:**5046-5055.

7. **Campo, N., M. L. Daveran-Mingot, K. Leenhouts, P. Ritzenthaler, and P. Le Bourgeois.** 2002. Cre-*loxP* recombination system for large genome rearrangements in *Lactococcus lactis*. Appl. Environ. Microbiol. **68:**2359-67.

8. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138:**179-207.

9. **Chacon, O., Z. Feng, N. B. Harris, N. E. Caceres, L. G. Adams, and R. G. Barletta.** 2002. *Mycobacterium smegmatis* D-Alanine Racemase Mutants Are Not Dependent on D-Alanine for Growth. Antimicrob. Agents. Chemother. **46:**47-54.

10. **Chen, J. D., and D. A. Morrison.** 1988. Construction and properties of a new insertion vector, pJDC9, that is protected by transcriptional terminators and useful for cloning of DNA from *Streptococcus pneumoniae.* Gene **64:**155-164.

11. **de Ruyter, P. G., O. P. Kuipers, M. M. Beerthuyzen, I. van Alen-Boerrigter, and W. M. de Vos.** 1996. Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*. J. Bacteriol. **178:**3434-3439.

12. **de Ruyter, P. G., O. P. Kuipers, and W. M. de Vos.** 1996. Controlled gene expression systems for *Lactococcus lactis* with the food- grade inducer nisin. Appl. Environ. Microbiol. **62:**3662-3667.

13. **de Ruyter, P. G., O. P. Kuipers, W. C. Meijer, and W. M. de Vos.** 1997. Food-grade controlled lysis of *Lactococcus lactis* for accelerated cheese ripening. Nature Biotechnol. **15:**976-979.

14. **de Vos, W. M.** 1999. Safe and sustainable systems for food-grade fermentations by genetically modified lactic acid bacteria. Int. Dairy J. **9:**3-10.

15. **de Vos, W. M., P. Vos, H. de Haard, and I. Boerrigter.** 1989. Cloning and expression of the *Lactococcus lactis* ssp. cremoris SK11 gene encoding an extracellular serine protease. Gene **85:**169-176.

16. **Eichenbaum, Z., M. J. Federle, D. Marra, W. M. de Vos, O. P. Kuipers, M. Kleerebezem, and J. R. Scott.** 1998. Use of the lactococcal *nisA* promoter to regulate gene expression in gram-positive bacteria: comparison of induction level and promoter strength. Appl. Environ. Microbiol. **64:**2763-2769.

17. **Emond, E., R. Lavall Ee, G. Drolet, S. Moineau, and G. LaPointe.** 2001. Molecular characterization of a theta replication plasmid and its use for development of a two-component food-grade cloning system for *Lactococcus lactis*. Appl. Environ. Microbiol. **67:**1700-1709.

18. **Ferain, T., D. Garmyn, N. Bernard, P. Hols, and J. Delcour.** 1994. *Lactobacillus plantarum ldhL* gene: overexpression and deletion. J. Bacteriol. **176:**596-601.

19. **Ferain, T., J. N. Hobbs, Jr., J. Richardson, N. Bernard, D. Garmyn, P. Hols, N. E. Allen, and J. Delcour.** 1996. Knockout of the two *ldh* genes has a major impact on peptidoglycan precursor synthesis in *Lactobacillus plantarum*. J. Bacteriol. **178:**5431-5437.

20. **Fernández, M., M. Kleerebezem, O. P. Kuipers, R. J. Siezen, and R. van Kranenburg.** 2002. Regulation of the *metC-cysK* operon, involved in sulfur metabolism in *Lactococcus lactis*. J. Bacteriol. **184:**82-90.

21. **Ferrari, E., D. J. Henner, and M. Y. Yang.** 1985. Isolation of an alanine racemase gene from *Bacillus subtilis* and its use for plasmid maintenance in *B. subtilis.* Bio/Technology **3:**1003-1007.

22. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:**1-9.

23. **Gilbert, C., K. Robinson, R. W. Le Page, and J. M. Wells.** 2000. Heterologous expression of an immunogenic pneumococcal type 3 capsular polysaccharide in *Lactococcus lactis*. Infect. Immun. **68:**3251-60.

24. **Grangette, C., H. Muller-Alouf, D. Goudercourt, M. C. Geoffroy, M. Turneer, and A. Mercenier.** 2001. Mucosal immune responses and protection against tetanus toxin after intranasal immunization with recombinant *Lactobacillus plantarum*. Infect. Immun. **69:**1547-53.

25. **Hashiba, H., R. Takiguchi, K. Jyoho, and K. Aoyama.** 1992. Establishment of a host-vector system in *Lactobacillus helveticus* with beta-galactosidase activity as a selection marker. Biosci. Biotechnol. Biochem. **56:**190-194.

26. **Hayward, A. C., and G. H. G. Davis.** 1956. The isolation and classification of *Lactobacillus* strains from italian saliva samples. Br. dent. J. **101:**2733-2741.

27. **Hoffmann, K., E. Schneider-Scherzer, H. Kleinkauf, and R. Zocher.** 1994. Purification and characterization of eucaryotic alanine racemase acting as key enzyme in cyclosporin biosynthesis. J. Biol. Chem. **269:**12710-12714.

28. **Hols, P., C. Defrenne, T. Ferain, S. Derzelle, B. Delplace, and J. Delcour.** 1997. The alanine racemase gene is essential for growth of *Lactobacillus plantarum*. J. Bacteriol. **179:**3804-3807.

29. **Hols, P., M. Kleerebezem, A. N. Schanck, T. Ferain, J. Hugenholtz, J. Delcour, and W. M. de Vos.** 1999. Conversion of *Lactococcus lactis* from homolactic to homoalanine fermentation through metabolic engineering. Nature biotechnol. **17:**588-592.

30. **Inagaki, K., K. Tanizawa, B. Badet, C. T. Walsh, H. Tanaka, and K. Soda.** 1986. Thermostable alanine racemase from *Bacillus stearothermophilus*: molecular cloning of the gene, enzyme purification, and characterization. Biochemistry **25:**3268-3274.

31. **Josson, K., T. Scheirlinck, F. Michiels, C. Platteeuw, P. Stanssens, H. Joos, P. Dhaese, M. Zabeau, and J. Mahillon.** 1989. Characterization of a gram-positive broad-host-range plasmid isolated from *Lactobacillus hilgardii*. Plasmid **21:**9-20.

32. **Kanda-Nambu, K., Y. Yasuda, and K. Tochikubo.** 2000. Isozymic nature of spore coat-associated alanine racemase of *Bacillus subtilis*. Amino Acids **18:**375-387.

33. **Kleerebezem, M., M. M. Beerthuyzen, E. E. Vaughan, W. M. de Vos, and O. P. Kuipers.** 1997. Controlled gene expression systems for lactic acid bacteria: transferable nisin-inducible expression cassettes for *Lactococcus*, *Leuconostoc*, and *Lactobacillus* spp. Appl. Environ. Microbiol. **63:**4581-4584.

34. **Koebmann, B. J., D. Nilsson, O. P. Kuipers, and P. R. Jensen.** 2000. The membrane-bound H(+)-ATPase complex is essential for growth of *Lactococcus lactis*. J. Bacteriol. **182:**4738-43.

35. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, A. Danchin *et al.*** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature**:**249-56.

36. **Lambert, M. P., and F. C. Neuhaus.** 1972. Mechanism of D-cycloserine action: alanine racemase from *Escherichia coli* W. J. Bacteriol. **110:**978-987.

37. **Lopez de Felipe, F., M. Kleerebezem, W. M. de Vos, and J. Hugenholtz.** 1998. Cofactor engineering: a novel approach to metabolic engineering in *Lactococcus lactis* by controlled expression of NADH oxidase. J. Bacteriol. **180:**3804-3808.

38. **Pavan, S., P. Hols, J. Delcour, M. C. Geoffroy, C. Grangette, M. Kleerebezem, and A. Mercenier.** 2000. Adaptation of the nisin-controlled expression system in *Lactobacillus plantarum*: a tool to study *in vivo* biological effects. Appl. Environ. Microbiol. **66:**4427-4432.

39. **Preston, R. A., and H. A. Douthit.** 1984. Germination of *Bacillus cereus* spores: critical control by DL-alanine racemase. J. Gen. Microbiol. **130:**3123-3133.

40. **Reusch, V. M., Jr., S. G. Hale, and B. J. Hurly.** 1982. Levels of cell wall enzymes in endospores and vegetative cells of *Bacillus subtilis*. J. Bacteriol. **152:**1147-1153.

41. **Rosso, G., K. Takashima, and E. Adams.** 1969. Coenzyme content of purified alanine racemase from *Pseudomonas*. Biochem. Biophys. Res. Commun. **34:**134-140.

42. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

43. **Shaw, J. P., G. A. Petsko, and D. Ringe.** 1997. Determination of the structure of alanine racemase from *Bacillus stearothermophilus* at 1.9-A resolution. Biochemistry **36:**1329-1342.

44. **Soda, K., and K. Tanizawa.** 1990. Thermostable alanine racemase. Its structural stability. Ann. N. Y. Acad. Sci. **585:**386-393.

45. **Steidler, L., W. Hans, L. Schotte, S. Neirynck, F. Obermeier, W. Falk, W. Fiers, and E. Remaut.** 2000. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin- 10. Science **289:**1352-1355.

46. **Stewart, B. T., and H. O. Halvorson.** 1953. Studies on the spores of aerobic bacteria. I. The occurence of alanine racemases. J. Bacteriol. **65:**160-166.

47. **Tanizawa, K., A. Ohshima, A. Scheidegger, K. Inagaki, H. Tanaka, and K. Soda.** 1988. Thermostable alanine racemase from *Bacillus stearothermophilus*: DNA and protein sequence determination and secondary structure prediction. Biochemistry **27:**1311-1316.

48. **van der Meer, J. R., J. Polman, M. M. Beerthuyzen, R. J. Siezen, O. P. Kuipers, and W. M. De Vos.** 1993. Characterization of the *Lactococcus lactis* nisin A operon genes *nisP*, encoding a subtilisin-like serine protease involved in precursor processing, and *nisR*, encoding a regulatory protein involved in nisin biosynthesis. J. Bacteriol. **175:**2578-88.

49. **van der Vossen, J. M., J. Kok, and G. Venema.** 1985. Construction of cloning, promoter-screening, and terminator-screening shuttle vectors for *Bacillus subtilis* and *Streptococcus lactis*. Appl. Environ. Microbiol. **50:**540-542.

50. **Wang, E., and C. Walsh.** 1978. Suicide substrates for the alanine racemase of *Escherichia coli* B. Biochemistry **17:**1313-1321.

51. **Wasserman, S. A., C. T. Walsh, and D. Botstein.** 1983. Two alanine racemase genes in *Salmonella typhimurium* that differ in structure and function. J.Bacteriol. **153:**1439-1450.

52. **Wijsman, H. J.** 1972. The characterization of an alanine racemase mutant of *Escherichia coli*. Genet. Res. **20:**269-277.

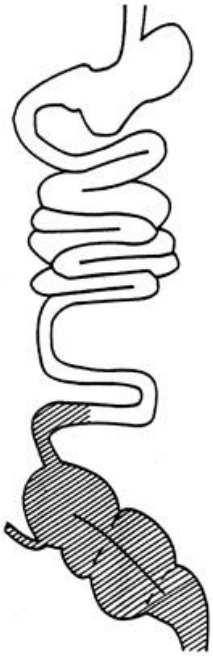
53. **Wild, J., J. Hennig, M. Lobocka, W. Walczak, and T. Klopotowski.** 1985. Identification of the *dadX* gene coding for the predominant isozyme of alanine racemase in *Escherichia coli* K12. Mol. Gen. Genet. **198:**315-322.

54. **Yasuda, Y., K. Kanda, S. Nishiola, Y. Tanimoto, C. Kato, A. Saito, S. Fukuchi, and Y. Nakanishi.** 1993. Regulation of L-alanine-initiated germination of *Bacillus subtilis* spores by alanine racemase. Amino Acids **4:**89-99.

55. **Yonaha, K., T. Yorifuji, T. Yamamoto, and K. Soda.** 1975. Alanine racemase of *Bacillus subtilis* var. *atterimus*. J. Ferment. Technol. **53:**579-587.

Chapter 3

Selection and Characterization of Conditionally Active Promoters in *Lactobacillus plantarum*, Using Alanine Racemase as a Promoter Probe



PETER A. BRON,[1,2] SALLY M. HOFFER,[1,2] IRIS I. VAN SWAM,[1,2] WILLEM M. DE VOS,[1] AND MICHIEL KLEEREBEZEM.[1,2]

[1] Wageningen Centre for Food Sciences, [2] NIZO food research, P.O. Box 20, 6710 BA Ede, The Netherlands.

Published in Appl. Environ. Microbiol. (2004) 70: 310-317.

Abstract

This paper describes the use of the *alr* gene, encoding alanine racemase, as a promoter-screening tool for the identification of conditional promoters in *Lactobacillus plantarum*. Random fragments of the *L. plantarum* WCFS1 genome were cloned upstream of the promoterless *alr* gene of *Lactococcus lactis* in a low copy number plasmid vector. The resulting plasmid library was introduced into a *L. plantarum*Δ*alr* strain (MD007) and 40,000 clones were selected. The genome coverage of the library was estimated to be 98% based on nucleotide insert-sequence and restriction analyses of the inserts of randomly selected clones. The library was screened for clones that were capable of complementing the D-alanine auxotroph phenotype of MD007 in media containing up to 10, 100 or 300 μg/ml of the competitive Alr inhibitor D-cycloserine. Western blot analysis using polyclonal antibodies raised against lactococcal Alr revealed that the Alr production level required for growth increased in the presence of increasing concentrations of D-cycloserine, adding a quantitative factor to the primarily qualitative nature of the *alr* complementation screen. Screening of the *alr* complementation library for clones that could grow only in the presence of 0.8M NaCl resulted in the identification of 8 clones that upon western blot analysis showed significantly higher Alr production under high salt conditions than under low salt conditions. These results established the effectiveness of the alanine racemase complementation screening method for the identification of promoters on their conditional or constitutive activity.

Introduction

Lactic acid bacteria (LAB) are a group of gram-positive bacteria which are of major economical importance. LAB are applied extensively in both the production and preservation of a wide variety of food products. During industrial processes LAB often face stress conditions such as changes in temperature, acidity, osmolarity and oxidative conditions. To improve the industrial performance or implement desirable properties in LAB it is of importance to gain insight in the complex regulatory processes that occur in these microbes during stress. Moreover, various LAB have developed into bacterial model systems because of their easy genetic accessibility, availability of genetic tools, and their relatively small and known genomes (4, 26). Hence, insight in gene expression and its control allows for further understanding of genome-wide regulation and the development of tools for the *in situ* and controlled expression of desirable functions. This justifies the increasing effort that is put in the analysis of gene expression signals and the characterization of regulatory elements in LAB (13, 14, 18, 22, 24).

Promoter-probe vectors are suitable genetic tools for the identification and characterization of promoters and the effective evaluation of their activity. Promoters in LAB have been studied mainly by insertion of chromosomal DNA fragments upstream of a promoterless reporter gene, such as the antibiotic resistance marker chloramphenicol acetyltransferase (Cat). The genes *cat-194* from *Staphylococcus aureus* (1) and *cat-86* from *Bacillus pumilis* (37), both encoding Cat, have been exploited as promoter-probes for the identification of streptococcal and lactococcal promoters, respectively. These systems are based on the selection of promoter activities by monitoring growth in the presence of chloramphenicol. In addition, promoter activities can be quantified by measuring Cat activity in a simple assay. Unfortunately, only relatively strong promoters were identified in these screening efforts, indicating that the Cat production levels required for selection are high (1, 37).

A second group of frequently used reporter genes encode sugar hydrolases. These genes generally allow qualitative detection of the encoded enzyme activity by addition of a chromogenic substrate to plates and quantitative assessment of promoter strength by a simple assay. Examples of sugar hydrolases employed in promoter screening procedures in LAB include the *Lactococcus lactis lacG* gene (35) , encoding phospho-ß-galactosidase, and the *lacZ* genes from *Leuconostoc mesenteroides* (24), *Streptococcus thermophilus* (18) and *Escherichia coli* (33), encoding ß-galactosidases. The application possibilities of these genes in promoter screens remain limited to LAB that do not express endogenous genes encoding these (phospho-)ß-galactosidases. Since many LAB are generally known to effectively ferment lactose it is not surprising that several (phospho-) ß-galactosidases have been found in these bacteria (11, 19, 29). Alternatively, the β-glucuronidase-encoding *gusA* genes originating from *E. coli* and *Lactobacillus gasseri*, have been used to study

promoter activities in LAB (30, 31). Although sugar hydrolases have been successfully applied in promoter screening strategies in several LAB, the selection of promoter activities using these systems is not based on conditional growth, resulting in laborious procedures, involving evaluation of enzyme activity levels in large numbers of colonies under various conditions (33).

A third group of reporter systems involves the emission of light. A mutant green fluorescent protein (GFP) from *Aequoria victoria* was optimized for bacterial expression (10, 21). However, very high *gfp* expression levels appeared to be required for effective detection of light emitting LAB, thereby limiting the applicability of this system in these bacteria (21). Alternative strategies using the luciferase (*luxAB*) genes from *Vibrio harveyi* and *Vibrio fischeri* were shown to be more sensitive (9, 16). Unfortunately, nonyl aldehyde is required as a substrate for luciferase in the light emission reaction. Therefore, enzyme activities can only be visualized using disrupted cells. In analogy with the sugar hydrolase-based systems, screens involving light emission are not based on conditional growth, disallowing the efficient use of this group of promoter-probes for genome-wide screens for conditionally active promoters.

In previous work we established the use of *alr* as a food-grade selection marker (5). Here we describe the utilization of the same gene as a promoter-probe to study global gene expression and its control in *L. plantarum*. It is demonstrated that the allosteric Alr inhibitor D-cycloserine can be used during the screening procedures to add a quantitative factor to the primarily qualitative nature of the screen. Moreover, the experiments demonstrate that *alr* allows rapid and highly selective identification of conditionally active, high salt inducible promoters.

Material and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids that were used in this study are listed in Table 1. *E. coli* strains MC1061 (7) and BL21 (Novagen, Madison, USA) were used as cloning hosts during construction of pNZ7119 and the overproduction of alanine racemase from pNZ7122, respectively. *E. coli* was grown aerobically in TY medium (32). *Lactococcus lactis* MG1363 (20) was used as a cloning host during construction of pNZ7120, pNZ7121 and the *L. plantarum* promoter library. *Lactococcus lactis* MG1363 was grown without aeration at 30 ^{o}C in M17 (Merck, Darmstadt, Germany), supplemented with 0.5% (wt/vol) glucose (GM17). *L. plantarum* WCFS1 (26) and its *alr* mutant derivative MD007 (5) were grown at 37 ^{o}C in MRS (Difco, Surrey, U.K.) without aeration. Varying concentrations of D-cycloserine (from freshly prepared, filter sterilized stock solutions) or 200 μg/ml D-alanine were added to the media when indicated. When appropriate, antibiotics were added to the media; for *E. coli* ampicillin (50 μg/ml); for *Lactococcus lactis* and *L. plantarum* erythromycin (5 μg/ml).

DNA techniques and DNA sequence analysis

Plasmid DNA was isolated from *E. coli* on a small scale using the alkaline lysis method (3, 32). Large scale plasmid DNA isolations were performed using Jetstar columns according to the manufacturer's instructions (Genomed GmbH, Bad Oberhausen, Germany). DNA isolation and transformation in *Lactococcus lactis* and *L. plantarum* were performed as described previously (12, 17, 25). Standard procedures were applied for DNA manipulations in *E. coli* (32). Restriction endonucleases, *Taq* and *Pwo* polymerase, T4 DNA ligase and calf intestine alkaline phosphatase (CIAP) were used following the recommendations of the manufacturer (Promega, Leiden, the Netherlands and Boehringer, Mannheim, Germany). Primers were purchased from Pharmacia Biotech (Roosendaal, the Netherlands). The sequences of the inserts present in the pNZ7120 derivatives (see below), were amplified by PCR using primers PB1 and PB2 (Table 1), followed by mini Elute PCR purification (Qiagen, Westburg, Germany). Partial insert sequences were determined using approximately 25 ng of the PCR product and 1 pmol of fluorescently labeled primer PB3 (Table 1) in the Thermo Sequenase cycle-sequence protocol provided by the manufacturer (Pharmacia Biotech, Roosendaal, The Netherlands). Sequence reaction products were analyzed using an ALF*red* DNA sequencer (Pharmacia Biotech). The determined insert sequences were assigned to chromosomal loci using Blast-N and the *L. plantarum* genome sequence (26) as database.

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

Material	Relevant features	Source or reference
Strains		
E. coli		
MC1061	cloning host	(7)
BL21	cloning host for the utilization of T7 promoter regulated expression	Novagen
Lactococcus lactis		
MG1363	cloning host	(20)
L. plantarum		
WCFS1	wild-type for which the genome sequence is available	(26)
MD007	*L. plantarum* WCFS1 Δ*alr*, D-alanine auxotroph	(5)
Plasmids		
pNZ7110	Ap^R, pUC18 derivative containing *cre* and T_{pepN}	(5)
pNZ7119	Ap^R, pNZ7110 derivative with *cre* replaced by *Lactococcus lactis alr*	this work
pIL252	Em^R	(34)
pNZ7120	Em^R, pIL252 derivative containing *Lactococcus lactis alr* from pNZ7119	this work
pNZ7121	Em^R, pNZ7120 derivative containing *L. plantarum* P_{ldhL1} promoter upstream of *alr*	this work
pET-16B	Ap^R, vector for his-tagged overexpression of heterologous proteins in *E. coli*	Novagen
pNZ7122	Ap^R, pET16-B derivative containing his-tagged fragment of *alr*	this work
Primers		
PB1	5'-ACCGCTACGGATCACATC-3'	
PB2	5'-CTCGGGGCAGTAAGACTA-3'	
PB3	5'-GTGGTGAAGTTTTCATGG-3'	
LLALR-TF-F [a]	5'-AGAGAAAGGTTTAAATCATGAAAAC -3'	
LLALR-TF-R [a]	5'- ACGTTCTAGATTATTTATAGATTCTTTTGATTC –3'	
PldhL-F [a]	5'-GAAGATCTTCAATCTTCTCACCGTCTTG-3'	
PldhL-R [a]	5'-GAAGATCTTCAATAAGTCATCCTCTCGT-3'	
SH4 [a]	5'-CCGCTCGAGGAAATTTGGCCAGCAGTGAAAGC-3'	
SH5 [a]	5'-CGCGGATCCCTTATGGGATTTATCTTCC-3'	

[a] underlined sequences indicate restriction sites subsequently used in cloning procedures

[b] Ap^R, ampicillin resistant; Em^R, erythromycin resistant

Construction of promoter-probe vector pNZ7120

Plasmid pGIP012 (5) was used as a template to amplify the *alr* gene from *Lactococcus lactis* MG1363 (20) using *Pwo* polymerase and the primers LLALR-TF-F and LLALR-TF-R (Table 1). The resulting 1.1 kb amplicon was digested with *Bsp*HI and *Xba*I (both restriction sites introduced with the primers) and cloned into *Nco*I-*Nhe*I digested pNZ7110 (5). The resulting plasmid was designated pNZ7119 and was checked by automatic double strand sequence analysis using an ALF*red* DNA sequencer. Sequence reactions were performed with an Autoread kit, were initiated using Cy5-labeled universal and reverse primers, and were continued with synthetic primers in combination with Cy13-dATP, following the instructions of the manufacturer (Pharmacia Biotech, Roosendaal, The Netherlands). A single point mutation was found in the coding sequence of *alr*, which was probably introduced during the *Pwo* polymerase reaction. However, this mutation did not result in a change in the alanine racemase protein sequence. pNZ7119 was digested with *Ecl*136II and *Sal*I, and the resulting 1.5 kb fragment, containing the *alr* coding sequence preceded by the pNZ7110 derived ribosome binding site (5), was ligated into *Sal*I-*Sma*I digested pIL252 (34), resulting in pNZ7120 (Fig. 1).

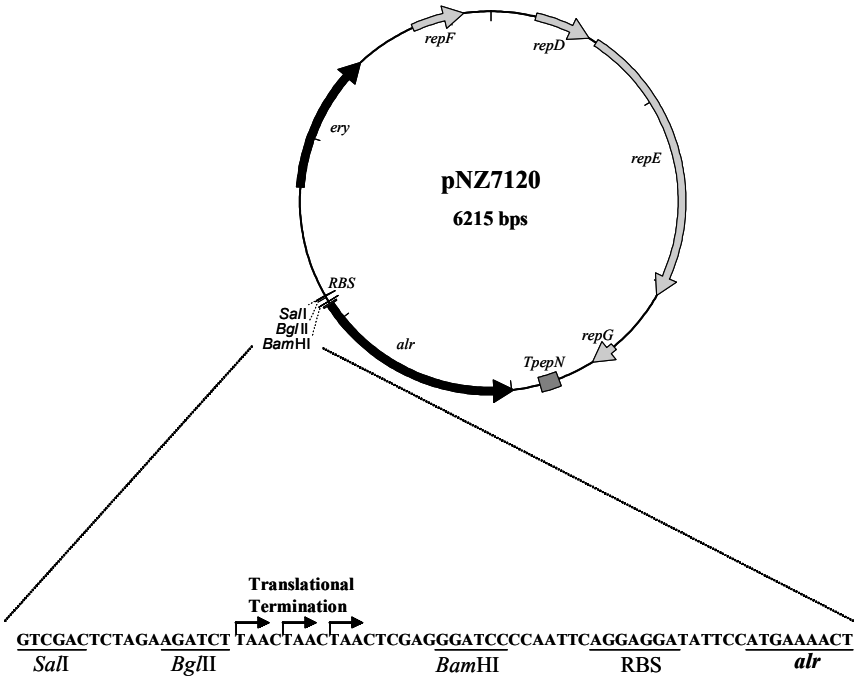


Fig. 1: Plasmid map of the promoter-probe vector pNZ7120. The vector is a derivative of the low-copy vector pIL252 (34) and carries an erythromycin resistance gene (*ery*) for selection in *L. plantarum*. pNZ7120 harbors a promoterless copy of the *Lactococcus lactis* MG1363 *alr* gene which is transcribed in the opposite orientation relative to all other genes encoded on pNZ7120. The DNA sequence visualizes the multiple cloning site and the lactococcal *prtP* ribosome binding site (18). Furthermore, stopcodons in all 3 reading frames (three black arrows) upstream of *alr* prevent translational fusions when chromosomal fragments are cloned in the *Bgl*II or *Sal*I sites of pNZ7120.

Vector validation and genomic library construction

The promoter region of the *ldhL1* gene of *L. plantarum* WCFS1 was amplified using chromosomal DNA of this strain (26) as template and the primers PldhL-F and PldhL-R (Table 1). The 0.5 kb amplicon was digested with *Bgl*II and cloned into similarly digested pNZ7120. The resulting plasmid was designated pNZ7121 and contains the *alr* gene under control of the *ldhL1* promoter.

To construct a *L. plantarum* WCFS1 library in pNZ7120, chromosomal DNA of this strain was partially digested with *Sau*3AI. The partial digests were size-fractionated on 1% agarose gel and fragments ranging from 1-2 kb were purified using Sephaglas^{TM} Bandprep (Pharmacia Biotech, Roosendaal, The Netherlands). These purified fragments were cloned into *Bgl*II digested and CIAP dephosphorylated pNZ7120. Ligation mixtures were transformed to *Lactococcus lactis* MG1363 (20) and approximately 50,000 of the obtained colonies were collectively resuspended in GM17 medium. Plasmid DNA was isolated from these pooled cells and was introduced into *L. plantarum*Δ*alr* (MD007) cells. Transformants were selected on MRS medium containing D-alanine and erythromycin to obtain all plasmid-containing cells, independent of *alr* expression. Approximately 40,000 colonies were collectively pooled in MRS containing 15% glycerol and stored at –80 ^{o}C in small aliquots.

Alr antibodies and western blotting

Plasmid pNZ7119 was used as a template to amplify a 1002 bp fragment of *alr*, using SH4 and SH5 as primers (Table 1) in the PCR reaction. The resulting amplicon was digested with *Xho*I and *Bam*HI and cloned into similarly digested pET16-B (Novagen, Madison, USA). The resulting plasmid pNZ7122 was introduced in *E. coli* BL21 (Novagen, Madison, USA) and these cells were used for IPTG induced overproduction of a his-tagged 334 amino-acid C-terminal fragment of the lactococcal alanine racemase protein according to the induction protocol of the provider of the vector. Subsequently, cells were pelleted, stored overnight at –20 ^{o}C and resuspended in 20 ml French press buffer (25% sucrose, 50mM Tris HCl, pH 8, 1mM PMSF). Using a French press (1000 psi, 4^{o}C, 2 successive treatments) (Sim Aminco, Abcoude, The Netherlands) cell extracts were prepared and the suspension was centrifuged at 5000 rpm for 30 minutes at 4^{o}C. The pellet was resuspended in 10 ml buffer (50 mM NaPi pH7.6, 1mM PMSF, 0.5% TritonX-100, 4 M urea and 0.5% Tween-20) and recentrifuged. The supernatant was collected and used for the purification of the Alr protein. A Hitrap^{TM} Chelating column was preloaded with Nickel as described in the protocol of the manufacturer (Amersham biosciences). During purification on the Akta*explorer* (Amersham Pharmacia Biotech, Upsalla, Sweden), buffers containing 20 mM NaPi (pH 7.4), 0.5 M NaCl, 4M urea and varying concentrations of immidazole were used. Five column-volumes containing 10 mM immidazole were used for equilibration. Subsequently, 25 ml cell extract containing

3.5 mg/ml of total protein was loaded on the column, followed by washing with 30 column volumes containing 10 mM immidazole and with 10 column volumes containing 59 mM immidazole. Finally, elution was performed with 18 column volumes containing 500 mM immidazole. The elution fractions were analyzed on a SDS-15%-polyacrylamide gel and Alr purity was estimated to be >95%. The elution fractions with the highest purity and protein concentration (total yield of 3.6 mg) were pooled and used to raise polyclonal antibodies against Alr in a rabbit (Eurogentec, Seraing, Belgium).

The selectivity and sensitivity of these antibodies were demonstrated by the specific detection of Alr in wild-type *Lactococcus lactis* MG1363 (20) in a western blot experiment. Cells were cultured at 30 ^{o}C and harvested cells were disrupted using 1 g of zirconium glass beads in a Fastprep^{TM} (Qbiogene Inc, Cedex, France) by two treatments of 40 sec interspaced by 1 min cooling of the samples on ice. Proteins in the crude cell extract were separated by SDS-15% polyacrylamide gel electrophoresis. Proteins were transferred onto Nitrocellulose membranes (Jepson Bolton, Watford, United Kingdom) by electroblotting (LKB 2051 Midget Multiblot) and *Lactococcus lactis* Alr was probed using 1000x diluted Alr antibodies, followed by detection using secondary goat anti-rabbit antibodies conjugated to peroxidase (Pierce, Rockford, USA). Reactivity was visualized using the Supersignal West Pico Chemiluminescent Substrate according to the manufacturer's protocol (Perbio Science, Etten Leur, The Netherlands). To determine Alr production levels in clones of interest from the *alr* complementation library, these clones were grown in MRS with D-alanine and erythromycin, and subsequently 10^{-5} dilutions were plated. Full-grown colonies were harvested in 3 ml of water and equal amounts of cells were disrupted and further analyzed as described above.

Results

Promoter-probe vector pNZ7120

The main goal of the work described here was to identify and analyze conditionally active promoters in *L. plantarum* using a promoter-probe. The *gusA* gene, encoding β-glucuronidase, has been used to study promoters originating from the genome of *Lactococcus lactis* (30). Since no genes encoding β-glucuronidase were found in the *L. plantarum* WCFS1 genome (26), the potential of the *E. coli gusA* gene as a promoter-probe in *L. plantarum* was initially evaluated. However, *L. plantarum* harboring a plasmid carrying *gusA* under control of a constitutive promoter, showed severe fluctuation of blue-coloring on X-gluc containing plates, preventing the effective application of this gene as a promoter-probe vector in *L. plantarum* (data not shown). Since we already exploited the *alr* gene as a food-grade selection marker (5), we investigated the applicability of this gene as an alternative method for the selection of conditionally active promoters. Hence, based on the low-copy plasmid pIL252

(34), a vector was constructed carrying a promoterless copy of the *alr* gene of *Lactococcus lactis* MG1363 (Fig. 1). Both the empty vector (pNZ7120) and a derivative containing the *alr* gene under control of the *ldhL1* promoter from *L. plantarum* WCFS1 (pNZ7121) were introduced in *L. plantarum*Δ*alr* (MD007) cells and plated on MRS medium with or without D-alanine. On MRS plates with D-alanine similar amounts of colonies were obtained for MD007 harboring either pNZ7120 (no promoter) or pNZ7121 (*ldhL1* promoter), while on plates lacking D-alanine colonies were only obtained with cells harboring pNZ7121. These results indicate that pNZ7120 is a suitable promoter-probe vector for the detection of promoter activities in *L. plantarum*.

Construction and functionality of an *alr* complementation library in pNZ7120

To use pNZ7120 as a promoter-probe for the detection of promoters, a library of partially digested *Sau*3AI fragments derived from *L. plantarum* WCFS1 genomic DNA was constructed via the intermediate host *Lactococcus lactis* MG1363. Approximately 40,000 colonies were obtained in *L. plantarum* MD007 and the library was analyzed for completeness in several ways. First, 100 colonies were randomly picked from MRS plates and their plasmids were used as template for PCR analysis. This demonstrated that all investigated clones contain an insert, of which the average size was estimated to be 1.3 kb (data not shown). To assess insert redundancy, restriction patterns of all amplicons obtained with *Sau*3AI or *Hha*I were compared. This indicated that redundancy in the library was below 10%. Moreover, 30 of these amplicons were used for partial sequence analysis, which revealed them to be randomly derived from different chromosomal regions of the *L. plantarum* genome. These results support the randomness of the library and genome coverage was estimated to be approximately 98% (data not shown).

To verify the functionality of the *alr*-based selection system, the library was plated with and without D-alanine, revealing that approximately 25% of the total library was able to grow on MRS plates lacking D-alanine (Table 2). These clones likely harbored a plasmid that contains a fragment of *L. plantarum* chromosomal DNA with a promoter element properly oriented for the expression of *alr* to a level sufficient for complementation of the D-alanine auxotroph phenotype of MD007. This expectation was investigated by insert-sequence analysis of 16 randomly picked clones. All these inserts contained a 3'-truncated ORF according to the annotation of the *L. plantarum* genome sequence (26) and their preceding upstream regions potentially carried a properly oriented promoter element responsible for *alr* expression (data not shown). In contrast, 16 randomly picked clones from a plate containing D-alanine did not display any preference of insert orientation relative to *alr* (data not shown). These experiments indicate that *alr* can be used as a promoter-probe for the selection of active promoters from the *alr* complementation library.

Table 2: Decreasing colony forming units when clones are selected from the *L. plantarum alr* complementation library that are capable of growth on media containing increasing concentrations of D-cycloserine, without the addition of D-alanine. Percentages are relative to the number of colonies on a control plate containing D-alanine, but lacking D-cycloserine.

D-cycloserine (μg/ml)	D-ala (μg/ml)	cfu (%)	D-cycloserine (μg/ml)	D-ala (μg/ml)	cfu (%)
0	200	100	100	0	9.56
0	0	24.6	200	0	6.97
10	0	14.6	400	0	3.96
20	0	13.0	600	0	2.46
50	0	10.9	900	0	< 0.01

Quantitative selection of promoters using D-cycloserine

D-cycloserine has been described as an allosteric Alr inhibitor (5, 6, 28). Previous experiments have demonstrated that higher levels of Alr result in higher levels of D-cycloserine resistance (5). This observation suggests that D-cycloserine can be used for the selection of clones from the *alr* complementation library that produce Alr at a relatively high level. To evaluate this possibility, the library was plated on MRS containing a range of D-cycloserine concentrations. After 3 days of growth, plates with higher concentrations of D-cycloserine contained fewer colonies (Table 2), suggesting that these colonies represent clones harboring pNZ7120 derivatives which produced Alr at a level sufficient to overcome the inhibitory effect of this compound. To further investigate this observation, dilutions of the *alr* complementation library were plated and grown on MRS lacking D-cycloserine. Subsequently, 384 colonies were randomly selected and cultured overnight in a microtiter plate containing MRS lacking D-cycloserine. These cultures were used for replica-plating to MRS plates containing 0, 10, 100 or 300 μg/ml D-cycloserine. Growth on the plates containing D-cycloserine was compared to the control plate lacking D-cycloserine, leading to the identification of clones capable of growth on plates containing up to 10, 100 or 300 μg/ml D-cycloserine. From these three groups 28 clones were randomly selected and insert sequence analysis revealed the corresponding chromosomal loci, the corresponding *L. plantarum* genes, and their potential promoters (Table 3). The Alr production levels in individual clones were investigated in 8 randomly picked clones from the three groups. These clones were plated on MRS lacking D-cycloserine and after 48 h full-grown colonies were harvested and equal amounts of cells were used to prepare crude cell extracts. To investigate the Alr production in each D-cycloserine resistance group, the cell extracts were analyzed by western blotting using Alr-specific antibodies (Fig. 2). All clones that displayed MD007 complementation at 100 and 300 μg/ml D-cycloserine showed a clearly higher Alr production level compared with the clones that show growth only at 10 μg/ml D-cycloserine. However, using this

approach, no clear differences in Alr production level could be visualized comparing the clones that displayed MD007 complementation at 100 and 300 μg/ml D-cycloserine. Nevertheless, these experiments demonstrate that *L. plantarum* genomic fragments displaying high promoter activity can be selected from the *alr* complementation library using high concentrations of D-cycloserine, adding a quantitative factor to the primary qualitative nature of the *alr* complementation screen.

Table 3: Clones in the *alr* complementation library that are capable of growth in the presence of a maximum concentration of 10, 100 or 300 μg/ml D-cyloserine, without the addition of D-alanine.

[D-cycloserine] (ug/ml)	clone name	insert start coordinate [b]	estimated insert size (kb)	ORF in insert [c]	function of homologue(s)
10	pNZ7120-csl1	1037318	1.7	lp_1141 (*purK2*)	Phosphoribosylaminoimidazole carboxylase, ATPase subunit
10	pNZ7120-csl2	1216019	2.0	lp_1320	Conserved hypothetical protein
10	pNZ7120-csl3	1000151	2.5	lp_1101 (*ldhl2*)	lactate dehydrogenase
10	pNZ7120-csl4	2617565	2.5	lp_2937	Transcription regulator
10	pNZ7120-csl5 [a]	1309715	3.0	lp_1430	glycosyltransferase
10	pNZ7120-csl6 [a]	1904621	2.6	lp_2109 (*uvrC*)	Excinuclease ABC, subunit C
10	pNZ7120-csl7	416640	2.5	lp_0464	zinc/iron ABC transporter
10	pNZ7120-csl8 [a]	3008044	3.0	lp_3392	transport protein
100	pNZ7120-csm1	470883	1.7	lp_0525 (*kup1*)	potassium uptake protein
100	pNZ7120-csm2	3035271	1.7	lp_3418 (*pck*)	phosphoenolpyruvate carboxykinase
100	pNZ7120-csm3	1038952	1.2	lp_1144 (*pcrA*)	ATP-dependent DNA helicase
100	pNZ7120-csm4	3308262	1.6	lp_3688a (*rpmH*)	ribosomal protein L34
100	pNZ7120-csm5	2948260	1.6	lp_3312	conserved hypothetical protein
100	pNZ7120-csm6	2373288	1.8	lp_2671	integral membrane protein
100	pNZ7120-csm7 [a]	2019577	2	lp_2231a	hypothetical protein
100	pNZ7120-csm8 [a]	2855464	1.5	lp_3204 (*nupC*)	nucleoside transport protein
100	pNZ7120-csm9 [a]	1454904	3	lp_1599 (*folD*)	methylenetetrahydrofolate dehydrogenase and cyclohydrolase
100	pNZ7120-csm10	2039898	2.5	lp_2256 (*ccpA*)	catabolite control protein A
100	pNZ7120-csm11	90985	2.8	lp_0101	cobalt ABC transporter component
300	pNZ7120-csh1	2668545	1.7	lp_3000	ABC transporter, ATP-binding and permease protein
300	pNZ7120-csh2 [a]	3015121	2.5	lp_3399 (*cpd*)	2',3'-cyclic-nucleotide 2'-phosphodiesterase precursor
300	pNZ7120-csh3 [a]	2483803	2.5	lp_2793	hypothetical protein
300	pNZ7120-csh4	1383808	2	lp_1512 (*dnaB*)	replication initiation and membrane attachment protein DnaB
300	pNZ7120-csh5	676236	2.2	lp_0739 (*secA*)	preprotein translocase, SecA subunit
300	pNZ7120-csh6	2990240	0.5	lp_3363 (*copB*)	copper transporting ATPase
300	pNZ7120-csh7	493323	1.8	lp_0542	conserved hypothetical protein
300	pNZ7120-csh8	2326766	2.4	lp_2608	Na(+)/H(+) antiporter

[a] Clones used for western blot analysis (Fig. 2)

[b] Inserts start coordinate indicates the first basepair of the *L. plantarum* genome sequence in the insert of the pNZ7120 derivatives directly upstream of *alr*

[c] Gene names are indicated between brackets if present in the current annotation of the *L. plantarum* WCFS1 genome (26)

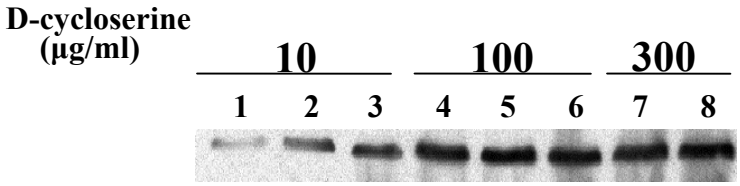


Fig. 2: Western blot analysis of Alr expression levels in colonies sensitive to different concentrations of D-cycloserine. Randomly selected individual clones that originally were selected on MRS plates containing 10 (lanes 1, 2, 3), 100 (lanes 4, 5, 6) or 300 (lanes 7, 8) µg/ml D-cycloserine.

Conditional promoter screen for 0.8M NaCl activated promoters

The possibility to select conditionally activated clones from the *alr* complementation library was investigated by studying the salt response. For this purpose the library was plated on MRS plates containing 0.8M NaCl. Subsequently, 768 colonies were randomly picked, organized in microtiter plates, and grown overnight in MRS containing D-alanine. All clones were replicated on MRS plates with or without 0.8M NaCl, and containing no or up to 200 µg/ml D-cycloserine. Growth was periodically compared between the different plates over 3 days and 46 clones were identified that showed conditional growth only in the presence of 0.8M NaCl. The corresponding chromosomal inserts present in the pNZ7120 derivatives were partially sequenced, revealing 23 unique loci. Subsequently, 20 of the 46 clones were found to grow only on plates containing 0.8M NaCl over a range of at least 8-fold different D-cycloserine concentrations (Table 4), indicating that the expression in the presence of NaCl is relatively high as compared to expression in the absence of NaCl. Sequence comparison of their inserts showed the presence of 8 different chromosomal loci (Table 4). Strikingly, an insert in pNZ7120-con1 harboring a 3'-truncated fragment of Lp_1459, encoding a gene with no homologues in the database, and its upstream sequence was found in 10 of the 20 identified clones. Similarly, a 3'-truncated fragment of Lp_1435, encoding an integral membrane protein and its upstream sequence, was found in two of the identified inserts. The latter two inserts differed in size (approximately 900 and 2200 bp), indicating that this promoter element was independently selected twice from the library. In pNZ7120-con4 and 6 no open reading frame and consequently no putative promoter region could be identified in the current genome annotation database of *L. plantarum* WCFS1 (26). However, manual inspection of these loci revealed the presence of a short ORF (162 and 165 bp, respectively) lacking any database homologue, that could be preceded by a high osmolarity induced promoter (Table 4).

Conditional, 0.8M NaCl-induced *alr* expression was further investigated in the 8 clones selected for conditional growth over a range of at least 8-fold different D-cycloserine concentrations. Overnight cultures in MRS containing D-alanine were diluted and plated on MRS lacking D-alanine and D-cycloserine, with or without 0.8 M NaCl. After 3 days colonies were harvested and cell extracts were analyzed by western blotting using the Alr antibodies (Fig. 3). It could clearly be established that Alr expression driven by 6 of these 8 chromosomal fragments is higher under high salt conditions compared to standard conditions. In this experiment, no Alr production could be detected under either condition for the clones pNZ7120-con2 and 5. When increased amounts of cell extract originating from these two clones were analyzed by western blotting, 0.8M NaCl dependent Alr production could be visualized (data not shown). However, the observation that pNZ7120-con5 is capable of growth on plates containing 200 μg/ml D-cycloserine (Table 4) appears to disagree with these relatively low production levels of Alr. A possible explanation for this phenomenon could be that the promoter element harbored by pNZ7120-con5 is induced by the combination of D-cycloserine and NaCl, but not by NaCl or by D-cycloserine alone. Overall, these experiments indicate that the *alr* complementation library can be used for the effective selection of *L. plantarum* chromosomal fragments displaying conditional promoter activity.

Table 4: Clones capable of complementing the D-ala auxotrophy of *L.plantarum*Δ*alr* MD007 only in the presence of 0.8M NaCl.

clone name	Insert start coordinate	estimated insert size (kb)	redundancy	gene in fragment	putative function	D-cycloserine (μg/ml)[a] 0 2.5 5 10 25 50 100 200
pNZ7120-con1	1335151	0.9	10	lp_1459	unknown	
pNZ7120-con2	1315041/ 1314390	0.9/2.2	2	lp_1435	integral membrane protein	
pNZ7120-con3	2905725	1.2	1	lp_3266 (*glyK)*	glycerate kinase	
pNZ7120-con4	2901280	2.0	2	ORF162[b]	unknown	
pNZ7120-con5	2242522	1.1	1	lp_2509	Permease, DMT superfamily	
pNZ7120-con6	427258	0.7	1	ORF165[b]	unknown	
pNZ7120-con7	2707493	3.0	1	lp_3045	short chain dehydrogenase	
pNZ7120-con8	2962996	3.5	2	lp_3330	unknown	

[a] Black bars indicate at which D-cycloserine concentrations growth was only observed in the presence of 0.8M NaCl. Grey bars indicate at which D-cycloserine concentration growth was detected independent of the presence of NaCl. White bars indicate that no growth was observed under either condition.

[b] Although in this pNZ7120 derivative no ORF is present on these chromosomal fragments in the current annotation database of *L. plantarum* WCFS1, manual inspection revealed the small ORFs indicated in the table.

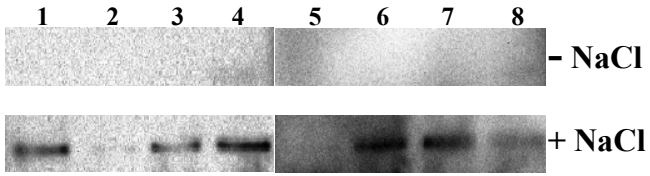


Fig. 3: Western blot analysis of 0.8M NaCl induced Alr expression in 8 clones. Alr production levels were clearly higher in all 8 clones (lanes 1 to 8) when cells were grown in media to which 0.8M NaCl was added (lower panel) as compared to standard MRS media (upper panel). It should be noted that using larger amounts of cell extract a more obvious signal for clones pNZ7120-con2 and 5 was detected (data not shown).

Discussion

Previous studies suggested that the alanine racemase encoding gene could be used as a complementation-based promoter-probe in an *alr* mutant of *L. plantarum* (5). This application was investigated using the lactococcal *alr* gene and analysis of the 3.3 Mbp genome of *L. plantarum* using an *alr* complementation library resulted in the identification of 8 chromosomal loci that show conditional promoter activity only under high salt conditions. Intrinsic to complementation-based screening procedures, growth was used as primary selection criterion. Therefore, the strategy described here has the same primary characteristics as the *cat* screening system, which also is based on growth in the presence of different amounts of chloramphenicol (1, 37). These types of growth-based screens are easy to perform and hence have advantages over screens based on sugar hydrolases (18, 24, 30, 31, 33, 35) or light emitting systems (9, 10, 16, 21), which are laborious when applied in genome-wide promoter screens. However, only promoters with a relatively high expression level have been detected in the *cat* system, because selection at low chloramphenicol concentrations is difficult to distinguish from endogenous resistance. In contrast, alanine racemase is only required at low levels to achieve complementation and can therefore be used for the identification of promoters with low activities. No revertants of the D-alanine auxotroph phenotype have been encountered in *L. plantarum* (data not shown). Moreover, using the *alr* system promoters with higher activity levels can also be selected by the application of the allosteric inhibitor D-cycloserine in the screening procedure. The addition of increasing concentrations of D-cycloserine allowed the direct selection of *L. plantarum* chromosomal fragments that drive *alr* expression to relatively high levels. Thereby, D-cycloserine adds a quantitative factor to this screening system. These features make the *alr*-based screening system suitable for identification of promoters in a relatively long dynamic range and convenient for sensitive, genome-wide promoter screening procedures.

The screen for high salt inducible promoter elements presented here demonstrates that the *alr* complementation library can be used for the identification of conditionally active promoters by selection of clones that grow independent of D-alanine only under a chosen condition. Replication of such clones to the control condition (normal laboratory conditions) allows direct selection of conditionally growing clones, indicating that *alr* expression is activated by the condition applied. Moreover, the clones identified in such a conditional screen can be analyzed in more detail by monitoring conditional capability of growth on plates with varying concentrations of D-cycloserine, without the addition of D-alanine. The conditional screen performed here resulted in the identification of 8 chromosomal *L. plantarum* loci that clearly possess high salt-inducible promoter activity. These promoters are of industrial relevance, since *L. plantarum* is frequently encountered in artisanal and industrial fermentations of vegetables, including unions, cucumbers, olives and

cabbage (8, 15), which in many cases involve high salt conditions. Therefore, the promoter fragments identified here can be employed to construct *in situ* expression systems for desirable proteins, including proteolytic and flavor-forming enzymes, by simply exchanging *alr* by the genes encoding such functions. Moreover, screens can be designed to identify conditional promoter elements for other niches in which *L. plantarum* is encountered. *L. plantarum* WCFS1 is known to actively pass the stomach (38), indicating that screens can be designed to construct *L. plantarum* strains for the delivery of desirable and potentially health-promoting proteins to the gastrointestinal tract.

Of the 8 most prominent salt-induced chromosomal loci (Table 4), two did not appear to contain an ORF and thus no putative promoter region in the current genome annotation database of *L. plantarum* WCFS1 (26). Nevertheless, manual inspection revealed small ORFs lacking any database homologue in both cases, possibly explaining the observed conditional promoter activity. Moreover, sequence comparison of all upstream regions of the 23 ORFs initially identified in the conditional screen using the web-based sequence alignment module MEME (2) revealed four loci that contain the same conserved motif (Table 5). A subsequent analysis using this 28 bp motif and the search program MAST (2) revealed that these are the only four loci in the *L. plantarum* genome where this motif is present with this high level of significance, and in an identical search using MAST and the genomes of the related species *Lactococcus lactis* and *B. subtilis* (4, 27) the same motif could not be found. These findings suggest that the *alr* system can be helpful in the identification of promoter activities that were not predicted in the primary annotation of a genome sequence, thereby allowing improvement of the annotation of such genomes using experimental data. Moreover, potential regulatory DNA sequences can be identified by *in silico* analysis of the identified conditional chromosomal loci.

Overall, this paper describes a promoter-probe vector that allows for convenient genome-wide analysis of *L. plantarum* promoter elements in a long dynamic range of promoter activity levels. Moreover, the *alr* system was used to identify high salt activated promoter elements in *L. plantarum*. D-alanine auxotroph mutants have been described in other bacteria (23, 36), allowing simple implementation of the *alr* promoter screening system in these microbes.

Acknowledgements

We are grateful to Douwe Molenaar for bio-informatics analysis and Jolanda Lambert, Armand Hermans and Saskia van Mil for technical assistance. The authors thank Masja Nierop Groot and Maria Marco for critically reading this manuscript, and Pascal Hols for fruitful discussions. Part of this work was supported by the EU project LABDEL (EU-QLRT-2000-00340).

Table 5: The motif that was found in the promoter regions of 4 ORFs that were harbored by the pNZ7120 derivatives originating from clones that display 0.8M NaCl dependent growth.

Gene	Putative function	P- value	motif sequence upstream of gene	distance to ATG (bp)
lp_0319	Transcription regulator	1.3 e-12	ATTAG G T G C G T T G T G G A C G G A C T T T T C G T G G A G TGCCG	205
lp_1435	Integral membrane protein	1.5 e-13	CCCGC G T G A G C G G T G G A C C C A G T G C A A G T C T G G GACCG	262
lp_2509	Permease, DMT superfamily	1.3 e-12	CAAAG G T G C G T C T G G G A A C C T A T G T T T A T G A G G AATCT	48
lp_3363 (*copB*)	copper transporting ATPase	6.0 e-16	CGATT G T G T G C C G G G G A C G C G G T G T T A A T G T G G TTATA	314
	Multilevel consensus sequence		G T G C G C C G G G G A C C C A G T G T T A A T G T G G A T T G T T A G G G A T C A C G C A A T T T C T C	

References

1. **Achen, M. G., B. E. Davidson, and A. J. Hillier.** 1986. Construction of plasmid vectors for the detection of streptococcal promoters. Gene **45:**45-49.

2. **Bailey, T. L., M. E. Baker, and C. P. Elkan.** 1997. An artificial intelligence approach to motif discovery in protein sequences: application to steriod dehydrogenases. J. Steroid Biochem. Mol. Biol. **62**(1)**:**29-44.

3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513-1523.

4. **Bolotin, A., P. Wincker, S. Mauger, O. Jaillon, K. Malarme, J. Weissenbach, S. D. Ehrlich, and A. Sorokin.** 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. lactis IL1403. Genome Res. **11:**731-753.

5. **Bron, P. A., M. G. Benchimol, J. Lambert, E. Palumbo, M. Deghorain, J. Delcour, W. M. de Vos, M. Kleerebezem, and P. Hols.** 2002. Use of the *alr* gene as a food-grade selection marker in lactic acid bacteria. Appl. Environ. Microbiol. **68:**5663-5670 (Chapter 2).

6. **Caceres, N. E., N. B. Harris, J. F. Wellehan, Z. Feng, V. Kapur, and R. G. Barletta.** 1997. Overexpression of the D-alanine racemase gene confers resistance to D- cycloserine in *Mycobacterium smegmatis.* J. Bacteriol. **179:**5046-5055.

7. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138:**179-207.

8. **Cocconcelli, P. S., E. Triban, M. Basso, and V. Bottazzi.** 1991. Use of DNA probes in the study of silage colonization by *Lactobacillus* and *Pediococcus* strains. J. Appl. Bacteriol. **71:**296-301.

9. **Corthier, G., C. Delorme, S. D. Ehrlich, and P. Renault.** 1998. Use of luciferase genes as biosensors to study bacterial physiology in the digestive tract. Appl. Environ. Microbiol. **64:**2721-2722.

10. **Crameri, A., E. A. Whitehorn, E. Tate, and W. P. Stemmer.** 1996. Improved green fluorescent protein by molecular evolution using DNA shuffling. Nat. Biotechnol. **14:**315-319.

11. **de Vos, W. M., I. Boerrigter, R. J. van Rooyen, B. Reiche, and W. Hengstenberg.** 1990. Characterization of the lactose-specific enzymes of the phosphotransferase system in *Lactococcus lactis*. J. Biol. Chem. **265:**22554-22560.

12. **de Vos, W. M., P. Vos, H. de Haard, and I. Boerrigter.** 1989. Cloning and expression of the *Lactococcus lactis* ssp. *cremoris* SK11 gene encoding an extracellular serine protease. Gene **85:**169-176.

13. **Derre, I., G. Rapoport, and T. Msadek.** 1999. CtsR, a novel regulator of stress and heat shock response, controls *clp* and molecular chaperone gene expression in gram-positive bacteria. Mol. Microbiol. **31:**117-131.

14. **Derzelle, S., B. Hallet, K. P. Francis, T. Ferain, J. Delcour, and P. Hols.** 2000. Changes in *cspL*, *cspP*, and *cspC* mRNA abundance as a function of cold shock and growth phase in *Lactobacillus plantarum*. J. Bacteriol. **182:**5105-5113.

15. **Duran Quintana, M. C., P. Garcia Garcia, and A. Garrido Fernandez.** 1999. Establishment of conditions for green table olive fermentation at low temperature. Int. J. Food Microbiol. **51:**133-43.

16. **Eaton, T. J., C. A. Shearman, and M. J. Gasson.** 1993. The use of bacterial luciferase genes as reporter genes in *Lactococcus*: regulation of the *Lactococcus lactis* subsp. *lactis* lactose genes. J. Gen. Microbiol. **139:**1495-1501.

17. **Ferain, T., D. Garmyn, N. Bernard, P. Hols, and J. Delcour.** 1994. *Lactobacillus plantarum ldhL* gene: overexpression and deletion. J. Bacteriol. **176:**596-601.

18. **Fernández, M., M. Kleerebezem, O. P. Kuipers, R. J. Siezen, and R. van Kranenburg.** 2002. Regulation of the *metC-cysK* operon, involved in sulfur metabolism in *Lactococcus lactis*. J. Bacteriol. **184:**82-90.

19. **Fernandez, M., A. Margolles, J. E. Suarez, and B. Mayo.** 1999. Duplication of the beta-galactosidase gene in some *Lactobacillus plantarum* strains. Int. J. Food Microbiol. **48:**113-23.

20. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:**1-9.

21. **Geoffroy, M. C., C. Guyard, B. Quatannens, S. Pavan, M. Lange, and A. Mercenier.** 2000. Use of green fluorescent protein to tag lactic acid bacterium strains under development as live vaccine vectors. Appl. Environ. Microbiol. **66:**383-391.

22. **Giard, J. C., N. Verneuil, Y. Auffray, and A. Hartke.** 2002. Characterization of genes homologous to the general stress-inducible gene *gls24* in *Enterococcus faecalis* and *Lactococcus lactis*. FEMS Microbiol. Lett. **206:**235-239.

23. **Hols, P., M. Kleerebezem, A. N. Schanck, T. Ferain, J. Hugenholtz, J. Delcour, and W. M. de Vos.** 1999. Conversion of *Lactococcus lactis* from homolactic to homoalanine fermentation through metabolic engineering. Nature biotechnol. **17:**588-592.

24. **Israelsen, H., S. M. Madsen, E. Johansen, A. Vrang, and E. B. Hansen.** 1995. Environmentally regulated promoters in Lactococci. Dev. Biol. Stand. **85:**443-448.

25. **Josson, K., T. Scheirlinck, F. Michiels, C. Platteeuw, P. Stanssens, H. Joos, P. Dhaese, M. Zabeau, and J. Mahillon.** 1989. Characterization of a gram-positive broad-host-range plasmid isolated from *Lactobacillus hilgardii*. Plasmid **21:**9-20.

26. **Kleerebezem, M., J. Boekhorst, R. Van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. De Vries, B. Ursing, W. M. De Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U.S.A. **100:**1990-1995.

27. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, A. Danchin *et al.*** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature **390:**249-56.

28. **Lambert, M. P., and F. C. Neuhaus.** 1972. Mechanism of D-cycloserine action: alanine racemase from *Escherichia coli* W. J. Bacteriol. **110:**978-987.

29. **Lee, L. J., J. B. Hansen, E. K. Jagusztyn-Krynicka, and B. M. Chassy.** 1982. Cloning and expression of the beta-D-phosphogalactoside galactohydrolase gene of *Lactobacillus casei* in *Escherichia coli* K-12. J. Bacteriol. **152:**1138-1146.

30. **Platteeuw, C., G. Simons, and W. M. de Vos.** 1994. Use of the *Escherichia coli* beta-glucuronidase (*gusA*) gene as a reporter gene for analyzing promoters in lactic acid bacteria. Appl. Environ. Microbiol. **60:**587-593.

31. **Russell, W. M., and T. R. Klaenhammer.** 2001. Identification and cloning of *gusA*, encoding a new beta-glucuronidase from *Lactobacillus gasseri* ADH. Appl. Environ. Microbiol. **67:**1253-1261.

32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

33. **Sanders, J. W., G. Venema, J. Kok, and K. Leenhouts.** 1998. Identification of a sodium chloride-regulated promoter in *Lactococcus lactis* by single-copy chromosomal fusion with a reporter gene. Mol. Gen. Genet. **257:**681-685.

34. **Simon, D., and A. Chopin.** 1988. Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*. Biochimie **70:**559-566.

35. **Simons, G., H. Buys, R. Roger, E. Koenhen, and W. M. De Vos.** 1990. Construction of a promoter-probe vector for lactic acid bacteria using the *lacG* gene of *Lactococcus lactis*. J. Ind. Microbiol. **31:**31-39.

36. **Tauch, A., S. Gotker, A. Puhler, J. Kalinowski, and G. Thierbach.** 2002. The alanine racemase gene *alr* is an alternative to antibiotic resistance genes in cloning systems for industrial *Corynebacterium glutamicum* strains. J. Biotechnol. **99:**79-91.

37. **van der Vossen, J. M., J. Kok, and G. Venema.** 1985. Construction of cloning, promoter-screening, and terminator-screening shuttle vectors for *Bacillus subtilis* and *Streptococcus lactis*. Appl. Environ. Microbiol. **50:**540-542.

38. **Vesa, T., P. Pochart, and P. Marteau.** 2000. Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. Aliment. Pharmacol. Ther. **14:**823-8.

Chapter 4

Genetic Characterization of the Bile Salt Response in *Lactobacillus plantarum* and Analysis of Responsive Promoters *in vitro* and *in situ* in the Gastrointestinal Tract

PETER A. BRON,[1,2] MARIA MARCO,[1,2] SALLY M. HOFFER,[1,2] ESTHER VAN MULLEKOM,[1,2] WILLEM M. DE VOS,[1] AND MICHIEL KLEEREBEZEM.[1,2]

[1] Wageningen Centre for Food Sciences, [2] NIZO food research, P.O. Box 20, 6710 BA Ede, The Netherlands.

Submitted to J. Bacteriol. (2004).

Abstract

This paper describes the growth, morphological and genetic response of *Lactobacillus plantarum* WCFS1 to bile. Growth experiments revealed that a stepwise increase in porcine bile concentrations led to a gradual decrease in maximal growth rate. Moreover, the final density reached by a *L. plantarum* culture growing in MRS containing 0.1% bile was approximately 3-fold lower than that in MRS lacking bile. The morphology of the cells grown in MRS containing 0.1% bile was investigated by scanning electron microscopy, which revealed that cells clump together, exhibited rough surfaces, and part of the cells displayed a shrunken and empty appearance, that contrasted with the characteristic rod-shaped, smooth-surfaced morphology of *L. plantarum* grown in MRS without bile. An *alr*-complementation-based genome-wide promoter screen was performed in *L. plantarum*, leading to the identification of 31 genes, of which the expression was potentially induced by 0.1% porcine bile. Remarkably, 11 membrane- and cell wall-associated functions appeared to be induced by bile, as well as 5 functions involved in redox reactions and 5 regulatory factors. Moreover, the genes lp_0237 and lp_0775, identified here as inducible by bile *in vitro* were previously identified in our laboratory as important for *L. plantarum in vivo* during passage of the mouse gastrointestinal tract. A quantitative reverse transcription PCR approach focused on these two genes and our results confirmed that the expression level of lp_0237 and lp_0775 was significantly higher in cells grown with bile and cells isolated from the mouse duodenum relative to cells grown on normal laboratory medium without bile.

Introduction

Several biological barriers are encountered by bacteria after ingestion, of which the first is the gastric acidity encountered in the stomach of the host. Bacteria able to survive these harsh conditions transit to the intestine where they encounter stresses associated with low oxygen availability, bile salts and competition with the commensal flora. Bile salts are synthesized in the liver by the conjugation of a heterocyclic steroid derived from cholesterol with glycine or taurine (18). The resulting conjugated bile salts are stored and concentrated in the gall bladder during the fasting state, and after consumption of a fat-containing meal these compounds are released into the duodenum where they play a major role in the dispersion and absorption of fats, including bacterial phospholipids and cell membranes (33). Bile salts are reintroduced in the liver following their reabsoprtion in the distal small intestine and colon after deconjugation by the resident microbial flora (17). This deconjugation reaction is performed by bacterial bile salt hydrolases (BSHs) which are encoded in the genomes of several intestinal bacteria, including *Bifidobacterium* and *Lactobacillus* species (9, 11, 20, 32).

Studies concerning gram-positive food-associated bacteria and their tolerance to digestive stress have focused mainly on physiological aspects such as determination of levels of acid- and bile salt-tolerance (8, 19) as well as the development of complex media in order to selectively enrich those bacteria which are digestive stress tolerant (29). Additionally, several studies describe defense mechanisms of gram-negative bacteria towards bile acids, which include the synthesis of porins, transport proteins, efflux pumps and lipopolysaccharides (16). A few genome-wide approaches in gram-positive bacteria aiming at the identification of proteins important for bile salt resistance have been described. In *Propionibacterium freudenreichii, Listeria monocytogenes* and *Enterococcus faecalis* 2D-gel electrophoresis led to the identification of several proteins that were higher expressed in the presence of bile salts than in control conditions (13, 21, 26). Only in *P. freudenreichii* these induced proteins were further characterized, leading to the identification of 11 proteins important in bile stress response. The induced proteins include general stress proteins such as ClpB and the chaperons DnaK and Hsp20 (21). Furthermore, random gene disruption strategies in *Listeria monocytogenes* and *E. faecalis* resulted in strains that were more susceptible to bile salts than the wild-type strains. Subsequent genetic analysis of the mutants revealed that the disrupted genes encode diverse functions, including an efflux pump homologue (3) and genes possibly involved in the biosynthesis of cell walls and fatty acids (4).

Lactic acid bacteria (LAB) are applied extensively in the production of fermented food products. Because of the frequent consumption of dairy, olives, sausages and other fermented food products, large amounts of LAB are ingested by humans. Moreover, LAB have the potential to serve as delivery vehicles of health-

promoting or therapeutic compounds to the human gastrointestinal tract (GI-tract) (14, 30). Among the LAB, *Lactobacillus plantarum* is encountered in many environmental niches, including dairy, meat and many vegetable fermentations (10, 12, 28). Next to the occurrence of *L. plantarum* in our diets, this microbe is frequently encountered as a natural inhabitant of the GI-tract (1). The complete 3.3 Mbp genome sequence of *L. plantarum* WCFS1 was recently determined (20). This strain is a single colony isolate of strain NCIMB 8826, a strain that effectively survives passage of the human stomach in an active form, reaches the ileum in high numbers as compared to other strains, and is detectable in the colon (34). The availability of its genome sequence allows effective investigation of the genes and regulation mechanisms underlying the observed persistence in the GI-tract. The research described here focuses on the response of *L. plantarum* to bile salt stress. Previously, we have described the utilization of alanine racemase as a promoter probe for the genome-wide identification of *L. plantarum* genes of which the expression is induced by high salt conditions (7). Here the same system was utilized, leading to the identification of 31 genes potentially induced by bile salts. Strikingly, two of the identified genes have previously been demonstrated to be *in vivo* induced in *L. plantarum* during passage of the mouse GI-tract (6). A quantitative PCR analysis focused on these two genes, demonstrating both their *in vitro* chromosomal induction by porcine bile and their *in vivo* expression in the duodenum of a mouse model.

Material and Methods

Bacterial strains, media and growth conditions

The bacterial strains, plasmids and primers that were used in this study are listed in Table 1. *L. plantarum* WCFS1 (20) and its *alr* mutant derivative MD007 (5) were grown at 37 ^{o}C in MRS (Difco, Surrey, U.K.) without aeration. Varying concentrations of D-cycloserine (from freshly prepared, filter sterilized stock solutions), 5 μg/ml erythromycin or 200 μg/ml D-alanine were added to the MRS medium when indicated.

Scanning electron microscopy

Nucleopore Polycarbonate membranes (Costar, Cambridge, USA) with 1 μm pores were incubated for 30 minute in a 0,01% poly-L-lysine solution in 0.1 M Tris/HCl buffer. Several drops of a logarithmically growing *L. plantarum* culture were positioned on the poly-L-lysine coated membranes and incubated for 5 minutes at 100% humidity. Afterwards the bacteria were fixated on the membranes for 30 minutes in 3% glutaraldehyde. Subsequently, the membranes were washed three times with MQ, dehydrated with ethanol, using 30%, 50%, 70%, 90% and finally 3 x 100% ethanol, and critical point dried by the CO_2 method (Balzers CPD 020, Balzers Union, Liechtenstein). The dried membranes were mounted on sample holders by carbon

adhesive tabs (Electron Microscopy Sciences, Washington, USA). Sample holders were positioned inside the preparation chamber (CT 1500 HF, Oxford Instruments, Oxon, England). The samples were sputter coated with 10 nm platinum and analyzed in a field emission scanning electron microscope (JEOL 6300F, Tokyo, Japan) at 2,5 KV. Images were recorded digitally, and photo processing was done with Adobe PhotoShop 5.5.

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

Material	Relevant features	Source or reference
Strains		
L. plantarum		
WCFS1	wild-type for which the genome sequence is available	(20)
MD007	*L. plantarum* WCFS1 *Δalr*, D-alanine auxotroph	(5)
Plasmids		
pNZ7120	Em^R, pIL252 derivative containing *Lactococcus lactis alr* promoter probe	(7)
BI1-BI96	PNZ7120 derivatives containing chromosomal *L. plantarum* fragments that were initially identified as inducible by 0.1% porcine bile	this work
Primers		
PB1	5'-ACCGCTACGGATCACATC-3'	(7)
PB2	5'-CTCGGGGCAGTAAGACTA-3'	(7)
PB3	5'-GTGGTGAAGTTTTCATGG-3'	(7)
16S-fo2	5'-TGATCCTGGCTCAGGACGAA-3'	this work
16S-re2	5'-TGCAAGCACCAATCAATACCA-3'	this work
237-fo	5'-CTACTGATATGGTTGTCGGGAATTA-3'	this work
237-re	5'-ACGGGTGCGTAGAAGAAGC-3'	this work
775-fo	5'-GCTCTTGCACCGGATATCAA-3'	this work
775-re	5'-TTTCTTCTTCCCGTGACCAGT-3'	this work
1898-fo	5'-GTGGCGACGGTTCTTACCAT-3'	this work
1898-re	5'-CCCTGGAAGACCAATCGTGT-3'	this work
1027-fo	5'-CCATGATGGTGCTTCACAA-3'	this work
1027-re	5'-TCGTGGCAGCAGAGGTAATG-3'	this work

Identification of bile salt inducible loci using the *alr* complementation library

Previously we have described the construction and utilization of a *L. plantarum* promoter probe library for the effective identification of conditionally active promoters (7). Here this library was exploited for the identification of clones that contain bile salt inducible chromosomal *L. plantarum* fragments. Appropriate dilutions of a -80 ^{o}C stock of the library were immediately plated on MRS plates containing erythromycin and 0.1% of porcine bile (Sigma, Zwijndrecht, The Netherlands, B-8631). After 2 days of growth approximately 4,000 individual colonies on these plates were used for replica-plating to plates containing erythromycin, with or without 0.1% porcine bile. Growth on both plates was periodically compared, leading to the primary identification of 96 colonies that could grow only in the presence of porcine bile (Table 1). The colonies displaying this bile-dependent growth were cultured overnight in a microtiterplate containing MRS with erythromycin and D-alanine. The full-grown cultures were used to reconfirm the initially observed conditional growth phenotype and to assess conditional promoter strength by observing the conditional growth phenotype in the presence of different concentrations of the Alr inhibitor D-cycloserine (7). The microtiterplate was replica-plated using a 96-pin replicator and MRS plates containing erythromycin and 0, 2.5, 5.0, 10, 25, 50, 100 or 200 µg/ml D-cycloserine, with or without 0.1% of bile salts. The bile-dependent, differential growth phenotype in the presence of different concentrations of D-cycloserine was determined by periodically comparing the growth on the plates with and without bile salts. From the clones for which the bile-dependent growth could be confirmed in this experiment, the chromosomal *L. plantarum* inserts harbored by their pNZ7120 derivatives were amplified by PCR using primers PB1 and PB2 (7). The resulting amplicons were used for partial DNA sequence analysis using primer PB3 (7) and the determined insert sequences were assigned to chromosomal loci using BlastN (2) and the *L. plantarum* genome sequence as database (20).

RNA isolations

Appropriate dilutions of an overnight culture of *L. plantarum* WCFS1 were plated on MRS with or without 0.1% porcine bile salts. After 3 days of growth colonies were rapidly collected from the plates in 3 ml MRS which was added to 12 ml quench buffer (60% methanol, 66.7 mM HEPES, pH 6.5, -40 ^{o}C) (27). Following quenching, the cells were immediately pelleted by centrifugation at 4500 rpm for 10 min and the cell pellets were resuspended in 0.4 ml ice-cold MRS. The cell suspensions were added to ice-cold tubes containing 1 g of zirconium glass beads, 0.4 ml phenol, 100 µl chloroform, 30 µl 10% SDS and 30 µl 3M NaAc pH 5.2. The cells were disrupted using two treatments of 40 seconds in a Fastprep^{TM} (Qbiogene Inc, Cedex, France) interspaced by 1 minute on ice. After centrifugation, 200 µl of the aqueous phase was

used for RNA isolation using the High Pure kit, which included a 1 hour treatment with DNaseI (Roche Diagnostics, Mannheim, Germany).

For detection of *in vivo* mRNA levels in an animal model, a mouse experiment was performed in an accredited establishment (N° A59107) according to guidelines N°86/609/CEE of the French government. Seven weeks-old female Balb/c mice were purchased from Iffa Credo (St Germain sur l'Arbresle, France) and had free access to tap water and standard mice chow during the experiments. After overnight culture, bacterial cells were pelleted by centrifugation and resuspended at 10^{10} cfu per ml in MRS. The mouse received a 100 µl (10^9 cfu) dose of these freshly prepared bacterial suspensions by intragastric administration and the next day this administration was repeated with a dose of 10^{10} cfu. Four hours later, the mouse was sacrificed and a section of mouse small intestine representing the duodenum (0.48g) was quickly collected and frozen in liquid nitrogen until it was processed using a liquid N_2-cooled BioPulverizer (BioSpec Products, Bartlesville, USA). The powdered sample was immediately used for cell disruption and RNA isolation essentially as described above for pure bacterial cell pellets.

cDNA synthesis and quantitative reverse transcription PCR

The expression levels of *L. plantarum* WCFS1 genes derived from cells grown on MRS plates was compared to MRS plates containing 0.1% porcine bile or to the mouse duodenum. First, cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen, Breda, the Netherlands), 2 pmol gene-specific primer (Table 1), 40 units RNaseOUT RNase inhibitor, 0.5 mM dNTPs, and either 0.11 µg or 2.1 µg total RNA from laboratory medium grown *L. plantarum* WCFS1 or mouse GI-tract sample, respectively. Reverse transcription was performed at 55 °C for 60 minutes, followed by inactivation of the reverse transcriptase by incubation at 70 ^{o}C for 15 minutes. Primers were designed using Primer 3 (ww-genome.wi.mit.edu) and the software package Primer Express (PE Applied Biosystems, Nieuwekerk a/d IJsel, The Netherlands). All primers were designed to have a Tm of 58-60 ^{o}C and the amplicon sizes ranged from 70-81 bp. A quantitative PCR was performed on the synthesized cDNAs in an ABI Prism 7700 using SYBR Green technology (Applied Biosystems, Nieuwekerk a/d IJsel, the Netherlands). Each 50 µl reaction contained 1x SYBR Green Master Mix (Applied Biosystems), 400 nM of each primer (Table 1), and 0.1 or 200 ng reverse transcribed RNA from either plate-grown WCFS1 cells or the mouse GI-tract sample, respectively. The reactions were initiated at 95 °C for 10 minutes followed by 40 amplification cycles of 95 °C for 15s and 55 °C for 60s. The specificity of the amplicons, resulting from the reactions using cDNA originating from culture- and mouse-derived templates, were checked post-amplification by melting curve analysis and amplicon DNA sequence analysis. Reactions containing either no template or DNase-treated RNA were included in each real-time PCR

experiment to assess for contamination and residual chromosomal DNA, respectively. Cycle threshold (Ct) values were obtained upon manually setting the baseline and threshold values at which fluorescence was appreciably above background fluorescence for each reaction and within the exponential phase of amplification for all reactions. Relative quantification transcript levels were calculated using the comparative $\Delta\Delta C_t$ method described by Pfaffl *et al*. (24, 25). Using this method, PCR efficiencies are calculated according to the equation E = 10 [-1/slope], whereby the slope was calculated from a standard curve of Ct values obtained for a dilution range of template cDNA. The average C_t values observed for the target genes transcripts (lp_0237, lp_0775, lp_1027 and lp_1898) were normalized to the average C_t values obtained for the reference gene transcripts (16S rRNA) from the same RNA sample. Three or four replicates of all samples and primer pairs were included in each quantitative PCR experiment and all experiments were performed in triplicate. Statistical analyses on the differences in expression between samples were assessed in group means for statistical significance by the Pair Wise Fixed Reallocation Randomization Test which was performed with the Relative Expression Software Tool (REST) (24, 25).

Results

Growth characteristics and morphology of *L. plantarum* in the presence of bile salts

The effects of different concentrations of bile salts on the morphology and growth of *L. plantarum* were investigated. After pregrowth in liquid MRS medium, *L. plantarum* WCFS1 was subcultured in MRS containing 0, 0.01, 0.05, 0.1 and 0.15 % porcine bile salts. Growth was monitored for 24 h by optical density measurement at 600 nm (OD_{600}) and used to calculate the maximal growth rate of *L. plantarum* in the presence of different concentrations of bile salts (Fig. 1). The maximal growth rate was found to decrease significantly with increasing bile concentrations. Moreover, the final OD_{600} reached by *L. plantarum* grown in medium containing 0.1% porcine bile was approximately 3-fold lower as compared to cells grown in standard MRS medium (data not shown). 4 h after inoculation logarithmically growing cells were collected from the cultures with different bile salt concentrations (Fig. 1) and the *L. plantarum* cell morphology was investigated by scanning electron microscopy (Fig. 2). Cells grown under standard conditions exhibited the characteristic rod-shaped, smooth-surfaced morphology of *L. plantarum*. When 0.05% of bile is present in the medium the cells displayed a slight tendency to clump together, their surface appeared to be less smooth and membrane vesicle structures are formed. At a concentration of 0.1% and 0.15% bile these changes in morphology were more pronounced. Moreover, at these higher concentrations of bile a proportion of the bacterial cells displayed a shrunken and empty appearance. Similar observations were made when the growth

rate and morphology on solid medium were investigated. Growth of colonies on MRS plates containing 0.1% bile salts was slightly retarded and colonies appeared very flat as compared to plates without added bile salts (data not shown). Since growth is the primary selection criterion in the conditional *alr* complementation screen described below, it is essential to perform such a screen comparing conditions that do not greatly differ in growth rate. Therefore, a concentration of 0.1% porcine bile was chosen for the identification of bile-inducible promoter elements in *L. plantarum*.

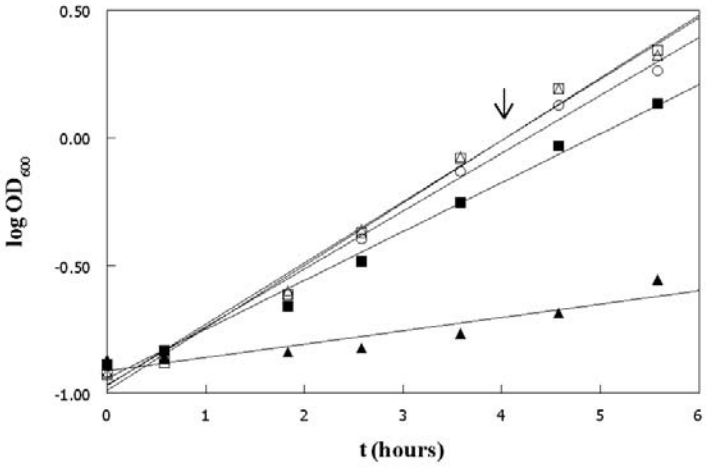


Fig. 1: Maximal growth rate determination of *L. plantarum* in the presence of increasing concentrations of bile salts. A full-grown culture was diluted 50-fold in MRS medium containing 0, 0.01, 0.05, 0.10 or 0.15% of porcine bile (squares, triangles, circles, solid squares or solid triangles, respectively) and growth was monitored by OD_{600} measurements. These data were used to calculate the maximal logarithmic growth rate (0.56, 0.54, 0.52, 0.44 and 0.14 h^{-1}, respectively). The arrow indicates the time point at which the morphology of the cells was investigated (Fig. 2).

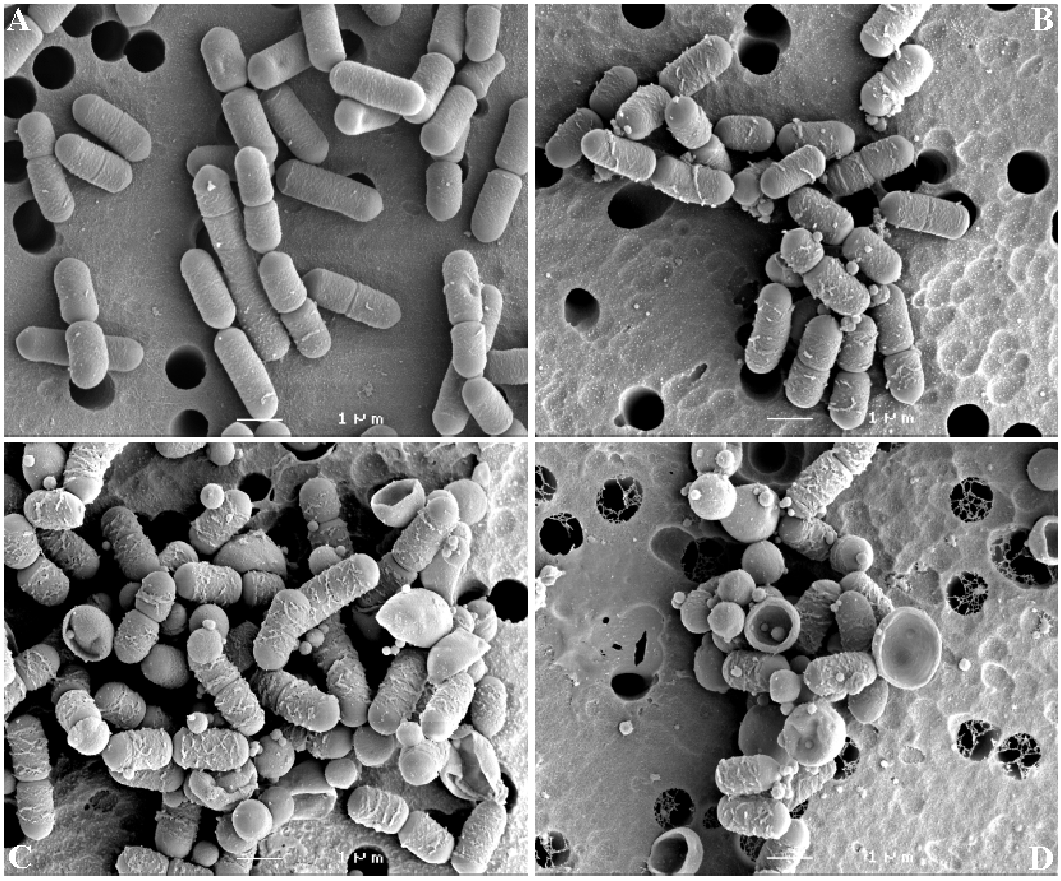


Fig. 2: Morphological changes of *L. plantarum* during bile salt stress. After 4 hours of exposure to 0, 0.05 , 0.10 or 0.15% of bile salts cells were investigated by scanning electron microscopy (Fig. 2A, B, C and D, respectively).

Identification of bile-inducible genes using the *alr* complementation library

Recently, we have described the exploitation of the essential, alanine racemase-encoding *alr* gene as a promoter probe in *L. plantarum* WCFS1 (7). This included the analysis of a chromosomal *L. plantarum* WCFS1 library in the *alr* promoter probe vector pNZ7120. Here, the same library was screened for pNZ7120 derivatives harboring chromosomal *L. plantarum* WCFS1 fragments that contain promoter elements conditionally activated by 0.1% porcine bile. During 72 h growth of approximately 4,000 colonies from the *alr* complementation library was compared on plates with and without bile, resulting in the initial identification of 96 (2.4%) colonies displaying conditional growth only in the presence of bile. This conditional growth phenotype could be confirmed for 46 of these colonies when growth with and without bile was monitored during 72 hours on plates containing a range of 0-200 μg/ml D-cycloserine. The partial sequence of the chromosomal inserts present in the pNZ7120 derivatives originating from 41 of the 46 clones was successfully determined. These sequences corresponded to 30 unique loci of the *L. plantarum* genome, since one locus was found six times, one locus was found three times and four loci were found twice. Two independent clones (BI21 and BI49) both contained a

different chromosomal fragment corresponding to lp_3415, encoding a transcription regulator homologue, and its upstream sequence (Table 2). Hence, the bile induction of lp_3415 was independently confirmed twice during the screening procedure. According to the current genome annotation database of *L. plantarum* WCFS1 (20) the loci harbor 31 unique genes and their upstream sequences in the proper orientation to explain the observed induction of *alr* expression (Table 2). Notably, 8 loci contained more than one putative 5'-end of an annotated ORF and their upstream region. All ORFs that were identified in this *alr* complementation screen were functionally categorized in groups involved in cell membrane function (8 ORFs), cell wall function (3 ORFs), redox reactions (5 ORFs), regulation (5 ORFs), and others (4 ORFs). The remaining 6 genes encoded (conserved) hypothetical proteins of unknown function (Table 2).

Clone BI41 harbors three genes, including a homologue of the bile acid 7α-dehydratase of *Eubacterium* sp. strain VPI 12708 and *Clostridium* sp. strain TO-931 (22, 35). In these intestinal organisms the *baiE* gene, encoding bile acid 7α-dehydratase, is localized in an operon. In *Eubacterium* the expression level of this operon appeared to be induced in the presence of bile (22). A similar operon structure is not found in *L. plantarum*. Nevertheless, the fact that this *baiE* homologue was identified in the *alr* complementation screen strongly suggests that the expression of this single gene is also regulated by bile in *L. plantarum* WCFS1. Clone BI87 harbors a 3'-truncated fragment of lp_1435, encoding an integral membrane protein, which was previously identified in an *alr* complementation screen for high salt inducible promoters of *L. plantarum* (7). These findings suggest a partial overlap in the response of this organism to NaCl and bile salts, possibly caused by the membrane stress induced under both these conditions. The most striking observation was the identification of lp_0237 and lp_0775, encoding an integral membrane protein and an argininosuccinate synthase, on the clones BI19 and BI79, respectively (Table 2). Both genes were previously identified in our laboratory in a resolvase-based *in vivo* expression technology (R-IVET) approach as being important for *L. plantarum* during passage of the GI-tract of mice (6).

Table 2: Identification of clones in the *alr* complementation library that display conditional growth only in the presence of 0.1% porcine bile. When multiple ORFs in the correct orientation to drive *alr* expression are present on a clone, the ORF that is located closest to *alr* gene is presented first. Black bars indicate at which D-cycloserine concentrations growth was only observed in the presence of bile.

clone name:	insert start coordinate	estimated insert size (kb)	lp-nr (gene):	gene product:	D-cycloserine (ug/ml) 0 2.5 5 10 25 50 100 200
BI1	79159	1.0	lp_0082[3]	oxidoreductase	
BI84	80981	2.3	lp_0085[1]	cation efflux protein	
BI19	215774	1.5	lp_0237[1]	integral membrane protein	
			lp_0240[6]	hypothetical protein	
BI79	711316	2.0	lp_0775 (*argG*)[5]	argininosuccinate synthase	
			lp_0774 (*luxS*)[5]	autoinducer production protein	
BI26,81	800854	2.0	lp_0858[3]	redox prot., reg. of disulfide bond form.	
BI65	1057803	1.5	lp_1158 (*lys*)[2]	lysozyme	
			lp_1157[4]	transcription regulator (RpiR family)	
BI87	1315054	0.9	lp_1435[1]	integral membrane protein	
BI14,BI95	1335147	1.5	lp_1459[6]	conserved hypothetical protein	
BI55	2013511	1.5	lp_2230[6]	conserved hypothetical protein	
BI9	2218907	1.5	lp_2484[4]	transcription regulator (MarR family)	
BI96	2284309	1.5	lp_2564[1]	integral membrane protein	
BI31	2528088	1.5	lp_2835[5]	2-haloacid dehalogenase	
BI64	2814574	2.0	lp_3145[3]	oxidoreductase, N-terminal fragment	
BI40	2819823	1.5	lp_3155[1]	cell surface protein, ErfK family	
			lp_3154 (*acm3-C*)[2]	muramidase, C-terminal fragment	
			lp_3153 (*acm3-M*)[2]	muramidase, middle fragment	
BI41	2821528	1.7	lp_3158[3]	oxidoreductase	
			lp_3159 (*baiE*)[5]	bile acid 7α-dehydratase	
			lp_3160[1]	multidrug transport protein	
BI28	2832336	2.0	lp_3175[1]	integral membrane protein	
BI45	2962988	2.0	lp_3330[6]	conserved hypothetical protein	
BI50	2976142	2.0	lp_3343[6]	conserved hypothetical protein	
			lp_3344[4]	transcription regulator (marR family)	
			lp_3345 (*nrpR4*)[4]	negative regulator of proteolysis	
BI21,BI49	3032470	1.5	lp_3415[6]	conserved hypothetical protein	
BI29	3100458	1.5	lp_3488 (*galR2*)[4]	galactose operon repressor (LacI family)	
			lp_3489[3]	oxidoreductase	
BI2,7,8,44,63,69	3240643	1.5	lp_3626[1]	sugar transport protein	

The identified genes were functionally categorized as [1]membrane-associated function, [2]cell wall-associated function, [3]redox reaction,[4]regulation, [5]other and [6]hypothetical protein.

Expression analysis of lp_0237 and lp_0775

The bile-inducible characteristics of lp_0237 and lp_0775 were further investigated by quantitative reverse transcription PCR. RNA was isolated from *L. plantarum* cells grown on plates with and without bile. The isolated RNA samples were used for gene-specific synthesis of cDNA which was used as template for quantitative real-time PCR using specific primers for the bile-induced genes lp_0237 and lp_0775 and the 16S rRNA gene. The latter RNA was used to correct for the total amount of *L. plantarum* specific RNA added to the different reactions. Negative control reactions containing the *L. plantarum* specific16S rRNA primers and DNase-treated RNA were included in each PCR. These reactions never produced any detectable amplicon, indicating the absence of DNA-contamination in the RNA samples. Moreover, the specificity of the PCR reactions was confirmed by a combination of melting curve analysis and DNA sequence analysis of the amplicons (data not shown). All signals

were correlated to the 16S rRNA signal derived from the corresponding cDNA samples. The *in vitro* induction of lp_0237 and lp_0775 by the presence of porcine bile was investigated. This experiment showed that the expression levels of lp_0237 and lp_0775 are induced (24- and 4-fold, respectively) in cells grown on plates containing 0.1% porcine bile relative to control plates lacking bile (Table 3). These data demonstrate that the bile-inducible regulatory characteristics obtained for these two genes using the plasmid-based *alr* promoter probe can be extrapolated to the single copy situation on the chromosome. Moreover, the observed induction by bile suggests that the previously observed *in vivo* induction (6) of lp_0237 and lp_0775 occurs in the duodenum, as this is the site of bile release in the host. Therefore, RNA was isolated from duodenum samples from a mouse fed with *L. plantarum* and a second quantitative reverse transcriptase PCR experiment was performed to assess the *in vivo* expression levels of lp_0237 and lp_0775 in the duodenum. This experiment revealed a significantly higher expression level (13- and 29-fold, respectively) for these genes during passage of the mouse duodenum as compared to expression levels in *L. plantarum* grown on MRS, while in an identical experiment the expression levels of two *L. plantarum* household genes (lp_1027; *fusA2*, encoding an elongation factor and lp_1898; *pfk*, encoding 6-phosphofructokinase), were not significantly increased *in vivo* relative to MRS (Table 3). These data demonstrate that the *in vitro* regulatory characteristics observed for lp_0237 and lp_0775 can be translated to the *in vivo* situation in the mouse duodenum.

Table 3: Induction levels of *L. plantarum* genes *in vitro* by 0.1% bile and *in situ* in the duodenum of a mouse model relative to MRS without bile. The data are presented as fold induction.

lp-nr (gene)	Function	MRS + 0.1% bile	Duodenum
lp_0237	integral membrane protein	23.7*	12.5*
lp_0775 (*argG*)	argininosuccinate synthase	4.3*	28.9*
lp_1027 (*fusA2*)	elongation factor	ND	2.5
lp_1898 (*pfk*)	6-phosphofructokinase	ND	1.4

*Relative expression level of the target gene is significantly different from that observed in MRS medium (lacking bile) according to the Pair Wise Fixed Reallocation Randomisation Test ($p<0.05$). ND = Not determined. Coefficient of variation among replicates (n=3) < 25%.

Discussion

This paper describes the growth, morphological and genetic response of *L. plantarum* WCFS1 to bile salts. A stepwise increase in porcine bile concentrations resulted in a stepwise decrease of maximal growth rate and final OD_{600} of *L. plantarum*. The observed gradual decrease in growth rate coincides with gradually increasing severeness of changes in morphology, including bulky structures on the cell surface, the formation of membrane vesicles and clumping of the cells. Moreover, the observed formation of ghost cells suggests that cell wall integrity is lost by the addition of bile, possibly leading to leakage of intracellular material from the cells and a disturbed energy balance. The bile-induced morphological changes of *L. plantarum* are very similar to those observed in *P. freudenreichii* (21). Furthermore, leakage of proteins from cells after bile treatment was previously observed in other LAB, including *P. freudenreichii* and *L. acidophilus* (21, 23). Since a concentration of 0.1% porcine bile only had a marginal effect on growth rate but, nevertheless, resulted in severe morphological changes, this concentration was used during the rest of the experiments.

The previously constructed *alr* complementation library (7) was exploited for the identification of clones containing *L. plantarum* chromosomal fragments that harbor promoter elements conditionally activated by bile. This approach resulted in the identification of 30 unique loci harboring 31 putative genes of which the expression is potentially induced by bile. The putative genes identified as bile-inducible were organized in six functional categories (Table 2). Strikingly, 11 of these ORFs encode proteins involved in membrane- and cell wall-associated functions. The induction of this relatively high number of genes involved in cellular integrity is in agreement with the observed morphological changes of *L. plantarum* in the presence of bile. Notably, genes encoding putative functions involved in fatty acid and cell wall biosynthesis have previously been identified as important for the bile resistance of *Enterococcus faecalis* (4). The group of membrane-associated functions includes three possible exporter proteins, namely lp_0085, lp_2564 and lp_3160. The latter is annotated as a multidrugtransporter, suggesting a possible role in the export of bile compounds. Similarly, lp_2564, encoding a protein with significant homology to a permease in *Bacillus* cereus, could play a role in the export of bile. Finally, lp_0085, encoding a putative efflux protein, might be involved in maintenance of the electrochemical membrane potential under bile induced stress conditions. Remarkably, the importance of efflux pumps in bile resistance was previously demonstrated in several bacteria, including *Escherichia coli* and *Listeria monocytogenes* (3, 16). The three cell wall-associated functions identified here include a putative lysozyme and two genes annotated to encode fragments of a possible muramidase. Moreover, BlastP analysis of lp_3154 demonstrates a 35% identity with a choline binding protein from *Streptococcus pneumoniae*. The chemical

structure of choline is similar to that of bile. Therefore, the protein encoded by lp_3154 could be important in the defense mechanism of *L. plantarum* to bile salts.

Five genes identified here as bile-inducible are annotated as functions involved in redox reactions, namely four oxidoreductases and a redox protein acting as a regulator of disulfide bond formation, suggesting bile-induced redox balance disturbance and/or oxidative stress. Notably, a gene encoding a function involved in a redox reaction was previously recognized as important during bile stress in *E. faecalis* (4). Another group of five genes encoding regulatory functions was identified as bile-inducible in *L. plantarum*. Remarkably, two of these genes (lp_2484 and lp_3344) belong to the marR family of regulators. In several bacteria, including *E. coli* and *Salmonella typhimurium*, MarA and MarR proteins mediate the expression of a diverse set of genes involved in multidrug resistance, including multidrug efflux proteins (15, 31). The lp_3344 gene product shares 28% identity with MarR from *S. typhimurium*. Moreover, the conserved hypothetical protein encoded by lp_3415 shares 30% identity with MarA from *S. typhymurium*. Therefore, lp_3344 and lp_3415 might be involved in the regulation of multidrug transport in *L. plantarum*, possibly including the two aforementioned transporters encoded by lp_2564 and lp_3160 (Table 2). Next to lp_3415 four other conserved and one unique hypothetical protein were identified in the screen for bile-inducible *L. plantarum* ORFs. Their role in bile resistance remains to be determined.

Bile induction could reflect the *in situ* conditions encountered by *L. plantarum* during passage of the gastro-intestinal tract. A striking finding in this respect are lp_0237 and lp_0775 which we identified as bile inducible in the *in vitro alr* complementation screen presented here and have been identified previously in *L. plantarum* as being *in vivo* induced during passage of the mouse GI-tract (6). Using quantitative PCR, the *in vitro* induction by bile and the *in vivo* induction in the duodenum of a mouse model system relative to laboratory conditions could be established for these genes. Conclusively, these experiments demonstrate that *in vitro* mimicking of complex environmental niches can result in the identification of genes that are of relevance *in situ* in these niches. Overall, this paper provides valuable data on the *in vitro* genetic response of *L. plantarum* to bile. Moreover, the *in vivo* data provide clues that enable the future unraveling of the genetic behavior of *L. plantarum* during residence in (specific parts of) the GI-tract.

Acknowledgements

The authors gratefully acknowledge Corinne Grangette and Annick Mercenier from the Pasteur Institute (Lille, France) for the opportunity to perform the animal experiments in their laboratory. We thank Adriaan van Aelst for excellent technical assistance on the scanning electron microscopy experiment. Part of this work was supported by the EU project LABDEL (EU-QLRT-2000-00340).

References

1. **Ahrne, S., S. Nobaek, B. Jeppsson, I. Adlerberth, A. E. Wold, and G. Molin.** 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. J. Appl. Microbiol. **85:**88-94.

2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403-10.

3. **Begley, M., C. G. Gahan, and C. Hill.** 2002. Bile stress response in *Listeria monocytogenes* LO28: adaptation, cross-protection, and identification of genetic loci involved in bile resistance. Appl. Environ. Microbiol. **68:**6005-12.

4. **Breton, Y. L., A. Maze, A. Hartke, S. Lemarinier, Y. Auffray, and A. Rince.** 2002. Isolation and characterization of bile salts-sensitive mutants of *Enterococcus faecalis*. Curr. Microbiol. **45:**434-9.

5. **Bron, P. A., M. G. Benchimol, J. Lambert, E. Palumbo, M. Deghorain, J. Delcour, W. M. de Vos, M. Kleerebezem, and P. Hols.** 2002. Use of the *alr* gene as a food-grade selection marker in lactic acid bacteria. Appl. Environ. Microbiol. **68:**5663-5670 (Chapter 2).

6. **Bron, P. A., C. Grangette, A. Mercenier, W. M. de Vos, and M. Kleerebezem.** 2004. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. (Chapter 6).

7. **Bron, P. A., S. M. Hoffer, I.I. van Swam, W. M. de Vos, and M. Kleerebezem.** 2004. Selection and characterization of conditionally active promoters in *Lactobacillus plantarum*, using alanine racemase as a promoter probe. Appl. Environ. Microbiol. **70:**310-7 (Chapter 3).

8. **Chou, L. S., and B. Weimer.** 1999. Isolation and characterization of acid- and bile-tolerant isolates from strains of *Lactobacillus acidophilus*. J. Dairy Sci. **82:**23-31.

9. **Christiaens, H., R. J. Leer, P. H. Pouwels, and W. Verstraete.** 1992. Cloning and expression of a conjugated bile acid hydrolase gene from *Lactobacillus plantarum* by using a direct plate assay. Appl. Environ. Microbiol. **58:**3792-8.

10. **Duran Quintana, M. C., P. Garcia Garcia, and A. Garrido Fernandez.** 1999. Establishment of conditions for green table olive fermentation at low temperature. Int. J. Food Microbiol. **51:**133-43.

11. **Dussurget, O., D. Cabanes, P. Dehoux, M. Lecuit, C. Buchrieser, P. Glaser, and P. Cossart.** 2002. *Listeria monocytogenes* bile salt hydrolase is a PrfA-regulated virulence factor involved in the intestinal and hepatic phases of listeriosis. Mol. Microbiol. **45:**1095-106.

12. **Enan, G., A. A. el-Essawy, M. Uyttendaele, and J. Debevere.** 1996. Antibacterial activity of *Lactobacillus plantarum* UG1 isolated from dry sausage: characterization, production and bactericidal action of plantaricin UG1. Int. J. Food Microbiol. **30:**189-215.

13. **Flahaut, S., J. Frere, P. Boutibonnes, and Y. Auffray.** 1996. Comparison of the bile salts and sodium dodecyl sulfate stress responses in *Enterococcus faecalis*. Appl. Environ. Microbiol. **62:**2416-20.

14. **Grangette, C., H. Muller-Alouf, M. Geoffroy, D. Goudercourt, M. Turneer, and A. Mercenier.** 2002. Protection against tetanus toxin after intragastric administration of two recombinant lactic acid bacteria: impact of strain viability and *in vivo* persistence. Vaccine **20:**3304-9.

15. **Grkovic, S., M. H. Brown, and R. A. Skurray.** 2002. Regulation of bacterial drug export systems. Microbiol. Mol. Biol. Rev. **66:**671-701.

16. **Gunn, J. S.** 2000. Mechanisms of bacterial resistance and response to bile. Microbes Infect. **2:**907-13.

17. **Hofmann, A. F., G. Molino, M. Milanese, and G. Belforte.** 1983. Description and simulation of a physiological pharmacokinetic model for the metabolism and enterohepatic circulation of bile acids in man. J. Clin. Invest. **71:**1003-22.

18. **Hofmann, A. F., and K. J. Mysels.** 1992. Bile acid solubility and precipitation *in vitro* and *in vivo*: the role of conjugation, pH, and Ca^{2+} ions. J. Lipid Res. **33:**617-26.

19. **Hyronimus, B., C. Le Marrec, A. H. Sassi, and A. Deschamps.** 2000. Acid and bile tolerance of spore-forming lactic acid bacteria. Int. J. Food Microbiol. **61:**193-7.

20. **Kleerebezem, M., J. Boekhorst, R. Van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. De Vries, B. Ursing, W. M. De Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U.S.A. **100:**1990-1995.

21. **Leverrier, P., D. Dimova, V. Pichereau, Y. Auffray, P. Boyaval, and G. Jan.** 2003. Susceptibility and adaptive response to bile salts in *Propionibacterium freudenreichii*: physiological and proteomic analysis. Appl. Environ. Microbiol. **69:**3809-18.

22. **Mallonee, D. H., W. B. White, and P. B. Hylemon.** 1990. Cloning and sequencing of a bile acid-inducible operon from *Eubacterium* sp. strain VPI 12708. J. Bacteriol. **172:**7011-9.

23. **Noh, D. O., and S. E. Gilliland.** 1993. Influence of bile on cellular integrity and beta-galactosidase activity of *Lactobacillus acidophilus*. J. Dairy Sci. **76:**1253-9.

24. **Pfaffl, M. W.** 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29:**e45.

25. **Pfaffl, M. W., G. W. Horgan, and L. Dempfle.** 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. **30:**e36.

26. **Phan-Thanh, L., and T. Gormon.** 1997. Stress proteins in *Listeria monocytogenes*. Electrophoresis **18:**1464-71.

27. **Pieterse, B., R. H. Jellema, and M. van der Werf.** Quenching of microbial samples for increased reliability of micro-array data. Manuscript in preparation.

28. **Ruiz-Barba, J. L., J. C. Piard, and R. Jimenez-Diaz.** 1991. Plasmid profiles and curing of plasmids in *Lactobacillus plantarum* strains isolated from green olive fermentations. J. Appl. Bacteriol. **71:**417-21.

29. **Shah, N. P.** 2000. Probiotic bacteria: selective enumeration and survival in dairy foods. J. Dairy Sci. **83:**894-907.

30. **Steidler, L., W. Hans, L. Schotte, S. Neirynck, F. Obermeier, W. Falk, W. Fiers, and E. Remaut.** 2000. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin- 10. Science **289:**1352-1355.

31. **Sulavik, M. C., M. Dazer, and P. F. Miller.** 1997. The *Salmonella typhimurium mar* locus: molecular and genetic analyses and assessment of its role in virulence. J. Bacteriol. **179:**1857-66.

32. **Tanaka, H., K. Doesburg, T. Iwasaki, and I. Mierau.** 1999. Screening of lactic acid bacteria for bile salt hydrolase activity. J. Dairy Sci. **82:**2530-5.

33. **Tannock, G. W., A. Tangerman, A. Van Schaik, and M. A. McConnell.** 1994. Deconjugation of bile acids by lactobacilli in the mouse small bowel. Appl. Environ. Microbiol. **60:**3419-20.

34. **Vesa, T., P. Pochart, and P. Marteau.** 2000. Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. Aliment. Pharmacol. Ther. **14:**823-8.

35. **Wells, J. E., and P. B. Hylemon.** 2000. Identification and characterization of a bile acid 7alpha-dehydroxylation operon in *Clostridium* sp. strain TO-931, a highly active 7alpha-dehydroxylating strain isolated from human feces. Appl. Environ. Microbiol. **66:**1107-13.

Chapter 5

DNA Micro-Array Based Identification of Bile-Responsive Genes in *Lactobacillus plantarum*

PETER A. BRON,[1,2] DOUWE MOLENAAR,[1,2] WILLEM M. DE VOS,[1] AND MICHIEL KLEEREBEZEM.[1,2]

[1] Wageningen Centre for Food Sciences, [2] NIZO food research, P.O. Box 20, 6710 BA Ede, The Netherlands.

Manuscript in preparation.

Abstract

Clone-based DNA micro-arrays were employed to describe the global transcriptional response of *Lactobacillus plantarum* WCFS1 towards porcine bile. Comparison of the differential transcript profiles obtained during growth of *L. plantarum* on plates with and without bile revealed 29 and 62 putative genes of which the expression was at least 2.5-fold up- or down-regulated by bile, respectively. Approximately 50% of these genes appeared genetically linked and 12 bile-responsive gene clusters were identified. Seven bile-responsive genes and gene clusters encode typical stress-related functions, including glutathione reductase and glutamate decarboxylase, involved in oxidative and acid stress, respectively. Moreover, 14 bile-responsive genes and gene clusters were identified that encode proteins that are located in the cell envelope, including the *dlt operon* and the F1F0 ATPase, indicating a major impact of bile acids on the integrity and/or functionality of the cytoplasmic membrane and cell wall.

Introduction

The human gastrointestinal tract (GI-tract) represents an important ecological niche for food-associated micro-organisms. Following their consumption, food-grade bacteria, as well as food-borne pathogens, meet their host. Several biological barriers are encountered by bacteria during travel through, and residence in the different parts of the host's GI-tract, such as the gastric acidity in the stomach, the presence of bile salts in the duodenum and stress conditions associated with oxygen gradients that are steep at the mucosal surface, while the colon is virtually anoxic (5). Moreover, considerable bacterial competition is encountered throughout the entire GI-tract and notably in the colon where the bacterial population reaches densities of up to 10^{12} cells per gram of luminal content (16). Due to the complex nature of host-specific and chemical conditions that are met by bacteria in the GI-tract, many studies describe the *in vitro* response of bacteria to simplified models that mimic these harsh conditions. These include many physiological and biochemical studies that have recently been reviewed (3).

Lactobacillus plantarum is a flexible and versatile lactic acid bacterium that inhabits a variety of environmental niches, including the human GI-tract. *L. plantarum* WCFS1 (10) is a single colony isolate of strain NCIMB 8826, which displayed high activity and survival rates in the human intestine in pharmacokinetic studies (20). Previously, the observed GI-tract persistence of *L. plantarum* has been studied by a genome-wide genetic screen, resulting in the identification of 31 putative genes of which the expression appeared to be induced on plates containing porcine bile (3). In analogy with random gene disruption strategies applied in *Listeria monocytogenes* (1) and *Enterococcus faecalis* (2), this genetic screen in *L. plantarum* revealed that efflux pumps and changes in the architecture in the cell envelope are important for bile resistance of these bacteria (3). In addition, it was observed that exposure to bile greatly affected the morphology of *L. plantarum* WCFS1 (3). Notably, these findings are in good agreement with several physiological studies in other bacteria such as *Propionibacterium freudenreichii* and *L. reuteri* that demonstrated that bile salts induce severe changes in the morphology of their cell envelope (12, 18).

Here we describe the utilization of clone-based DNA micro-arrays for the analysis of the response of *L. plantarum* WCFS1 towards porcine bile during growth on plates. This approach resulted in the identification of 29 and 62 potential genes of which the expression appeared at least 2.5-fold up- or down-regulated in the presence of bile. Moreover, the expression levels of twelve gene clusters appeared to be regulated by bile. The observation that many of these genes and gene clusters encode cell wall and cell membrane associated functions strongly suggests that bile acids have a major impact on the integrity of the cell envelope.

Material and Methods

Growth conditions and RNA isolations

L. plantarum WCFS1 (10) was grown at 37 ^{o}C in MRS (Difco, Surrey, U.K.) without aeration. Appropriate dilutions of a full-grown culture were plated on MRS containing 1% agarose, with or without 0.1% porcine bile salts (Sigma, Zwijndrecht, The Netherlands, B-8631). After 3 days of growth at 37 ^{o}C, colonies on each plate were rapidly collected by resuspension in 3 ml MRS, which was immediately added to twelve ml quench buffer (60% methanol, 66.7 mM HEPES, pH 6.5, -40 ^{o}C)(14). Following quenching, the cells were pelleted by centrifugation at 4500 rpm for 10 min and cell pellets were resuspended in 0.4 ml ice-cold MRS. The cell suspensions were added to ice-cold tubes containing 1 g of zirconium glass beads, 0.4 ml phenol, 100 µl chloroform, 30 µl 10% SDS and 30 µl 3M NaAc pH 5.2. The cells were disrupted using two treatments of 40 seconds in a Fastprep^{TM} (Qbiogene Inc, Cedex, France) interspaced by 1 min on ice. After centrifugation, 200 µl of the aqueous phase was used for RNA isolation using the High Pure system, which included a 1 h treatment with DNaseI (Roche Diagnostics, Mannheim, Germany).

Array design

The DNA micro-arrays were based on clones derived from the genomic library that was previously constructed for the determination of the complete genome sequence of *L. plantarum* WCFS1 (10). In total, 3692 genomic fragments were amplified by PCR from the genomic library using Supertaq (SphaeroQ, Leiden, the Netherlands), and vector-derived universal forward and reverse primers with 5'-C6 aminolinkers to facilitate cross-linking to the aldehyde-coated glass slides. The resulting amplicons were purified by ethanol precipitation and dissolved in 3x SSC (1x SSC contains 150mM NaCl and 17mM sodiumcitrate, pH 7.2). Subsequently, the purified amplicons were arrayed in a controlled atmosphere on CSS-100 silylated aldehyde glass slides with quill pins (Telechem, SMP3, USA) in a SDDC 2 Eurogridder (ESI, Toronto, Canada). Afterwards the slides were dried and blocked with borohydride.

Fluorescent labelling and hybridization

Differential transcript levels were determined by two-color (Cy5 and Cy3) fluorescent hybridizations of the corresponding cDNAs on the *L. plantarum* WCFS1 clone-based DNA micro-array. The RNA samples were labelled during reverse transcription using random hexamers primers and either Cy5- or Cy3-labeled dUTP (Amersham Biosciences). Unincorporated dyes were removed from the synthesized cDNA using autoseq G50 columns (Amersham Biosciences). The arrays were prehybridized for 45 minutes at 42 °C in prehybridization solution (1% BSA, 5x SCC and 0.1% SDS). Co-hybridizations of the labelled cDNA samples were performed overnight at 42 ºC in Easyhyb buffer (Roche) according to the manufacturer's protocol. The slides were

subsequently washed twice in 1x SSC and 0.2% SDS, once in 0.5x SSC, and twice in 0.2x SSC at 37 ^{o}C.

Scanning and data analysis

After the washing steps, slides were dried, and scanned with a ScanArray Express 4000 scanner for both dyes (Perkin Elmer). Images were analyzed by using ImaGene 4.2 software (BioDiscovery). Criteria for flagging spots were as follows: (i) empty spots threshold 2.0 (ii) poor spots threshold 0.4 (iii) negative spots on. The resulting ImaGene output files were further processed by discarding spots flagged as empty, poor or negative by ImaGene software, and the Cy5 and Cy3 signals being less than two times above the local background. Routinely over 80% of all spots passed all these quality criteria and the remaining, high-quality spots were normalized. For each array the raw spot intensities in both channels (I_1 and I_2) were converted to M-A coordinates, where $M= {}^{2}\log(I_1 / I_2)$ and $A= {}^{10}\log(I_1 + I_2)/2$. Subsequently a fit of M as a function of A was calculated using a robust local linear regression algorithm (LOWESS). This fit was used as the new M=0 axis by subtracting from each M-value the corresponding fitted value. To calculate a regulatory ratio for each gene, as far as they were represented by clones on the micro-array, a weighted average of the M-values of all clones that overlapped with the gene of interest was calculated. The weight used for each clone was equal to the square of the overlap between gene and clone divided by the total length of the gene. Hence, this method weighs small overlapping fragments less than proportional compared to larger overlapping fragments.

Results and Discussion

Clone-based transcriptome analysis

Clone-based DNA micro-arrays were used to investigate the genetic bile-response of *L. plantarum* WCFS1. The development of these clone-based arrays was initiated following the random sequencing of the *L. plantarum* genomic library and its primary sequence assembly during the genome sequence project (10). Subsequently, 3692 inserts from this genomic library were amplified. The resulting amplicons had an average size of approximately 1.2 kb, cover 80.8% of the *L. plantarum* WCFS1 genome, and represent 2683 of the 3052 annotated genes (88 %). The overlap of clones in the part of the genome that is covered resulted in an average 1.6-fold redundancy. Notably, for the construction of ORF-based arrays a partial, pre-annotated genome sequence is a minimum requirement. Therefore, one advantage of clone-based arrays lies within the fact that its construction can be initiated earlier in time as compared to ORF-based arrays. An intrinsic disadvantage of clone-based arrays is that certain genes will be incompletely represented or completely lacking on such an array. Moreover, certain clones will represent two or more ORFs, which

potentially results in masking of regulation of a specific gene by neighboring genes that are regulated in the opposite direction. Consequently, interpretation of the regulatory factors for individual genes requires relative complex data processing and in some situations is simply impossible. On the other hand, multiple clones can represent individual genes or gene clusters on a clone-based DNA micro-array. Although multiple clones complicate the assessment of the exact magnitude of regulation factors, they provide independent duplicate measurements that intrinsically validate the conditional response of specific genes or gene clusters. Therefore, our analyses exploited the latter advantages of clone-based DNA micro-arrays and have mainly focused on the bile-response of gene clusters and individual genes that are represented by multiple clones.

Global transcriptome analysis and selection of bile-regulated genes and clusters

The genome-wide transcription profiles of *L. plantarum* WCFS1 grown on plates with or without 0.1% porcine bile were compared. The experimental set-up that was used during these transcriptome analyses was highly similar to those used during a previous genetic screen in our laboratory (3). An independent biological duplicate was performed using two DNA micro-arrays and RNA derived from *L. plantarum* colonies grown on different batches of plates. Moreover, technical variations introduced by cDNA synthesis and/or dye-swap effects were assessed by a third array experiment in which two cDNA samples were synthesized using the same RNA template derived from *L. plantarum* cells grown without bile (Fig. 1).

The differences in gene expression in the technical duplicate appeared to be relatively small as compared to those found in the arrays that assessed the differential transcript profile of cells grown with or without bile (biological variation). These global analyses indicate that the specific response of *L. plantarum* towards bile is drastically larger than the experimental variation (Fig. 1). Nevertheless, the variation between biological duplicates appeared relatively large (data not shown). Therefore, this first study focused on those responses that were consistently observed in both experiments, and the primary selection of regulated clones was performed using stringent criteria: (i) consistent direction of regulation in both arrays (ii) at least 2.5-fold up or down regulation in both arrays (iii) the same order of magnitude of regulatory factor (maximum 10-fold difference between the two slides). Using these stringent criteria, 62 and 29 ORFs were found to be down- and up-regulated during growth of *L. plantarum* on plates containing bile as compared to control plates lacking bile. (Table 1 and 2, respectively). Approximately 25% of these individual genes are represented by a single clone and, consequently, significant assessment of their bile induction or repression levels requires expansion of the number of arrays. The rest of the genes are represented by two or more clones, allowing more trustworthy assessment of their induction levels.

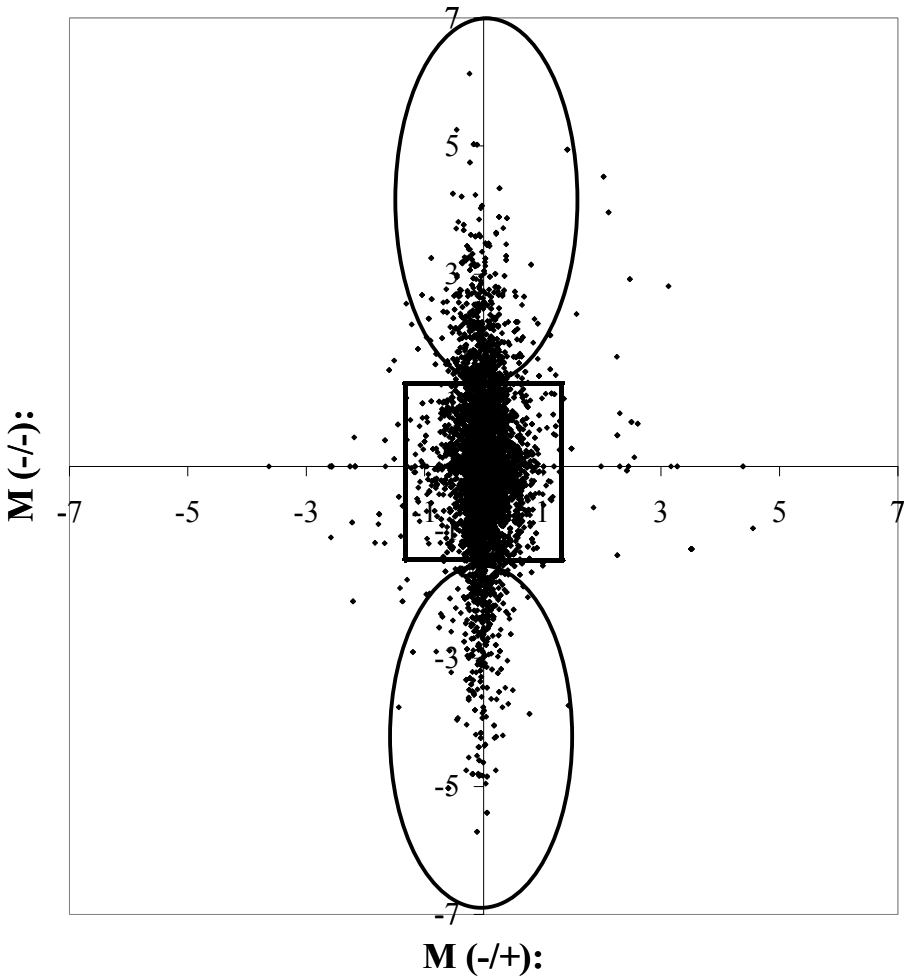


Fig. 1: Scatter plot of the M-values of two array experiments. The M-value is defined as the 2-log of the ratio of the Cy5 and Cy3 signal intensities per spot determined for one array. The X-axis represents the M-values from an array hybridized with two cDNAs derived from a single RNA sample, originating from *L. plantarum* cells grown without bile (M-/-). The Y-axis represents the M-values from two RNAs originating from cells grown with and without bile (M-/+). The square indicates 2.5-fold regulation which was used as a cut-off value in the analyses, and the circles enclose bile-responsive clones.

Table 1: *L. plantarum* genes that are at least 2.5-fold down-regulated by bile. The gene clusters are indicated in bold and are presented in more detail in Fig. 2. The two ratios represent the ratio of signal (Cy5 and Cy3) of the individual arrays.

Gene	Product	nr. of clones	ratio 1	ratio 2
lp_0005	**DNA repair and genetic recombination protein RecF**	**3**	**-3.7**	**-2.6**
lp_0006	**DNA gyrase, B subunit**	**3**	**-3.5**	**-2.5**
lp_0007	**DNA gyrase, A subunit**	**4**	**-9.8**	**-3.5**
lp_0064	conserved hypothetical protein	1	-2.8	-3.0
lp_0175	**maltose/maltodextrin ABC transporter, substrate binding protein**	**3**	**-4.0**	**-4.0**
lp_0176	**maltose/maltodextrin ABC transporter, permease protein**	**3**	**-3.2**	**-3.0**
lp_0177	**maltose/maltodextrin ABC transporter, permease protein**	**2**	**-3.0**	**-2.5**
lp_0178	**maltose/maltodextrin ABC transporter subunit (putative)**	**2**	**-4.9**	**-3.7**
lp_0179	**alpha-amylase**	**2**	**-4.6**	**-5.3**
lp_0180	**multiple sugar ABC transporter, ATP-binding protein**	**1**	**-11.3**	**-6.1**
lp_0239	hypothetical protein	2	-5.3	-3.0
lp_0240	conserved hypothetical protein	2	-6.1	-3.2
lp_0242	nucleoside-diphosphate kinase	2	-5.3	-3.0
lp_0266	hypothetical protein	2	-4.9	-5.7
lp_0286	cellobiose PTS, EIIC	4	-6.5	-2.8
lp_0287	hypothetical protein	2	-3.2	-2.6
lp_0289	hypothetical protein	1	-3.0	-2.6
lp_0852	pyruvate oxidase	3	-7.5	-3.0
lp_1069	NADH dehydrogenase	2	-5.3	-3.2
lp_1168	hypothetical protein	1	-10.6	-4.9
lp_1169	glutamate dehydrogenase (NAD(P)+)	4	-4.0	-3.7
lp_1171	serine-type D-Ala-D-Ala carboxypeptidase	3	-8.6	-2.8
lp_1362	hypothetical protein	2	-3.7	-2.8
lp_1420	nisin resistance protein (putative)	1	-3.0	-4.0
lp_1544	response regulator	2	-7.0	-3.2
lp_1545	histidine protein kinase; sensor protein	3	-9.2	-3.0
lp_1558	phenylalanine--tRNA ligase, alpha chain	3	-3.5	-3.7
lp_1643	cell surface protein precursor	11	-9.8	-3.2
lp_1956	**ABC transporter, permease protein**	**1**	**-3.7**	**-3.2**
lp_1957	**ABC transporter, permease protein**	**3**	**-3.2**	**-3.0**
lp_1958	**ABC transporter, ATP-binding protein**	**2**	**-3.0**	**-3.2**
lp_1959	**transcription regulator**	**2**	**-5.3**	**-4.9**
lp_1960	hypothetical protein	2	-2.8	-5.7
lp_1961	hypothetical protein	1	-2.8	-5.7
lp_1962	DNA-directed RNA polymerase, sigma factor 42	2	-2.5	-4.3
lp_2143	integral membrane protein	1	-4.0	-3.0
lp_2173	**extracellular protein**	**3**	**-5.3**	**-3.2**
lp_2174	**cell surface protein precursor**	**3**	**-4.6**	**-3.2**
lp_2647	**N-acetylglucosamine/galactosamine PTS, EIIA**	**3**	**-36.8**	**-4.3**
lp_2648	**N-acetylgalactosamine PTS, EIID**	**3**	**-48.5**	**-6.1**
lp_2649	**N-acetylgalactosamine PTS, EIIC**	**2**	**-9.8**	**-4.6**
lp_2776	D-serine dehydratase	2	-4.0	-3.5
lp_2936	thiamin biosynthesis lipoprotein ApbE	1	-4.0	-4.6
lp_2937	transcription regulator	2	-8.6	-3.7
lp_2975	**extracellular protein**	**3**	**-4.9**	**-3.2**
lp_2976	**cell surface protein precursor (putative)**	**2**	**-6.5**	**-3.2**
lp_2977	**cell surface protein precursor**	**2**	**-2.8**	**-2.5**
lp_2978	**extracellular protein**	**1**	**-4.3**	**-2.6**
lp_3091	conserved hypothetical protein	1	-3.7	-3.7
lp_3092	succinate-semialdehyde dehydrogenase (NAD(P)+)	3	-5.3	-4.6
lp_3172	xylose operon regulator	4	-4.9	-2.8
lp_3219	**sucrose PTS, EIIBCA**	**3**	**-3.5**	**-2.6**
lp_3220	**alpha-glucosidase**	**6**	**-5.3**	**-2.5**
lp_3221	**transcription regulator**	**4**	**-3.5**	**-2.8**
lp_3222	**hypothetical protein**	**3**	**-2.6**	**-3.0**
lp_3360	integral membrane protein	1	-4.9	-2.8
lp_3362	choloylglycine hydrolase	4	-9.8	-3.0

lp_3397	conserved hypothetical protein	1	-2.8	-3.7
lp_3553	**maltose O-acetyltransferase**	**3**	**-8.0**	**-4.3**
lp_3554	**L-arabinose isomerase**	**5**	**-18.4**	**-7.5**
lp_3555	**L-ribulose 5-phosphate 4-epimerase**	**2**	**-97.0**	**-16.0**
lp_3556	**L-ribulokinase (putative)**	**6**	**-13.9**	**-8.6**

Table 2: *L. plantarum* genes that are at least 2.5-fold up-regulated by bile. The gene clusters are indicated in bold and are presented in more detail in Fig. 2. The two ratios represent the ratio of signal (Cy5 and Cy3) of the individual arrays.

Gene	Product	nr. of clones	ratio 1	ratio 2
Lp_0254	**serine O-acetyltransferase**	**3**	**2.8**	**3.0**
Lp_0255	**cystathionine beta-lyase**	**3**	**9.2**	**9.2**
Lp_0256	**cysteine synthase**	**3**	**8.6**	**8.6**
Lp_0512	ribosomal protein L31	1	9.2	5.3
Lp_0547	cell division protein FtsH, ATP-dependent zinc metallopeptidase	4	4.0	3.2
Lp_0609	glutamate--tRNA ligase	4	4.3	3.5
Lp_0779	**nucleotide kinase (putative)**	**3**	**6.1**	**4.0**
Lp_0780	**conserved hypothetical protein**	**4**	**4.9**	**4.6**
Lp_0781	**conserved hypothetical protein**	**2**	**5.3**	**5.3**
Lp_1026	ribosomal protein S7	1	3.2	3.7
Lp_1253	glutathione reductase	1	4.3	4.9
Lp_1541	phosphogluconate dehydrogenase (decarboxylating)	3	5.7	3.0
Lp_2002	hypothetical protein	1	3.2	4.3
Lp_2003	transcription regulator (putative)	1	3.2	4.0
Lp_2019	**D-alanine activating enzyme DltA**	**3**	**4.0**	**3.5**
Lp_2020	**D-Ala-teichoic acid biosynthesis protein (putative)**	**1**	**4.6**	**6.1**
Lp_2364	**H(+)-transporting two-sector ATPase, beta subunit**	**3**	**3.7**	**11.3**
Lp_2365	**H(+)-transporting two-sector ATPase, gamma subunit**	**3**	**3.0**	**12.1**
Lp_2366	**H(+)-transporting two-sector ATPase, alpha subunit**	**2**	**4.0**	**11.3**
Lp_2367	**H(+)-transporting two-sector ATPase, delta subunit**	**3**	**3.0**	**9.2**
Lp_2368	**H(+)-transporting two-sector ATPase, B subunit**	**2**	**2.6**	**8.6**
Lp_2369	**H(+)-transporting two-sector ATPase, C subunit**	**2**	**2.6**	**8.6**
Lp_3014	extracellular protein	2	2.8	3.5
Lp_3420	glutamate decarboxylase	2	6.5	4.9
Lp_3421	gamma-D-glutamate-meso-diaminopimelate muropeptidase (putative)	2	6.5	3.0
Lp_3536	choloylglycine hydrolase	2	4.9	4.6
Lp_3537	hydrolase, HAD superfamily, Cof family	1	4.9	3.7
Lp_3687	cell division protein	1	5.7	4.6

Approximately 50% of the regulated genes appeared genetically linked (Table 1 and 2), suggesting clustered organization of these genes in operon-like structures, which could explain their concerted regulation. Therefore, these entire clusters, as based on gene annotation in the *L. plantarum* genome (10), were analyzed in more detail (Fig. 2). To increase confidence, only clusters represented by at least 3 clones were used in the analyses, which in some cases included flanking genes that were not necessarily identified by our initial, stringent selection. Notably, some clones represent multiple genes (Fig. 2C, lp_1956), some genes are only partly represented (Fig. 2H, lp_3554 and lp_3557), and absolute induction levels obtained vary for different clones representing the same gene. The latter is especially true for clones that are located at the termini of the gene clusters identified and partially overlap with genes up- or downstream of the gene cluster (Fig. 2H, three clones representing lp_3553). On the other hand the direction and magnitude of bile-mediated regulation is very similar for all clones representing the twelve complete gene clusters, thereby allowing multiple, independent measurements of the bile-response of these gene clusters.

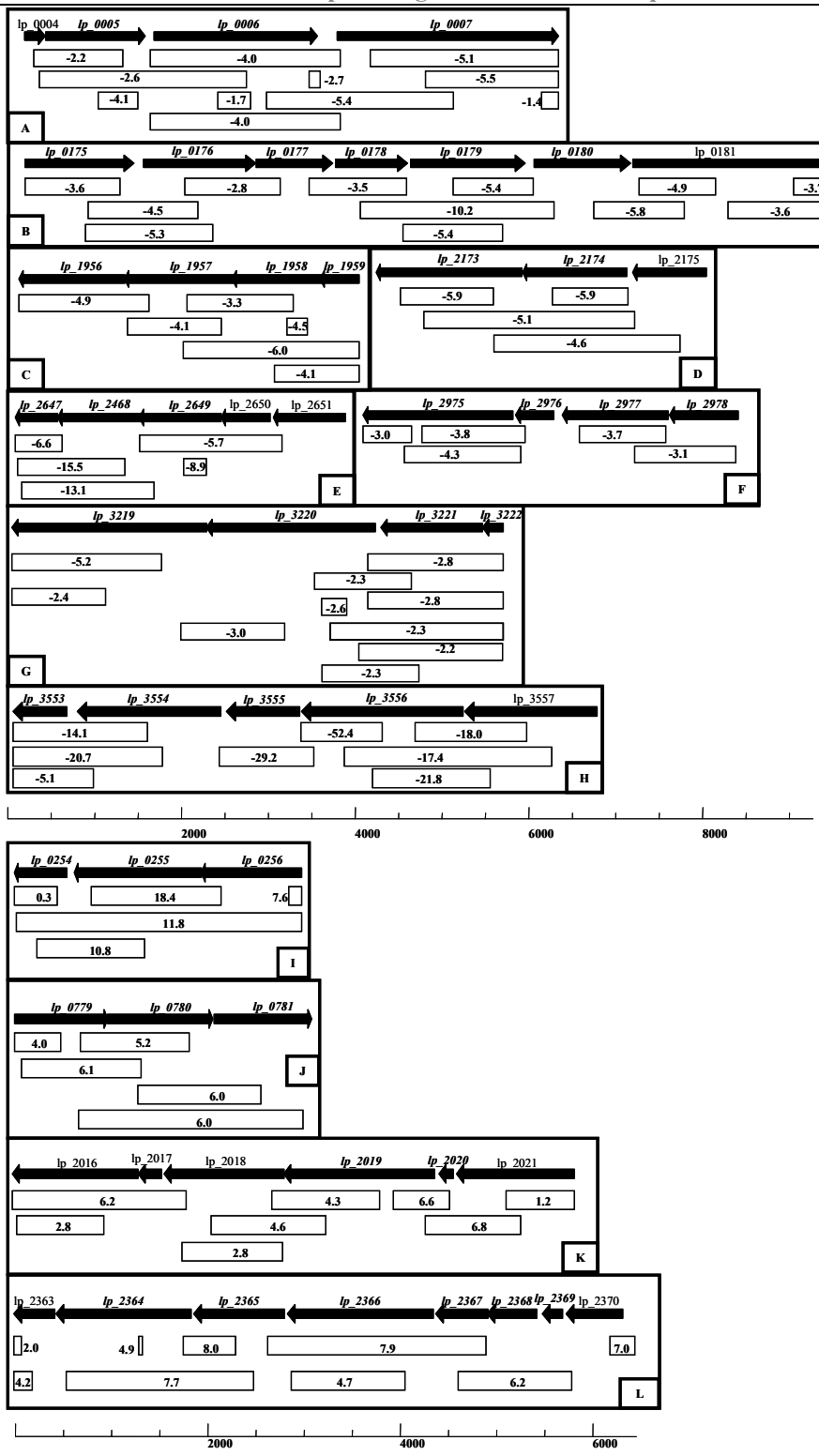


Fig. 2: Schematic overview of the identified bile-responsive gene clusters. Figure 2 A-H represent the bile-repressed clusters lp_0004-lp_0007 (DNA gyrase), lp_0175-lp_0181 (maltose ABC transporter), lp_1956-lp_1959 (ABC transporter), lp_2173-lp_2175 (extracellular proteins), lp_2647-lp_2651 (N-acetylgalactosamine PTS-system), lp_2975-lp_2978 (extracellular proteins), lp_3219-lp_3222 (sucrose PTS-system) and lp_3553-lp_3557 (arabinose transporter), respectively. Figure 2 I-L represent the bile-induced gene clusters lp_0254-lp_0256 (*metC-cysK* operon), lp_0779-lp_0781 (nucelotide kinase), lp_2016-lp_2021 (*dlt* operon) and lp_2363-lp_2370 (F1F0 ATPase), respectively. Notably, representative clones are truncated at the first start or stop codon of the genes encompassed by the gene clusters and the presented fold-induction levels are the average of the values obtained in the two arrays performed. Genes presented as bold-italic were initially identified using stringent selection criterion

Bile-regulated gene expression

Seven genes or gene clusters encoding typical stress related functions appeared regulated by bile. Remarkably, the operon encoding the RecF protein and DNA gyrase, involved in the repair of point mutations during oxidative stress in several bacteria (15) is down-regulated, while bile acids are considered to induce oxidative stress. This finding is unexpected, especially since several other bile-mediated responses appear to reflect the oxidative stress induced by bile. As an example, the increased expression of glutathione reductase and the *metC-cysK* operon upon bile-treatment could be involved in the protection against the oxidative stress imposed on *L. plantarum* by bile (12, 13). Notably, one of the bile-induced proteins that was identified in *Propionibacterium freudenreichii* in a differential proteome analysis is the cysteine synthase encoded by *cysK* (12), suggesting that at least part of the defense against bile appears to be conserved among different Gram-positive bacteria. The increased *ftsH* transcript levels upon treatment with bile could also relate to stress adaptation in *L. plantarum*. The *ftsH* gene encodes an ATP-dependent zinc metallopeptidase involved in cell-cycle control and membrane function in *Escherichia coli* and *Bacillus subtilis* (8, 9, 19). Moreover, in *Bacillus subtilis*, the *ftsH* gene is induced upon osmotic up shift and has a general role in stress adaptation (9). It is remarkable that the expression of one of the four choloylglycine hydrolase encoding genes of *L. plantarum* (lp_3536, *bsh1*) is approximately 5-fold induced by bile, while the expression of another *bsh* gene is reduced (lp_3362, *bsh3*). This observation suggests differential regulation of *bsh* genes by different bile compounds present in the porcine bile mixture used during our experiments. Therefore, it would be interesting to perform experiments in which at a chosen moment a chemically-defined bile compound is added and transcript profiles are determined following bile addition. Finally, the DNA micro-array experiments demonstrated that transcription of the glutamate decarboxylase encoding *gadB* gene was significantly induced by bile. In addition, two other genes encoding proteins involved in glutamate metabolism are regulated by bile, namely glutamate dehydrogenase and glutamate tRNA-ligase (lp_1169 and lp_0609, Table 1 and 2, respectively). It has been demonstrated in several GI-tract related bacteria, including *E. coli* (6) and *Listeria monocytogenes* (4), that intracellular glutamate accumulation enhances the survival of these microbes during osmotic and acid stress. This protective effect is directly correlated to the glutamate decarboxylase activity that is involved in the conversion of glutamate into γ-aminobutyrate. The γ-aminobutyrate formed is subsequently exchanged for another extracellular glutamate via the *gadC* encoded antiporter (4), thereby the combination of these reactions results in the consumption of an internal proton, which is thought to generate the observed stress tolerance (4). The *gadB* and *gadC* genes of *E. coli* and *Listeria monocytogenes* are genetically linked and tandemly transcribed, which is a feature that is not conserved in *L. plantarum.* Nevertheless, the *L. plantarum* genome

appears to encode a GadC homologue (lp_2799). While the expression of this gene did not appear to be affected by bile in our experiments, a protective effect of *L. plantarum* GadB in maintenance of the relative intracellular pH and proton motive force, possibly involving a constitutively expressed glutamate-γ-aminobutyrate antiporter, can certainly not be excluded.

Six bile-repressed genes and gene clusters encode transporters located in the cell membrane, namely two ABC-transporters, of which one is annotated to be specific for maltose/maltodextrin, three PTS-systems specific for N-acetylglucosamine, cellobiose and sucrose, and an arabinose transport protein. The observation that the transcription of all these transporters is down-regulated by bile could reflect the major impact of this lipid-active compound on membrane integrity and stability. Possibly, bile stress-induced loss of membrane integrity can partially be compensated by the down-regulation of the genes encoding non-essential membrane proteins. A similar rationale might explain the reduced expression of the membrane associated protein encoded by lp_2143. Moreover, it should be noted that the absolute regulatory factors found for the gene clusters containing the N-acetylgalactosamine/glucosamine PTS-system and the arabinose transporter, were among the highest in the array analyses. Uptake of N-acetyl-D-glucosamine via its PTS transporter leads to N-acetyl-D-glucosamine-6P intracellular, which is a precursor in the biosynthesis of both UDP-N-acetyl-D-glucosamine and UDP-N-acetylmuramate that are both required for peptidoglycan biosynthesis. The results appear to suggest that *L. plantarum* could exploit this pathway under non-stressed conditions, while bile induced stress leads to repression of this metabolic shortcut for cell-wall precursor biosynthesis, which could be related to the membrane stress caused by bile. The only membrane-associated function of which the transcript levels are enhanced during bile stress is the F1F0 ATPase system, which is involved in maintenance of the proton motif force at the expense of ATP (17). The expression of the *atp* operon in *L. acidophilus*, encoding F1F0 ATPase, appeared to be induced at low pH ranges (11). The increased transcript levels of the complete F1F0 ATPase encoding gene cluster in *L. plantarum* in the presence of bile strongly suggests proton motive force dissipation in the presence of bile. Apparently, maintenance of proton motif force is a crucial factor during bile stress, as two systems potentially contributing to this process appear to be induced, generating proton motif force at the expense of ATP (F1F0 ATPase system) or glutamate (glutamate decarboxylase system, see above).

Several changes in the expression levels of genes encoding cell-wall associated proteins were observed. The expression of lp_1643 and lp_3421, encoding two individual cell surface proteins, appear to be down- and up-regulated by bile, respectively. Moreover, the transcript levels of two clusters encoding multiple extracellular proteins were reduced (lp_2173-lp_2175 and lp_2975-lp_2978). The

exact role of these extracellular proteins in *L. plantarum* during bile stress remains to be established. Nevertheless, the remarkable changes in transcription level of several genes encoding cell wall-associated functions clearly indicate a changed cell wall composition during bile stress. A striking observation in this respect is the increased expression of the *dlt* operon, involved in D-alanylation of teichoic acids (7). This observation suggests an increased molecular decoration of lipoteichoic acid in the presence of bile, which would influence the charge of the cell wall and might provide a defense mechanism against bile. Besides the increase in expression of the serine-type D-Ala-D-Ala carboxypeptidase encoded in the *dlt* operon, a second gene encoding this function appeared reduced during bile stress. In conclusion, the fact that fourteen genes and gene clusters encoding cell envelop associated functions display altered expression levels indicate prominent changes in cell envelope architecture during bile stress.

Concluding remarks

This paper describes the first transcriptome analysis of the bile response in *L. plantarum*. In parallel with a genetic screen performed previously in our lab (3), the response towards bile was investigated during growth on plates. Clone-based arrays were used and stringent selection of differentially expressed genes led to the identification of 29 and 62 putative genes that were up- and down-regulated in the presence of bile, respectively. Genetic linkage of several of these individual bile-responsive genes resulted in the identification of twelve bile-responsive gene clusters. Several genes and gene clusters encode functions that have previously been associated with stress response and/or adaptation in different bacteria (4, 9, 13). The major impact of bile acids on cell envelope integrity is clearly exemplified by the change in expression of fourteen genes and gene clusters encoding cytoplasmic membrane and cell wall-associated functions. In analogy, a genetic screen that was previously performed in our laboratory already elucidated the importance of efflux pumps and cell envelope architecture in the response towards bile (3). Moreover, these findings are in good agreement with the dramatic changes in morphology of *L. plantarum* cells upon bile stress (3), which has also been observed for other Gram-positive bacteria like *Propionibacterium freudenreichii* (12) and *L. reuteri* (18). The aforementioned genetic screen (3) and the DNA micro-arrays presented here, both aim at analysis of the bile response in *L. plantarum* during growth on solid media. However, the genes identified by the genetic screen (3) did not appear to be consistently up-regulated by bile according to the array analyses presented here. This apparent discrepancy might be explained by the fact that the genetic screen primarily identifies differentially active promoter elements in a multi-copy plasmid system, while the DNA micro-array studies aim at differential transcript profiling. Moreover, since the genetic screen employed was based on complementation of a mutation in the essential alanine

racemase encoding gene (*alr*), it is likely that this screen primarily identifies genes that are differentially expressed for several days during growth on plates. In contrast, the transcript levels assessed with DNA micro-arrays represent the abundance of mRNAs at one specific time point and thus reflect the response to the physico-chemical conditions present at that time point. Consequently, these analyses provide a snap-shot of the bacterial expression profile, namely at the time of RNA isolation. Hence, within the experimental set-up used here, it could very well be that the *alr* complementation screen has identified genes involved in the initial stress response of *L. plantarum* upon bile treatment, while DNA micro-array analyses have unraveled the later, bile adaptive response. These subtle differences could explain the low level of similarity found in the outcome of these two screens. Nevertheless, the complementary approaches used (genetic screen and DNA micro-arrays) have provided valuables clues towards the defense mechanisms that play a role during bile stress in *L. plantarum*.

References

1. **Begley, M., C. G. Gahan, and C. Hill.** 2002. Bile stress response in *Listeria monocytogenes* LO28: adaptation, cross-protection, and identification of genetic loci involved in bile resistance. Appl. Environ. Microbiol. **68:**6005-12.

2. **Breton, Y. L., A. Maze, A. Hartke, S. Lemarinier, Y. Auffray, and A. Rince.** 2002. Isolation and characterization of bile salts-sensitive mutants of *Enterococcus faecalis*. Curr. Microbiol. **45:**434-9.

3. **Bron, P. A., M. Marco, S. M. Hoffer, E. van Mullekom, W. M. de Vos, and M. Kleerebezem.** 2004. Genetic characterization of the bile salt response in *Lactobacillus plantarum* and analysis of responsive promoters *in vitro* and *in situ* in the gastrointestinal tract (Chapter 4).

4. **Cotter, P. D., C. G. Gahan, and C. Hill.** 2001. A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. Mol. Microbiol. **40:**465-75.

5. **Cummings, J. H., and G. T. Macfarlane.** 1991. The control and consequences of bacterial fermentation in the human colon. J. Appl. Bacteriol. **70:**443-59.

6. **De Biase, D., A. Tramonti, F. Bossa, and P. Visca.** 1999. The response to stationary-phase stress conditions in *Escherichia coli*: role and regulation of the glutamic acid decarboxylase system. Mol. Microbiol. **32:**1198-211.

7. **Delcour, J., T. Ferain, M. Deghorain, E. Palumbo, and P. Hols.** 1999. The biosynthesis and functionality of the cell-wall of lactic acid bacteria. Antonie Van Leeuwenhoek **76:**159-84.

8. **Deuerling, E., A. Mogk, C. Richter, M. Purucker, and W. Schumann.** 1997. The *ftsH* gene of *Bacillus subtilis* is involved in major cellular processes such as sporulation, stress adaptation and secretion. Mol. Microbiol. **23:**921-33.

9. **Deuerling, E., B. Paeslack, and W. Schumann.** 1995. The *ftsH* gene of *Bacillus subtilis* is transiently induced after osmotic and temperature upshift. J. Bacteriol. **177:**4105-12.

10. **Kleerebezem, M., J. Boekhorst, R. Van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. De Vries, B. Ursing, W. M. De Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U.S.A. **100:**1990-1995.

11. **Kullen, M. J., and T. R. Klaenhammer.** 1999. Identification of the pH-inducible, proton-translocating F1F0-ATPase (*atpBEFHAGDC*) operon of *Lactobacillus acidophilus* by differential display: gene structure, cloning and characterization. Mol. Microbiol. **33:**1152-61.

12. **Leverrier, P., D. Dimova, V. Pichereau, Y. Auffray, P. Boyaval, and G. Jan.** 2003. Susceptibility and adaptive response to bile salts in *Propionibacterium freudenreichii*: physiological and proteomic analysis. Appl. Environ. Microbiol. **69:**3809-18.

13. **Li, Y., J. Hugenholtz, T. Abee, and D. Molenaar.** 2003. Glutathione protects *Lactococcus lactis* against oxidative stress. Appl. Environ. Microbiol. **69:**5739-45.

14. **Pieterse, B., R. H. Jellema, and M. van der Werf.** 2004. Quenching of microbial samples for increased reliability of micro-array data. Manuscript in preparation.

15. **Rui, S., and Y. C. Tse-Dinh.** 2003. Topoisomerase function during bacterial responses to environmental challenge. Front. Biosci. **8:**d256-63.

16. **Savage, D. C.** 1977. Microbial ecology of the gastrointestinal tract. Annu. Rev. Microbiol. **31:**107-33.

17. **Senior, A. E.** 1990. The proton-translocating ATPase of *Escherichia coli*. Annu. Rev. Biophys. Biophys. Chem. **19:**7-41.

18. **Taranto, M. P., M. L. Fernandez Murga, G. Lorca, and G. F. de Valdez.** 2003. Bile salts and cholesterol induce changes in the lipid cell membrane of *Lactobacillus reuteri*. J. Appl. Microbiol. **95:**86-91.

19. **Tomoyasu, T., T. Yuki, S. Morimura, H. Mori, K. Yamanaka, H. Niki, S. Hiraga, and T. Ogura.** 1993. The *Escherichia coli* FtsH protein is a prokaryotic member of a protein family of putative ATPases involved in membrane functions, cell cycle control, and gene expression. J. Bacteriol. **175:**1344-51.

20. **Vesa, T., P. Pochart, and P. Marteau.** 2000. Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. Aliment. Pharmacol. Ther. **14:**823-8.

Chapter 6

Identification of *Lactobacillus plantarum* Genes that are Induced in the Gastrointestinal Tract of Mice

PETER A. BRON,[1,2] CORINNE GRANGETTE,[3] ANNICK MERCENIER,[3] WILLEM M. DE VOS,[1] AND MICHIEL KLEEREBEZEM.[1,2]

[1] Wageningen Centre for Food Sciences, [2]NIZO food research, P.O. Box 20, 6710 BA Ede, The Netherlands, [3] Laboratoire de Bactériologie des Ecosystemes, Institut Pasteur de Lille, 59019 Lille Cedex, France.

Submitted to J. Bacteriol. (2004).

Abstract

Lactobacillus plantarum **is a flexible and versatile microorganism that inhabits a variety of environmental niches, including the human gastrointestinal tract (GI-tract). Moreover, this lactic acid bacterium has been shown to be able to survive passage of the human and mouse stomach in an active form. To investigate the genetic background of this persistence, resolvase-based *in vivo* expression technology (R-IVET) was performed in *L. plantarum* WCFS1, using the mouse GI-tract as a model system. This approach identified 72 *L. plantarum* genes of which the expression is induced during passage of the GI-tract as compared to laboratory media. Nine of these genes encode sugar-related functions, including ribose, cellobiose, sucrose and sorbitol transporter genes. Another nine genes encode functions involved in acquisition and synthesis of amino acids, nucleotides, cofactors and vitamins, indicating their limited availability in the GI-tract. Four genes involved in stress-related functions were identified, reflecting the harsh conditions that *L. plantarum* encounters in the GI-tract. The four extracellular protein encoding genes identified could potentially be involved in interaction with host specific factors. The rest of the genes are part of several functionally unrelated pathways or encode (conserved) hypothetical proteins. Comparison of the R-IVET screen with several *in vitro* experiments might provide clues to geographical differentiation of *L. plantarum* gene expression along the host's GI-tract. Remarkably, a large number of the functions or pathways identified here have previously been identified in pathogens as being important *in vivo* during infection, strongly suggesting that survival rather than virulence is the explanation for the importance of these genes during host residence.**

Introduction

Traditionally lactic acid bacteria (LAB) are applied extensively in the production of a wide variety of fermented food and feed products. In addition, certain LAB species, in particular from the genus *Lactobacillus*, are natural inhabitants of the gastrointestinal tract (GI-tract) and may have probiotic effects in man and animals (2). Moreover, LAB have great potential to serve as delivery vehicles of health-promoting or therapeutic compounds to the human GI-tract (23, 49). Among the different species encompassed by this genus, *Lactobacillus plantarum* is encountered in many environmental niches, including some dairy, meat and many vegetables fermentations (31). Because of the long tradition of utilization of *L. plantarum* in industrial and artisanal fermentations, this microbe is generally regarded as safe (GRAS). Next to the occurrence of *L. plantarum* in our diets, this microbe is frequently encountered as a natural inhabitant of the human GI-tract (2). The complete 3.3 Mbp genome sequence of *L. plantarum* WCFS1 has been determined (31). This strain is a single colony isolate of strain NCIMB8826, which effectively survives passage of the human stomach in an active form, reaches the ileum in high numbers as compared to other strains, and is detectable in the colon (52). Intriguingly, genome sequence comparison revealed that the closest relatives of *L. plantarum* include *Listeria innocua* and *monocytogenes*, which also naturally inhabit and persist the human GI-tract (22). The availability of its genome sequence allows effective investigation of the genes and regulation mechanisms underlying the observed persistence of *L. plantarum* in the GI-tract.

Three main strategies have been developed for the identification of genes that are highly expressed *in vivo* as compared to laboratory conditions, namely selective capture of transcribed sequences (SCOTS; 13, 17), signature tagged mutagenesis (STM, for reviews see 43, 54) and *in vivo* expression technology (IVET). The original IVET strategy involves a tandem set of promoterless reporter genes which were used to identify promoters that are specifically switched on in *Salmonella typhimurium* during infection (38). Variations to IVET utilizing auxotrophic markers, antibiotic resistance genes and site-specific recombinases have been employed to trap promoters specifically activated during infection of several pathogens (for reviews see 3, 37). Although many papers describe the *in vivo* behavior of pathogens during infection in various animal models, the bacterial factors that allow survival and persistence of food-associated microorganisms remain largely unknown. Only a single study describes an *in vivo* approach in *L. reuteri*, resulting in the identification of 3 genes that are induced during colonization of *Lactobacillus*-free mice on an antibiotic-containing diet (53). Unfortunately, the administration of antibiotics dramatically disturbs the intestinal microflora present in these mice, probably leading to GI-tract conditions that differ significantly from those in a conventional mouse. This disadvantage does not apply to recombination-based IVET (R-IVET) strategies (3).

Here we describe the exploitation of such a R-IVET approach in the food-grade organism *L. plantarum* WCFS1, leading to the identification of 72 genes of which the expression is induced in this LAB during passage of the GI-tract of conventional mice as compared to laboratory media. Many of the genes identified here have previously been identified in pathogenic bacteria using a variety of SCOTS, STM and (R)-IVET approaches. In addition, several novel *in vivo* induced genes of *L. plantarum* have been identified that potentially contribute to specific host-microbe interactions.

Material and Methods

Bacterial strains and plasmids

The bacterial strains and plasmids that were used in this study are listed in Table 1. *Escherichia coli* strains MC1061 (11) was used as cloning host during construction of pNZ7109 (see below) and was grown aerobically in TY medium (46). *Lactococcus lactis* MG1363 (21) was used as a cloning host during construction of pNZ7125, pNZ7126 and the *L. plantarum* promoter library (see below). *Lactococcus lactis* was grown without aeration at 30 ^{o}C in M17 medium (Merck, Darmstadt, Germany), supplemented with 0.5% (wt/vol) glucose (GM17). *L. plantarum* WCFS1 (31) and its *loxP-ery-loxP* derivative NZ7109 (see below) were grown at 37 ^{o}C in MRS medium (Difco, Surrey, U.K.) without aeration. When appropriate, antibiotics were added to the media; for *E. coli* ampicillin (50 μg/ml); for *Lactococcus lactis* chloramphenicol (5 μg/ml); for *L. plantarum* chloramphenicol (5 μg/ml), erythromycin (5 or 30 μg/ml, for selection after transformation or replica-plating, respectively), lincomycin (10 μg/ml) and rifampicin (50 μg/ml).

DNA techniques and sequence analysis

Plasmid DNA was isolated from *E. coli* on a small scale using the alkaline lysis method (4, 46). Large scale plasmid DNA isolations were performed using Jetstar columns according to the manufacturer's instructions (Genomed GmbH, Bad Oberhausen, Germany). DNA isolation and transformation in *Lactococcus lactis* and *L. plantarum* were performed as described previously (14, 18, 26). Standard procedures were applied for DNA manipulations in *E. coli* (46). Restriction endonucleases, *Taq* and *Pwo* polymerase, T4 DNA ligase and calf intestine alakaline phosphatase (CIAP) were used following the recommendations of the manufacturer (Promega, Leiden, the Netherlands and Boehringer, Mannheim, Germany). Primers were purchased from Pharmacia Biotech (Roosendaal, the Netherlands). The sequences of the inserts present in the pNZ7125 derivatives (see below), were amplified by PCR using the primers cre-R2 and BglII-cre (Table 1), followed by amplicon purification using Sephadex-G50 and multiscreen HV 96-well plates (Millipore, Amsterdam, The Netherlands). Partial insert sequences were determined using primer cre-R2 or BglII-cre, approximately 100 ng of the purified amplicons and

the ABI Prism® BigDye (tm) Terminator Cycle Sequencing Ready Reaction Kit protocol (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). Sequence reaction products were analyzed using an ABI-prism 3700 DNA analyzer. The determined insert sequences were assigned to *L. plantarum* WCFS1 chromosomal loci using Blast-N (31).

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

Material	Relevant features	Source or reference
Strains		
E. coli		
MC1061	cloning host	(11)
Lactococcus lactis		
MG1363	cloning host	(21)
L. plantarum		
WCFS1	wild-type for which the genome sequence is available	(31)
WCFS1-R	Rif^R	this work
NZ7109	Rif^R, Em^R, *L. plantarum* WCFS1 derivative containing a chromosomally located *loxP-ery-loxP*-cassette	this work
Plasmids		
pUC18	Ap^R	(55)
pUC19	Ap^R	(55)
pNZ7105	Ap^R , pUC18 derivative containing a 5'-truncated fragment of lp_3503	this work
pNZ7106	Ap^R , pUC19 derivative containing a 5'-truncated fragment of lp_3504	this work
pUC18ery	Ap^R, Em^R	(51)
pNZ7109	Ap^R, Em^R, contains *ery* originating from pUC18ery flanked by two synthetic *loxP* sites and 5'-truncated fragments of lp_3503 and lp_3504 originating from pNZ7105 and pNZ7106, respectively (Fig. 1)	this work
pNZ7110	Ap^R, pUC18 derivative containing *cre* and T_{pepN}	(6)
pJIM2246	low copy cloning vector, Cm^R	(45)
pNZ7124	Cm^R, pJIM2246 derivative containing *cre* and T_{pepN} originating from pNZ7110	this work
pNZ7125	Cm^R, pNZ7124 derivative containing T_{las} upstream of *cre*	this work
pNZ7126	Cm^R, pNZ7125 derivative containg P_{ldhL1} upstream of *cre*	this work
Primers		
cre-R2	5'-GTCCATCAGGTTCTTGCG-3'	
BglII-cre	5'-ATAGTTTACCCCGTCAGC-3'	
lp_3503F	5'-TGCTTTCCAAGAGCAAGCTG–3'	
lp_3503R [a]	5'-ACGTCTGCAGTCAGGTGTGAAGTTGGCACT-3'	
lp_3504F [a]	5'-ACGTCTGCAGCCACTCACCGACTAACACTC-3'	
lp_3504R [a]	5'-ACGTAAGCTTGACCCACGAGTTACCAACACG-5'	
lasF2 [a]	5'-ACGTGGATCCGGACAATATGGGGTAAGCG-3'	
lasR [a]	5'-AAGAAGATCT CTAAAGCTGACGGGGTAAAC-3'	
PldhL-F [a]	5'-GAAGATCTTCAATCTTCTCACCGTCTTG-3'	
PldhL-R [a]	5'-GAAGATCTTCAATAAGTCATCCTCTCGT-3'	

[a] underlined sequences indicate restriction sites subsequently used in cloning procedures

[b] Ap^R, ampicillin resistant; Cm^R, chloramphenicol resistant; Em^R, erythromycin; Rif^R, rifampicin resistant

Construction of *L. plantarum* NZ7109

To allow highly selective recovery of *L. plantarum* WCFS1 from faecal samples, this strain was cultured with increasing concentrations of rifampicin (up to 50 µg/ml) and the resulting rifampicin resistant strain was designated *L. plantarum* WCFS1-R (Table 1). The intergenic locus flanked by the convergently transcribed genes lp_3503 and lp_3504 (Fig. 1) was chosen for the integration of a *loxP-ery-loxP* cassette into the chromosome of *L. plantarum* WCFS1-R using a two step double cross-over strategy (18). For this purpose pNZ7109, a pUC18 (55) derivative which harbors a *loxP-ery-loxP* cassette flanked by PCR amplified 5'-truncated fragments of lp_3503 (using primers lp_3503F and R) and lp_3504 (using primers lp3504F and R) was constructed and integrated into the chromosome of *L. plantarum* WCFS1-R (Fig. 1). A selected integrant possessing the anticipated genotype, as analyzed by PCR and Southern blotting, was designated NZ7109 (Table 1).

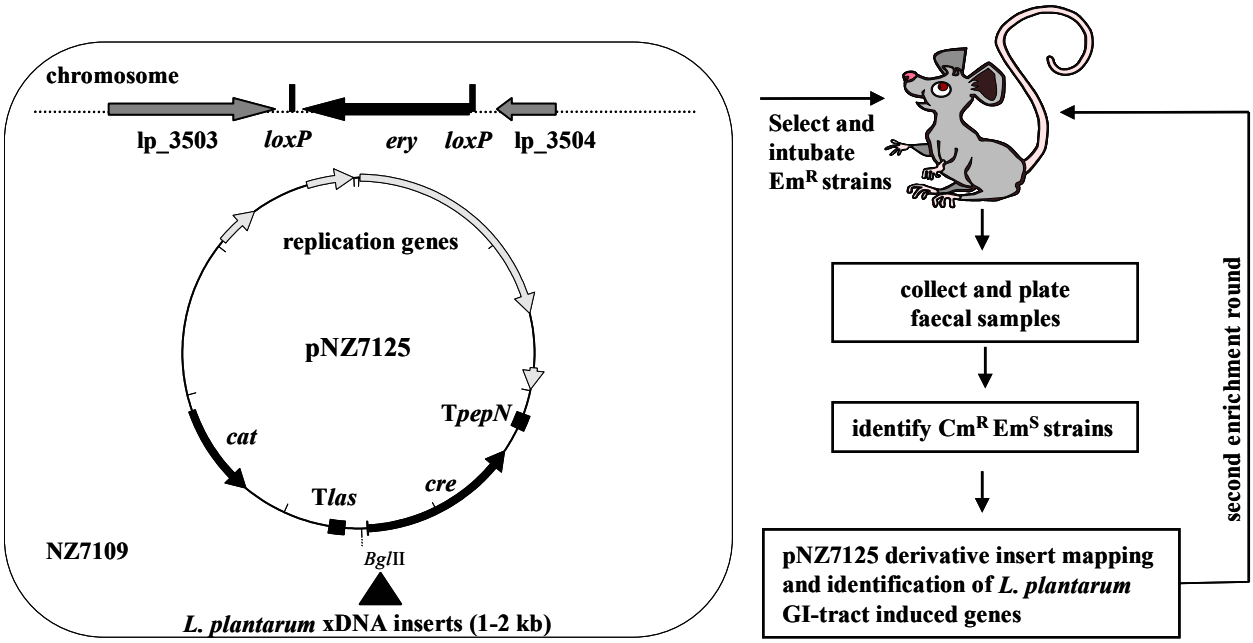


Fig. 1: Basic principles of R-IVET. *L. plantarum* strain NZ7109 harbors a chromosomally located *loxP-ery-loxP* cassette and pNZ7125. Chromosomal loci of the *L. plantarum* genome (black triangle) are cloned upstream of *cre* in pNZ7125 and promoter activities in the resulting library can be trapped by monitoring the erythromycin phenotype, since *cre* expression will lead to excision of the erythromycin marker from the chromosome by a homologous recombination event between the two *loxP* sites. The colonies in the library appearing as erythromycin resistant in the laboratory (no active promoters under laboratory conditions) are administrated to mice and changes in the erythromycin phenotype (promoter activations) can be monitored in faecal samples.

pNZ7125, pNZ7126 and R-IVET library construction

To implement R-IVET in *L. plantarum* the low-copy vector pNZ7125 was constructed. First, a fragment harboring *cre*, originating from pNZ7110 (7), was cloned into the low copy variant of pJIM2246 (45), yielding pNZ7124. Genomic DNA of *Lactococcus lactis* MG1363 (21) was used as a template to amplify the terminator of the *las* operon, using primers lasF2 and lasR (Table 1). The resulting 0.35 kb amplicon was cloned into pNZ7124 and a plasmid containing the terminator of the *las* operon properly oriented upstream of *cre* to prevent read-through transcription was designated pNZ7125. This vector harbors the *prtP* ribosome binding site originating from pUC-*Nco*I (19) linked to a promoterless copy of the *cre* gene followed by the *pepN* terminator, both originating from pNZ8048-Cre (10). To confirm correct chromosomal *loxP-ery-loxP* resolution (1) upon *cre* expression, a pNZ7125 derivative containing the *L. plantarum ldhL1* promoter (18) upstream of the *cre* gene was constructed. The promoter region of the *ldhL1* gene of *L. plantarum* WCFS1 was amplified using chromosomal DNA of this strain (31) as template and the primers PldhL-F and PldhL-R (Table 1). The resulting 0.5 kb amplicon was digested with *Bgl*II and cloned into similarly digested pNZ7125. The constructed plasmid was designated pNZ7126 and contains the *cre* gene under control of the *ldhL1* promoter. Finally, a *L. plantarum* WCFS1 chromosomal R-IVET library was constructed in pNZ7125. Chromosomal DNA was partially digested with *Sau*3AI and size-fractionated on 1% agarose gels. Fragments ranging from 1-2 kb were purified using Sephaglas^{TM} Bandprep (Pharmacia Biotech, Roosendaal, The Netherlands). These purified fragments were cloned into *Bgl*II digested and CIAP dephosphorylated pNZ7125 (Fig. 1). Ligation mixtures were transformed to *Lactococcus lactis* MG1363 (21) and approximately 50,000 of the obtained colonies were collectively resuspended in GM17. Plasmid DNA was isolated from these cells and introduced into NZ7109. The approximate 37,000 colonies obtained were collectively resuspended in MRS containing 15% glycerol, and stored in aliquots at –80 ^{o}C.

R-IVET animal experiments

To counter select against clones in the R-IVET library that harbor pNZ7125 derivatives containing a promoter element that is active under the laboratory conditions applied, the collective library was subcultured for approximately 20 generations in MRS containing 5 μg/ml chloramphenicol, 30 μg/ml erythromycin and 50 μg/ml rifampicin. Subsequent animal experiments were performed in an accredited establishment (N° A59107) according to guidelines N°86/609/CEE of the French government. Seven weeks-old female Balb/c mice were purchased from Iffa Credo (St Germain sur l'Arbresle, France) and had free access to tap water and standard mice chow during the experiments. After overnight culture, bacterial cells were pelleted by centrifugation and resuspended at 10^{10} CFU per ml in MRS. The 4 mice received a

100 µl oral dose of these freshly prepared bacterial suspensions by intragastric administration at 2 consecutive days, and 24 h after the last administration individual faecal samples were collected and resuspended in MRS medium. After extensive homogenization, the complete R-IVET library was recovered from the faecal samples by plating appropriate dilutions of the suspensions on MRS plates containing 5 µg/ml chloramphenicol and 50 µg/ml rifampicin. After 72 h full grown colonies were replica-plated to plates containing 5 µg/ml chloramphenicol and 50 µg/ml rifampicin, with or without 30 µg/ml erythromycin. Another 24 h later the plates were compared, leading to the identification of cells displaying an erythromycin sensitive phenotype. The inserts present in the corresponding pNZ7125 derivatives harbored by these resolved clones were amplified by PCR and from the resulting amplicons the DNA sequences were analyzed.

Results

Implementation and functionality of R-IVET in *L. plantarum*

Previously R-IVET has been used exclusively for the identification of genes induced in pathogenic microorganisms during infection of host tissues (for reviews see 3, 37). Since it has been reported that the Cre resolvase, encoded by the *cre* gene, could be functionally implemented in the LAB *Lactococcus lactis* (10), the suitability of this resolvase for a R-IVET approach in *L. plantarum* was evaluated. Therefore, a *L. plantarum* WCFS1 derivative strain harboring a chromosomally located *loxP-ery-loxP* cassette was constructed and designated strain NZ7109 (Fig. 1). The growth rate of NZ7109 did not differ from that observed for wild-type *L. plantarum* WCFS1, and after 50 generations of growth without antibiotic selection pressure, replica-plating revealed that all NZ7109 cells were erythromycin resistant. Moreover, PCR analysis revealed that the chromosomal *loxP-ery-loxP* insertion could be amplified from all colonies (data not shown), indicating that the chromosomal insertion is stable. Both pNZ7125, a low-copy vector encoding a promoterless copy of *cre*, and a derivative containing the *cre* gene under control of the *L. plantarum* WCFS1 *ldhL1* promoter (pNZ7126) were introduced in NZ7109 cells and plated on MRS medium with chloramphenicol. Replica-plating of the transformants revealed that all NZ7109 colonies harboring pNZ7125 were erythromycin resistant, while all NZ7109 colonies harboring pNZ7126 were erythromycin sensitive. In addition, PCR analysis confirmed that the *loxP-ery-loxP* locus could only be amplified from erythromycin resistant colonies (data not shown). These results establish the functional implementation of the *cre-loxP* resolution system in *L. plantarum* WCFS1 and confirm the suitability of pNZ7125 as a R-IVET vector.

Construction of a *L. plantarum* R-IVET library in pNZ7125

A genomic library of *L. plantarum* WCFS1 was constructed in pNZ7125, using *Lactococcus lactis* MG1363 as an intermediate cloning host. Approximately 37,000 colonies were obtained in *L. plantarum* NZ7109 and the quality of this library was assessed in several ways. The pNZ7125 derivatives of 100 randomly picked colonies were used as template for insert amplification by PCR, demonstrating that over 95% of the investigated clones contain an insert with an estimated average size of 1.3 kb (data not shown). To assess insert redundancy, all amplicons were digested with *Sau*3AI and the resulting fragments were separated by 2% agarose gel electrophoresis. No common restriction profiles were detected, indicating that redundancy in the R-IVET library is low. Moreover, 28 of these amplicons were used for partial sequence analysis, which revealed no apparent over- or under-representation of a specific region of the *L. plantarum* genome. These results support the randomness of the library and genome coverage was calculated to be approximately 98% (data not shown). The R-IVET library was replica-plated to plates with and without erythromycin, which indicated that 10% of the R-IVET clones contain a pNZ7125 derivative harboring a properly oriented promoter element that is active under the laboratory conditions applied and drives *cre* expression at a sufficient level to excise the *loxP-ery-loxP* cassette from the chromosome of NZ7109.

R-IVET screen in mice

To counter select against clones displaying *cre* expression under laboratory conditions, the R-IVET library was cultured for 20 generations in the presence of erythromycin. Subsequently, full-grown cultures were used for gastric administration to 4 Balb/c mice. After recovery from faecal samples 6,000 of the R-IVET clones were analyzed for their erythromycin phenotype by replica-plating, revealing 198 (3.3%) clones that displayed an erythromycin sensitive phenotype. The partial sequence of 132 of the chromosomal inserts present in the pNZ7125 derivatives originating from these clones was determined and corresponded to 119 unique loci of the *L. plantarum* genome, since one locus was found three times, while 11 loci were found twice. According to the current genome annotation database of *L. plantarum* WCFS1 (31) these loci harbor 72 unique genes and their upstream sequences in the proper orientation to explain the observed induction of *cre* expression (Table 2). Notably, 9 loci contained more than one putative 5'-end of an annotated ORF and their potential promoter. Remarkably, two independent *Sau*3AI clones corresponding to lp_0291 and its upstream sequence were found that differ in size (1.0 and 1.5 kb). Hence, the *in vivo* induction of this gene was independently confirmed twice during the R-IVET procedure. The identified genes appeared to be randomly located within the *L. plantarum* genome. Moreover, the genes appeared to be randomly distributed among the main functional categories. The ORFs identified in this R-IVET screen

were functionally grouped in genes involved in nutrient acquisition and synthesis (18 ORFs), stress response and adaptation (4 ORFs), extracellular proteins (4 ORFs), regulation (3 ORFs) and others (19 ORFs). The remaining 24 genes encoded (conserved) hypothetical proteins of unknown function (Table 2).

To verify the primary R-IVET results the 132 partially sequenced clones were divided in four groups of 33 clones which were used for collective plasmid DNA isolation. These mixtures of pNZ7125 derivatives were reintroduced into *L. plantarum* NZ7109 and the resulting colonies were collectively stored. Replica-plating of the transformants revealed that all tested colonies displayed an erythromycin resistant phenotype, confirming the absence of *cre* expression in these clones under laboratory conditions. These four groups were separately subjected to a second round passage through 8 mice (2 animals per group), following the same procedure as applied in the first round passage, including recovery from faecal samples and analysis of the erythromycin phenotype. This second round passage revealed a dramatic increase in the percentage of erythromycin sensitive colonies that was recovered from the faecal samples of all individual mice (Table 3). The observation that the second round passage resulted in an average of 38.1% erythromycin sensitive clones as compared to 3.3% in the experimentally identical first round passage confirmed the R-IVET based enrichment of *L. plantarum* chromosomal fragments harboring *in vivo* induced promoter elements.

Table 2: *L. plantarum* genes that are induced during passage of the mouse GI-tract as compared to laboratory conditions.

class	ORF (gene)	product or function	relevant reference(s)
nutrient acquisition and synthesis			
sugar	lp_0185 (*pts1BCA*)[a]	sucrose PTS EIIBCA	(30)[b]
	lp_1164 (*pts14C*)	cellobiose PTS, EIIC	(20, 30)
	lp_2647 (*pts19A*)	N-acetylglucosamine/galactosamine PTS, EIIA	
	lp_3473 (*ram2*)	alpha-L-rhamnosidase	
	lp_3522 (*pts32BC*)	sucrose PTS, EIIBC	(30)[b]
	lp_3526 (*pbg10*)[a]	6-phospho-beta-glucosidase	
	lp_3618[2] (*pts37A*)	sorbitol PTS EIIA	(25)
	lp_3659[3] (*rbsD*)	ribose transport protein	(13, 34)
	lp_3660[3] (*rbsK3*)	ribokinase	(13, 34)
non-sugar	lp_0017 (*proA*)	glutamate-5-semialdehyde dehydrogenase	(28, 48)
	lp_0228 (*pepD1*)	dipeptidase	
	lp_0696	cytosine/adenosine deaminase	(44)
	lp_0775 (*argG*)	argininosuccinate synthase	(9)
	lp_0854 (*birA2*)[a]	biotin--[acetyl-CoA-carboxylase] ligase and biotin operon repressor	(12, 29)
	lp_1058 (*adk*)	adenylate kinase	
	lp_1319 (*rsuA*)	pseudouridylate synthase	
	lp_1602[7]	geranyltranstransferase / farnesyl di P transferase	
	lp_2031 (*ribC2*)	bifunctional protein: riboflavin kinase and FMN adenylyltransferase	(36)
stress			
	lp_1019 (*clpC*)	ATP-dependent Clp protease	(27, 35)
	lp_3055[4] (*copA*)	copper transporting ATPase	(24, 37)
	lp_3288	cation efflux protein	
	lp_3303[5]	putative multidrug transport protein	(28)
extracellular			
LPQTNE containing	lp_0800	cell surface protein precursor	
	lp_2940	cell surface protein precursor	
other	lp_0141	extracellular protein	
	lp_1403[a]	cell surface protein	
regulators			
	lp_3176 (*pkn2*)	serine/threonine protein kinase	
	lp_3514 (*bglG4*)	transcription antitermination	(39)
	lp_3646[a]	transcription regulator	
other			
	lp_0237	integral membrane protein	
	lp_0299	ABC transporter, ATP-binding protein	
	lp_0305 (*gcsH1*)	glycine cleavage system, H protein	
	lp_0393[1] (*thgA1*)	galactoside O-acetyltransferase	
	lp_0394[1]	transport protein	
	lp_0419 (*plnI*)[a]	immunity protein	
	lp_0489 (*tkt1-N*)	transketolase	
	lp_0698 (*dnaX*)[a]	DNA-directed DNA polymerase III, gamma/tau subunit	(30)
	lp_0740[6] (*prfB-N*)	peptide chain release factor 2, N-terminal fragment	
	lp_0741[6] (*prfB-C*)	peptide chain release factor 2, C-terminal fragment	
	lp_1603[7] (*ispA)*	hemolysin homologue	
	lp_1847 (*codV*)	integrase/recombinase	
	lp_3051 (*dhaT*)	1,3-propanediol dehydrogenase	(40)
	lp_3082	transport protein	
	lp_3281	transport protein	
	lp_3500	short-chain dehydrogenase/oxidoreductase	(40)
	lp_3505 (*est2*)	acetylesterase	
	lp_3617[2] (*tal3*)	transaldolase	
	lp_3662 (*adhE*)	bifunctional protein: alcohol and acetaldehyde dehydrogenase	(41)
hypothetical			
conserved	lp_0026, lp_0139, lp_0190, lp_0291[a], lp_0476[8], lp_0477[8], lp_0907, lp_1969, lp_2337, lp_2507[9], lp_2508[9], lp_2713, lp_2718, lp_3057[4], lp_3058[4] and lp_3312		(53) for lp_2718, see text)
not conserved	lp_0118[a], lp_0292, lp_1788, lp_1872, lp_2112, lp_2584, lp_3246 and lp_3305[5]		

[a] These genes were identified twice during the R-IVET screen

[b] In the original paper 13 *in vivo* induced loci were identified in *Streptococcus gordonii*. However, these loci were only partially sequenced and for six loci this was not sufficient to identify a promoter element. At present, using more detailed sequence analysis and the partially available genome sequence, these loci have been assigned a promoter element (Lin Tao, personal communication)

[1-9] genes with the same tag originate from one clone

Table 3: A second round passage of R-IVET positives through mice results in an increased percentage of erythromycin sensitive NZ7109 colonies as compared to the first round passage (3.3%).

mouse	promoter group	ery^S (%)
1	1	58.0
2	1	71.7
3	2	52.9
4	2	28.8
5	3	34.2
6	3	33.3
7	4	31.5
8	4	37.5
average (1-8)	1-4	38.1

Discussion

Three major strategies have been utilized for the *in vivo* identification of promoters. The major disadvantages of SCOTS are the instability of bacterial mRNA for the construction of cDNA libraries, the low abundance of mRNA from transiently expressed genes, and the difficulty in isolation of sufficient high-quality mRNA from small populations of bacteria *in vivo* (37). Using STM only limited numbers of mutants can be screened per animal model. Moreover, mutants that are slow-growing, unviable, contain mutations in genes encoding redundant functions, or that can be complemented in a mixed population may be underrepresented (37). The disadvantages mentioned above for SCOTS and STM do not apply to IVET and R-IVET strategies (37). For reasons mentioned in the introduction we preferred a R-IVET screening in *L. plantarum*. To our knowledge, this is the first R-IVET approach in a food-grade bacterium, which resulted in the identification of 72 genes that are induced *in situ* in *L. plantarum* WCFS1 during passage through the mouse GI-tract. The identified genes appeared to be randomly distributed over the chromosome. Moreover, genes from many functional classes were identified and grouped into 6 functional domains (Table 2).

Nine of the genes identified using R-IVET are involved in sugar transport and utilization (Table 2), including 5 (components of) PTS systems, specific for N-acetylglucosamine, sorbitol, sucrose (2x) and cellobiose, a ribose permease and a ribose kinase, and two di- and polysaccharide hydrolyzing enzymes. A diverse carbohydrate potential has been associated with several gram-positive microbes inhabiting the GI-tract, including *L. plantarum* (31), *Listeria innocua* and *monocytogenes* (22) and *Bifidobacterium longum* (47). The finding that several of these functions are induced *in situ* in the GI-tract confirms their importance for survival and persistence under GI-tract conditions. Moreover, genes involved in the metabolism of the same sugars have been described as being important for

pathogenesis in various bacteria, including *Klebsiella pneumoniae* (ribose, 34), *Salmonella typhi* (ribose, 13), *Pasteurella multocida* (sorbitol, 25), *Listeria monocytogenes* (cellobiose, 20) and *Streptococcus gordonii* (sucrose and cellobiose, 30; Lin Tao, personal communication). Remarkably, the IIC transport component of the cellobiose PTS system found (lp_1164) is not located in a typical PTS- operon structure. In *Listeria monocytogenes* similar "orphan" cellobiose-PTS-IIC components have been shown to play a role in host-specific signaling, leading to modulation of virulence gene expression (33), suggesting host-factor mediated gene regulation in bacteria, possibly including *L. plantarum*.

Nine genes were identified that are involved in the acquisition of non-sugar compounds, including factors involved in acquisition and synthesis of amino acids, nucleotides, cofactors and vitamins (Table 2). These results suggest that limiting amounts of these compounds are available under the starvation conditions in the GI-tract, leading to activation or derepression of these *L. plantarum* genes. Accordingly, *in vitro* studies in different bacteria confirm the induction of several of these metabolic pathways under limiting conditions of specific compounds (12, 36, 44). Moreover, *in vivo* approaches have demonstrated that genes in arginine, biotin and proline metabolism in *Vibrio cholerae* (9), *E. coli* (29) and *Helicobacter pylori* (28), respectively, are induced during mouse infection. In *Listeria monocytogenes*, one of the closest relatives of *L. plantarum*, proline metabolism is induced under high osmolarity conditions (48). Such conditions could potentially be found in the colon and might suggest differential colonic expression of lp_0017. In analogy, other experiments in our laboratory have identified lp_3473, encoding an α-rhamnosidase, as induced by high osmolarity (unpublished observation), suggesting its colonic induction.

The copper transporting ATPase identified here (lp_3055, Table 2) could be involved in copper acquisition. Alternatively, this transporter could act as an exporter, thereby preventing accumulation of copper in the cytoplasm. Arbitrarily, this gene was categorized as a stress-related protein, involved in copper detoxification. Three other genes were categorized as stress-related, namely *clpC*, a multidrug transporter and a cation efflux protein (Table 2). The fact that 3 possible exporters were identified suggests that the efficient transport of toxic compounds is important for GI-tract persistence of *L. plantarum*. Genes important in the transport of metals have been identified in many IVET screens (37), including a heavy metal transporter in *Salmonella typhimurium* possibly involved in Cu^{2+} homeostasis during infection (24). Moreover, in *Helicobacter pylori* an STM strategy revealed two genes encoding multidrug transporters to be essential for gastric colonization of mice (28). In several gram-positive microorganisms *clp* genes were demonstrated to be involved in stress response (15). In *Streptococcus mutants* the *clpC* operon is induced at low pH (35), which could suggest that expression of this gene might be induced in *L. plantarum*

during passage through the mouse stomach. Moreover, mutations in the *ctsR* gene, encoding the *clpC* regulator, in *Listeria monocytogenes* displayed a lower ability to survive the initial stages of murine infection in mice (27).

Four genes encoding extracellular proteins were identified in the R-IVET screen (Table 2), including two proteins (lp_0800 and lp_2940) that contain an LPXTG-like motif (LPQTNE) involved in anchoring them to the bacterial cell wall (42). lp_0141 contains a high number of positive charges which could be involved in the interaction of the encoded protein with the cell wall. No putative binding domains were found in the protein encoded by lp_1403, suggesting secretion of this protein. The bacterial surface is the primary site of interaction with the host and numerous surface exposed adhesion factors have been described (32). Therefore, the surface anchored proteins identified here might represent factors that mediate interaction with host cells in the GI-tract or with components excreted in the GI-tract lumen of the host. lp_0800 encodes a protein that is extremely rich in serine and threonine. For a serine-rich surface protein encoded by *Streptococcus pneumoniae* it has been suggested that the serine residues might be glycosylated by glycosyltransferases that are encoded by genes flanking the surface protein encoding gene (50). These glycosylated serine residues could resemble mucin like structures that coat the bacterial surface or interact with host cell mucins (50). Although no glycosyltransferase encoding genes appear to be genetically linked to lp_0800, a similar role might be fulfilled in *L. plantarum* by the lp_0800 encoded protein.

Three regulators of different families were found to be induced *in vivo* (Table 2). The *bgl* operon in *L. plantarum* was previously shown to be down-regulated in the presence of glucose (39). Therefore, the BglG transcription antiterminator (lp_3514) might be involved in the regulation of the response to the different sugars *L. plantarum* ferments during passage of the GI-tract. Remarkably, among the best homologues of lp_3514 in the *Listeria monocytogenes* genome is the *bvrA* gene (33% identity), which encodes a BglG-family antiterminator involved in regulation of virulence gene expression (5).

Nineteen identified genes are involved in diverse pathways, including DNA and energy metabolism, protein fate and synthesis, and fermentation (Table 2). Several genes in these pathways have previously been described as being important for pathogenesis in various bacteria, including the 1,3-propanediol regulator and a short chain dehydrogenase of *Klebsiella pneumonia* (40), a bifunctional protein possessing alcohol and acetaldehyde dehydrogenase activity in *E. coli* K1 (41) and a DNA polymerase in *Streptococcus gordonii* (30). Another interesting observation is the apparent induction of PlnI, a plantaricin immunity protein (16), suggesting that the production of this bacteriocin is important for *L. plantarum* in the highly competitive environment in the GI-tract.

Finally, 24 hypothetical proteins (16 conserved and 8 unique) apparently play a role during passage of *L. plantarum* through the GI-tract (Table 2). Strikingly, the protein encoded by lp_2718 is a homologue (32% identity) of the only conserved hypothetical protein that was identified with IVET in *L. reuteri* (53; Christian Hertel, personal communication). Although the other putative genes were used for extensive analysis, using the available (R)-IVET literature and blast searches, no significant homologies could be found between these *L. plantarum* hypothetical proteins and other *in vivo* induced genes found in other species. One reason for this could be that a large amount of the nucleotide sequence data obtained for the hypotheticals found in *in vivo* screens in pathogens is not publicly available. The role of the aforementioned hypothetical genes in *L. plantarum* GI-tract persistence remains to be determined.

Overall, a striking amount of parallels can be drawn between the pathogenic and non-pathogenic *in vivo* response, strongly suggesting that survival rather than virulence is the explanation for the importance of these genes during host residence. This suggestion is further corroborated by the fact that the gene encoding peptide methionine sulfoxide reductase has previously been identified using IVET in the food-associated microbe *L. reuteri* during passage of the GI-tract (53) and IVET in the non-food-associated *Streptococcus gordonii* during endocarditis (30). The similarities found for the *L. plantarum* R-IVET screen presented here are most prominent with *in vivo* screens that are performed in the same host (mice) using pathogens that infect the same host-organ (GI-tract), suggesting that organ- and host-specific factors play a key role in the determination of the microbial response. In addition, a number of functions that are *in vivo* induced in *L. plantarum* during passage of the mouse GI-tract, which (so far) have not been identified in similar *in vivo* studies in other bacteria, might contribute to specific interactions between this bacterium and factors encountered in this niche. Moreover, parallels between the *in vivo* study presented here and *in vitro* studies performed in the same bacterium (osmolarity induction, see above) or closely related species (osmolarity induction or low pH, see above) might hint towards spatial differentiation of *L. plantarum* gene expression during passage of the mouse GI-tract, i.e. specific induction in the stomach, small intestine or colon. Interesting in this respect is the recent observation in our laboratory that expression of lp_0237 and lp_0775 is induced by bile (8), suggesting *in vivo* induction in the duodenum, as this is the site of bile release by the host. In conclusion, the R-IVET screen performed in *L. plantarum* is an important step towards understanding the behavior of this food-associated microbe related to stress, persistence, host-microbe- and microbe-microbe-interactions in the complex GI-tract environment.

Acknowledgements

Part of this work was supported by the EU project LABDEL (EU-QLRT-2000-00340). The authors thank Thamara Hesselink, René Klein Lankhorst and Michiel Wels for sequence and blast analysis, Catherine Daniel and Sally Hoffer for fruitful discussions, and Lin Tao, Cormac Gahan and Christian Hertel for sharing unpublished sequence information on their IVET clones.

References

1. **Abremski, K., R. Hoess, and N. Sternberg.** 1983. Studies on the properties of P1 site-specific recombination: evidence for topologically unlinked products following recombination. Cell **32:**1301-11.

2. **Ahrne, S., S. Nobaek, B. Jeppsson, I. Adlerberth, A. E. Wold, and G. Molin.** 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. J. Appl. Microbiol. **85:**88-94.

3. **Angelichio, M. J., and A. Camilli.** 2002. *In vivo* expression technology. Infect. Immun. **70:**6518-23.

4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513-1523.

5. **Brehm, K., M. T. Ripio, J. Kreft, and J. A. Vazquez-Boland.** 1999. The *bvr* locus of *Listeria monocytogenes* mediates virulence gene repression by beta-glucosides. J. Bacteriol. **181:**5024-32.

6. **Bron, P. A., M. G. Benchimol, J. Lambert, E. Palumbo, M. Deghorain, J. Delcour, W. M. de Vos, M. Kleerebezem, and P. Hols.** 2002. Use of the *alr* gene as a food-grade selection marker in lactic acid bacteria. Appl. Environ. Microbiol. **68:**5663-5670 (Chapter 2).

7. **Bron, P. A., S. M. Hoffer, I.I. van Swam, W. M. de Vos, and M. Kleerebezem.** 2004. Selection and characterization of conditionally active promoters in *Lactobacillus plantarum*, using alanine racemase as a promoter probe. Appl. Environ. Microbiol. **70:**310-7 (Chapter 3).

8. **Bron, P. A., M. Marco, S. M. Hoffer, E. van Mullekom, W. M. de Vos, and M. Kleerebezem.** 2004. Genetic characterization of the bile salt response in *Lactobacillus plantarum* and analysis of responsive promoters *in vitro* and *in situ* in the gastrointestinal tract (Chapter 4).

9. **Camilli, A., and J. J. Mekalanos.** 1995. Use of recombinase gene fusions to identify *Vibrio cholerae* genes induced during infection. Mol. Microbiol. **18:**671-83.

10. **Campo, N., M. L. Daveran-Mingot, K. Leenhouts, P. Ritzenthaler, and P. Le Bourgeois.** 2002. Cre-*loxP* recombination system for large genome rearrangements in *Lactococcus lactis*. Appl. Environ. Microbiol. **68:**2359-67.

11. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138:**179-207.

12. **Cronan, J. E., Jr.** 1988. Expression of the biotin biosynthetic operon of *Escherichia coli* is regulated by the rate of protein biotination. J. Biol. Chem. **263:**10332-6.

13. **Daigle, F., J. E. Graham, and R. Curtiss, 3rd.** 2001. Identification of *Salmonella typhi* genes expressed within macrophages by selective capture of transcribed sequences (SCOTS). Mol. Microbiol. **41:**1211-22.

14. **de Vos, W. M., P. Vos, H. de Haard, and I. Boerrigter.** 1989. Cloning and expression of the *Lactococcus lactis* ssp. *cremoris* SK11 gene encoding an extracellular serine protease. Gene **85:**169-176.

15. **Derre, I., G. Rapoport, and T. Msadek.** 1999. CtsR, a novel regulator of stress and heat shock response, controls *clp* and molecular chaperone gene expression in gram-positive bacteria. Mol. Microbiol. **31:**117-31.

16. **Diep, D. B., L. S. Havarstein, and I. F. Nes.** 1996. Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. J. Bacteriol. **178:**4472-83.

17. **Dozois, C. M., F. Daigle, and R. Curtiss, 3rd.** 2003. Identification of pathogen-specific and conserved genes expressed *in vivo* by an avian pathogenic *Escherichia coli* strain. Proc. Natl. Acad. Sci. U.S.A. **100:**247-52.

18. **Ferain, T., D. Garmyn, N. Bernard, P. Hols, and J. Delcour.** 1994. *Lactobacillus plantarum ldhL* gene: overexpression and deletion. J. Bacteriol. **176:**596-601.

19. **Fernández, M., M. Kleerebezem, O. P. Kuipers, R. J. Siezen, and R. van Kranenburg.** 2002. Regulation of the *metC-cysK* operon, involved in sulfur metabolism in *Lactococcus lactis*. J. Bacteriol. **184:**82-90.

20. **Gahan, C. G., and C. Hill.** 2000. The use of listeriolysin to identify *in vivo* induced genes in the gram- positive intracellular pathogen *Listeria monocytogenes*. Mol. Microbiol. **36:**498-507.

21. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:**1-9.

22. **Glaser, P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend, F. Baquero, P. Berche, H. Bloecker, P. Brandt, T. Chakraborty, A. Charbit, F. Chetouani, E. Couve, A. de Daruvar, P. Dehoux, E. Domann, G. Dominguez-Bernal, E. Duchaud, L. Durant, O. Dussurget, K. D. Entian, H. Fsihi, F. G. Portillo, P. Garrido, L. Gautier, W. Goebel, N. Gomez-Lopez, T. Hain, J. Hauf, D. Jackson, L. M. Jones, U. Kaerst, J. Kreft, M. Kuhn, F. Kunst, G. Kurapkat, E. Madueno, A. Maitournam, J. M. Vicente, E. Ng, H. Nedjari, G. Nordsiek, S. Novella, B. de Pablos, J. C. Perez-Diaz, R. Purcell, B. Remmel, M. Rose, T. Schlueter, N. Simoes, A. Tierrez, J. A. Vazquez-Boland, H. Voss, J. Wehland, and P. Cossart.** 2001. Comparative genomics of *Listeria* species. Science **294:**849-52.

23. **Grangette, C., H. Muller-Alouf, M. Geoffroy, D. Goudercourt, M. Turneer, and A. Mercenier.** 2002. Protection against tetanus toxin after intragastric administration of two recombinant lactic acid bacteria: impact of strain viability and *in vivo* persistence. Vaccine **20:**3304-9.

24. **Heithoff, D. M., C. P. Conner, P. C. Hanna, S. M. Julio, U. Hentschel, and M. J. Mahan.** 1997. Bacterial infection as assessed by *in vivo* gene expression. Proc. Natl. Acad. Sci. U.S.A. **94:**934-9.

25. **Hunt, M. L., D. J. Boucher, J. D. Boyce, and B. Adler.** 2001. *In vivo*-expressed genes of *Pasteurella multocida*. Infect. Immun. **69:**3004-12.

26. **Josson, K., T. Scheirlinck, F. Michiels, C. Platteeuw, P. Stanssens, H. Joos, P. Dhaese, M. Zabeau, and J. Mahillon.** 1989. Characterization of a gram-positive broad-host-range plasmid isolated from *Lactobacillus hilgardii*. Plasmid **21:**9-20.

27. **Karatzas, K. A., J. A. Wouters, C. G. Gahan, C. Hill, T. Abee, and M. H. Bennik.** 2003. The CtsR regulator of *Listeria monocytogenes* contains a variant glycine repeat region that affects piezotolerance, stress resistance, motility and virulence. Mol. Microbiol. **49:**1227-38.

28. **Kavermann, H., B. P. Burns, K. Angermuller, S. Odenbreit, W. Fischer, K. Melchers, and R. Haas.** 2003. Identification and characterization of *Helicobacter pylori* genes essential for gastric colonization. J. Exp. Med. **197:**813-22.

29. **Khan, M. A., and R. E. Isaacson.** 2002. Identification of *Escherichia coli* genes that are specifically expressed in a murine model of septicemic infection. Infect. Immun. **70:**3404-12.

30. **Kiliç, A. O., M. C. Herzberg, M. W. Meyer, X. Zhao, and L. Tao.** 1999. Streptococcal reporter gene-fusion vector for identification of *in vivo* expressed genes. Plasmid **42:**67-72.

31. **Kleerebezem, M., J. Boekhorst, R. Van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. De Vries, B. Ursing, W. M. De Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U.S.A. **100:**1990-1995.

32. **Klemm, P., and M. A. Schembri.** 2000. Bacterial adhesins: function and structure. Int. J. Med. Microbiol. **290:**27-35.

33. **Kreft, J., and J. A. Vazquez-Boland.** 2001. Regulation of virulence genes in *Listeria*. Int. J. Med. Microbiol. **291:**145-57.

34. **Lai, Y. C., H. L. Peng, and H. Y. Chang.** 2001. Identification of genes induced *in vivo* during *Klebsiella pneumoniae* CG43 infection. Infect. Immun. **69:**7140-5.

35. **Lemos, J. A., and R. A. Burne.** 2002. Regulation and Physiological Significance of ClpC and ClpP in *Streptococcus mutans*. J. Bacteriol. **184:**6357-66.

36. **Mack, M., A. P. van Loon, and H. P. Hohmann.** 1998. Regulation of riboflavin biosynthesis in *Bacillus subtilis* is affected by the activity of the flavokinase/flavin adenine dinucleotide synthetase encoded by *ribC*. J. Bacteriol. **180:**950-5.

37. **Mahan, M. J., D. M. Heithoff, R. L. Sinsheimer, and D. A. Low.** 2000. Assessment of bacterial pathogenesis by analysis of gene expression in the host. Annu. Rev. Genet. **34:**139-164.

38. **Mahan, M. J., J. M. Slauch, and J. J. Mekalanos.** 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259:**686-8.

39. **Marasco, R., I. Salatiello, M. De Felice, and M. Sacco.** 2000. A physical and functional analysis of the newly-identified *bglGPT* operon of *Lactobacillus plantarum*. FEMS Microbiol. Lett. **186:**269-73.

40. **Maroncle, N., D. Balestrino, C. Rich, and C. Forestier.** 2002. Identification of *Klebsiella pneumoniae* genes involved in intestinal colonization and adhesion using signature-tagged mutagenesis. Infect. Immun. **70:**4729-34.

41. **Martindale, J., D. Stroud, E. R. Moxon, and C. M. Tang.** 2000. Genetic analysis of *Escherichia coli* K1 gastrointestinal colonization. Mol. Microbiol. **37:**1293-305.

42. **Mazmanian, S. K., G. Liu, H. Ton-That, and O. Schneewind.** 1999. *Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall. Science **285:**760-3.

43. **Mecsas, J.** 2002. Use of signature-tagged mutagenesis in pathogenesis studies. Curr. Opin. Microbiol. **5:**33-7.

44. **Muse, W. B., C. J. Rosario, and R. A. Bender.** 2003. Nitrogen regulation of the *codBA* (cytosine deaminase) operon from *Escherichia coli* by the nitrogen assimilation control protein, NAC. J. Bacteriol. **185:**2920-6.

45. **Renault, P., G. Corthier, N. Goupil, C. Delorme, and S. D. Ehrlich.** 1996. Plasmid vectors for gram-positive bacteria switching from high to low copy number. Gene **183:**175-82.

46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

47. **Schell, M. A., M. Karmirantzou, B. Snel, D. Vilanova, B. Berger, G. Pessi, M. C. Zwahlen, F. Desiere, P. Bork, M. Delley, R. D. Pridmore, and F. Arigoni.** 2002. The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. Proc. Natl. Acad. Sci. U.S.A. **99:**14422-7.

48. **Sleator, R. D., C. G. Gahan, and C. Hill.** 2001. Identification and disruption of the *proBA* locus in *Listeria monocytogenes*: role of proline biosynthesis in salt tolerance and murine infection. Appl. Environ. Microbiol. **67:**2571-7.

49. **Steidler, L., W. Hans, L. Schotte, S. Neirynck, F. Obermeier, W. Falk, W. Fiers, and E. Remaut.** 2000. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin- 10. Science **289:**1352-1355.

50. **Tettelin, H., K. E. Nelson, I. T. Paulsen, J. A. Eisen, T. D. Read, S. Peterson, J. Heidelberg, R. T. DeBoy, D. H. Haft, R. J. Dodson, A. S. Durkin, M. Gwinn, J. F. Kolonay, W. C. Nelson, J. D. Peterson, L. A. Umayam, O. White, S. L. Salzberg, M. R. Lewis, D. Radune, E. Holtzapple, H. Khouri, A. M. Wolf, T. R. Utterback, C. L. Hansen, L. A. McDonald, T. V. Feldblyum, S. Angiuoli, T. Dickinson, E. K. Hickey, I. E. Holt, B. J. Loftus, F. Yang, H. O. Smith, J. C. Venter, B. A. Dougherty, D. A. Morrison, S. K. Hollingshead, and C. M. Fraser.** 2001. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. Science **293:**498-506.

51. **van Kranenburg, R., J. D. Marugg, S. van, II, N. J. Willem, and W. M. de Vos.** 1997. Molecular characterization of the plasmid-encoded *eps* gene cluster essential for exopolysaccharide biosynthesis in *Lactococcus lactis*. Mol. Microbiol. **24:**387-97.

52. **Vesa, T., P. Pochart, and P. Marteau.** 2000. Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. Aliment. Pharmacol. Ther. **14:**823-8.

53. **Walter, J., N. C. Heng, W. P. Hammes, D. M. Loach, G. W. Tannock, and C. Hertel.** 2003. Identification of *Lactobacillus reuteri* genes specifically induced in the mouse gastrointestinal tract. Appl. Environ. Microbiol. **69:**2044-51.

54. **West, N. P., P. J. Sansonetti, G. Frankel, and C. M. Tang.** 2003. Finding your niche: what has been learnt from STM studies on GI colonization. Trends Microbiol. **11:**338-44.

55. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved m13 phage cloning vectors and host strains; nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103-119.

Chapter 7

Competitive Population Dynamics of *ivi*-Gene Mutants of *Lactobacillus plantarum* in the Gastrointestinal Tract of Mice

PETER A. BRON,[1,2] MIEKE MEIJER,[1,2] ROGER BONGERS,[1,2]
WILLEM M. DE VOS,[1] AND MICHIEL KLEEREBEZEM.[1,2]

[1] Wageningen Centre for Food Sciences, [2] NIZO food research, P.O. Box 20, 6710 BA Ede, The Netherlands.

Manuscript in preparation.

Abstract

***In vivo* expression technology (IVET) and its resolvase-based variant (R-IVET) have been applied frequently for the identification of genes in pathogenic bacteria that are differentially expressed *in vivo* during infection. Recently, the first R-IVET screen in a food-grade organism, *Lactobacillus plantarum* WCFS1, was performed in our laboratory. This screen resulted in the identification of 72 genes that are *in vivo* induced (*ivi*) during passage of the gastrointestinal tract (GI-tract) as compared to laboratory conditions (3). Here, we have selected 12 of these 72 *L. plantarum ivi* genes, mainly focusing on genes that encode proteins with a predicted role in cell envelope functionality, stress response and regulation, for the construction of gene replacement mutants. The growth characteristics of the constructed *ivi* gene replacement mutants on MRS and chemically defined medium appeared highly similar to that of the wild-type strain, which is in agreement with the R-IVET-based suggestion that these genes are not transcribed in the laboratory. Quantitative PCR (qPCR) experiments were performed to monitor the relative population dynamics of the group of *L. plantarum cat*-replacement mutants in faecal samples after passage through the GI-tract of mice, in competition with each other and a wild-type control strain NZ7109. These competition experiments indicated that after GI-tract passage the abundance of the total mutant group is drastically decreased relative to the control strain. Moreover, the relative abundance of individual *ivi* gene mutants appeared to be highly variable, and led to the identification of three *ivi* gene mutants that displayed 100- to 1000-fold reduced abundance as compared to other mutant strains.**

Introduction

Three main strategies have been developed for the identification of genes that are either highly expressed, differentially expressed or specifically required *in vivo*, namely (recombination-based) *in vivo* expression technology ((R-)IVET), signature tagged mutagenesis (STM), and selective capture of transcribed sequences (SCOTS). The basic characteristics and relative (dis)advantages of these approaches have been addressed previously (Chapter 1) and will therefore not be discussed in detail here. Particularly the different variants of the (R-)IVET approach have been applied to a large number of pathogenic bacteria, including *Escherichia coli , Salmonella enterica*, and *Klebsiella pneumoniae* (1, 20). The number of genes identified as *in vivo* induced (*ivi*) in these (R-)IVET screens is extremely variable and ranges from 1 to more than 100 (20). After the initial identification of these *ivi* genes, quantitative reverse transcriptase PCR (qRT-PCR) has been used during follow-up experiments, allowing detailed analysis of the spatial and temporal expression patterns of (a subset of) these genes. In *E. coli* and *Lactobacillus plantarum* qRT-PCR experiments have revealed specific induction of gene expression in the liver and duodenum, respectively (4, 16). Another frequently applied follow-up strategy with *ivi* genes identified in pathogenic bacteria is the construction of gene deletion mutants followed by assessment of the relative virulence of the constructed mutants as compared to the wild-type strain (21, 30). In addition, several studies report the comparative abundance of the mutants in infected tissues relative to the wild-type strain, as determined by selective plate counts of both strains (10, 12, 19). These gene disruptions strategies have confirmed the importance of many *ivi* genes during the infection process of several pathogens, including *Staphylococcus aureus* (19), *Pseudomonas aeruginosa* (30), *Salmonella typhimurium* (20), *Yersinia enterocolitica* (12), and *Listeria monocytogenes* (10).

L. plantarum is a flexible and versatile lactic acid bacterium that inhabits a variety of environmental niches, including the human gastrointestinal tract (GI-tract). *L. plantarum* WCFS1 is a single colony isolate of strain NCIMB 8826, which displayed high activity and survival rates in the human intestine in pharmacokinetic experiments (29). To investigate the genetic events during this persistence, a R-IVET screen was performed in our laboratory, using the mouse GI-tract as a model system. This approach identified 72 *L. plantarum* WCFS1 genes of which the expression is induced during passage of the GI-tract as compared to laboratory media (3).

This paper describes the construction of *L. plantarum* gene replacement mutants of 12 of the 72 *ivi* genes that were identified using R-IVET. Growth of these mutants on MRS and chemically defined medium was investigated, revealing that all mutants displayed highly similar growth characteristics as compared to the wild-type strain. Equal amounts of cells of the individual chloramphenicol resistant mutants and the control strain NZ7109, which harbors a chromosomal copy of an erythromycin-resistance cassette (3), were administered to three mice and their relative population

dynamics were investigated by quantitative PCR (qPCR). Relative to the population ratios observed in the mixed bacterial population administered to the mice, the abundance of the mutant group as a whole appeared significantly reduced relative to the control strain in faecal samples. Moreover, qPCR analysis of the relative population dynamics within the mutant group revealed that the relative abundance of several of the mutants was significantly reduced in faecal samples as compared with the initial mutant population ratios administered to the mice.

Material and Methods

Bacterial strains, media and growth conditions

The parent strains and plasmids that were used in this study are listed in Table 1. The regular primers, the primers for *cat*-replacement mutant construction, and the primers and probes used for qPCR are presented in Tables 1, 2 and 3, respectively. The *L. plantarum* mutants that were constructed are listed in Table 4. *E. coli* strain MC1061 (6) was used as cloning hosts during construction of the pNZ7101 derived gene replacement vectors (see below). *E. coli* was grown aerobically in TY medium (25). *L. plantarum* WCFS1 (17), NZ7109 (3) and the gene disruption mutants were grown at 37 ^{o}C in MRS (Difco, Surrey, U.K.) or chemically defined medium (CDM)(23) without aeration. When appropriate, erythromycin (5 μg/ml or 30 μg/ml for primary selection or replica-plating, respectively) or chloramphenicol were added to the media (8 and 5 μg/ml for *E. coli* and *L. plantarum*, respectively).

Table 1: Strains and plasmids used in this study, and primers for the construction of pNZ7101.

Material	Relevant features[a]	Source or reference
Strains		
E.coli MC1061	cloning host	(6)
L.plantarum WCFS1	wild-type for which the genome sequence is available	(17)
L. plantarum NZ7109	Rif^R Em^R, *L. plantarum* WCFS1 containing chromosomal *ery* in neutral locus	(3)
Plasmids		
pNZ84	Cm^R, pACYC184 derivative	(26)
pGIZ850	Cm^R, contains P32-*cat* selectable as single copy in the chromosome of *L. plantarum*	Goffin and Hols, unpublished data
pUC18Ery	Amp^R Em^R	(28)
pNZ7101	Cm^R Em^R, vector for construction of *L. plantarum* gene replacement mutants	this study
Primers		
pNZ84F	5'-CGGGATCCCAACAGTACTGCGATGAG-3'	
pNZ84R	5'-GGGGTACCATCCAGTGATTTTTTTCTCC-3'	

[a] Ap^R, ampicillin resistant; Em^R, erythromycin resistant; Cm^R, chloramphenicol resistant; Rif^R, rifampicin resistant

Table 2: Primers used during construction of the *L. plantarum cat*-replacement mutants.

target locus	Forward primer	Reverse primer
5'-lp_0190	5'-TACGAAAACTAAAGGC-3'	5'-ATGAACAACTACCGTTTAAC-3'
3'-lp_0190	5'-TCTTCAAATACGGTAGGTCC-3'	5'-TTGCCTTAACTAAGATCGCC-3
5'-lp_0237	5'-TTTTGGATGTCGGGACCTTA-3'	5'-ATTGCCATTTTCAGCACATG-3'
3'-lp_0237	5'-CAGATAGGGTGTTGTCATAA-3'	5'-GTTTAGCCGGAATCTTCATC-3'
5'-lp_0682	5'-GTAGAATCGGCTGAAGAACA-3'	5'-CCCATTCATCCCAACCTAAT-3'
3'-lp_ 0682	5'-ACTCAGATCAATTACACCCA-3'	5'-CAACGTATATACGTTCAGGC-3'
5'-lp_1019	5'-GGAGAAATTAGTCTCAGAAC-3'	5'-TGAAATATTTGGCCTGTTCC-3'
3'-lp_1019	5'-TTAAAACCAGCCTAAGGCAG-3'	5'-TGTAGTTGCATGTCAGGTTG-3'
5'-lp_1164	5'-TCATAGCACAACTGGATAGG-3'	5'-GACCCGTTCAAAGGTCAATA-3'
3'-lp_1164	5'-TTGGCAAACCAGGTTGAGGA-3'	5'-CGTTTCAGCCAGTAAGTACT-3'
5'-lp_1403	5'-CTTTGACCATCGTTTTAGCA-3'	5'-TGGCTCTATGAAAACATCTC-3'
3'-lp_1403	5'-TAAGCTAATTGCGCCAATCG-3'	5'-GATTTTGCGACTTGGCAGAC-3'
5'-lp_2940	5'-GCTAAACTAAAAAACGGTCG-3'	5'-GGTTTTAGCAAGCAACAACG-3'
3'-lp_2940	5'-TAACATGGTGATTCCCATCA-3'	5'-TATCAGGATGCTGAGTATCA-3'
5'-lp_3055	5'- TCCCAAGCGACGTAAATCTA– 3'	5'-GTAGTCACGAGTTCGGTATTG-3'
3'-lp_3058	5'-CCCAATTGCTAACCCACCAA– 3'	5'-AACCAAGTCAACCAGAAGAG-3'
5'-lp_3281	5'-CGATTAAAGTACCAGACGAC-3'	5'-GTGACGTCTCAAATAGCGAA-3'
3'-lp_3281	5'-GTACAAGTCAACGTCATCCC-3'	5'-CGCGACTTTGCCAATAAGTT-3'
5'-lp_3514	5'-ACGACAATACCCAAACTGCC-3'	5'-ATCATGCACATTCAGCGGAT-3'
3'-lp_3514	5'-TTCAGCCAATAGGACGTTAT-3'	5'-AAGGAACAGTTAGAATCACG-3'
5'-lp_3646	5'-GGATGGATGAAGCCTATCAG-3'	5'-AGCAAGGTATTCAAGCGGTA-3'
3'-lp_3646	5'-GGCGTAAAGAACTTCCGGTT-3'	5'-CATGATTGAGTTGCTGGAAC-3'
5'-lp_3659	5'-TTGGTAGTCTTCTTTTGGCC-3'	5'-ACTGGTGAAATGACACCTTA-3'
3'-lp_3660	5'-GTTGATACTCCCAATAACGG-3'	5'-GAATCCCTTTTTTGGTGAGC-3'

Table 3: Primers used to check chromosomal integration events, and primers and probes used during qPCR.

Primer	sequence
Integration controls	
HTPcmF	5'-TAGTGACAAGGGTGATAAAC-3'
HTPcmR	5'- TCCTGACCACCATTATCAAG-3'
HTPeryF	5'-GATACCGTTTACGAAATTGG -3'
HTPeryR	5'- CCCATTTTGAAACAAAGTAC -3'
SmaI- ori	5'-TTAAGAAGATACTGGCCGAA-3'
PvuII-ori	5'-TAGTAAGCCAGTATACACTC-3'
PvuII- SCO	5'-TGATGGTGTTTTTGAGGTGC-3'
eryF	5'-AAGCAATGAAACACGCC-3'
qPCR primers	
TM-cat-F96	5'-TCAAATACAGCTTTTAGAACTGG-3'
TM-cat-R97	5'-ACCATCAAAAATTGTATAAAGTGGC-3'
TM-ery-F99	5'-TTCACCGAACACTAGGGTTGC-3'
TM-ery-R100	5'-CATTCCGCTGGCAGCTTAAG-3'
uniF	5'- CCGAGCCTAAGGAACAGGC-3'
qPCR_0190	5'- AATTTGGGGGACCTACCGTAT-3'
qPCR_0237	5'- AAGCTGCCCATGTGCTGAAAA-3'
qPCR_1019	5'- ACGACCATCTGCCTTAGGCT-3'
qPCR_1164	5'- CCTCCTCCTCAACCTGGTTT -3'
qPCR_1403	5'- TGGGTCACGATTGGCGCAAT-3'
qPCR_2940	5'-TAAGATAGTGATGGGAATCACC-3'
qPCR_3055	5'- ACTCGTGACTACGGACAATGA-3'
qPCR_3281	5'- TTTAAGCGGGATGACGTTGAC-3'
qPCR_3514	5'- TAATAATAACGTCCTATTGGCTG-3'
qPCR_3646	5'- GTGAATTTGAACCGGAAGTTCT-3'
qPCR_3659	5'- ACGGTAACCGTTATTGGGAGT-3'
qPCR_0682	5'-CATTTAACTGGGTGTAATTGATC-3'
qPCR probes	
TM-cat-VIC98	5'-VIC-GCGACGGAGAGTTAGGTTATTGGG-TAMRA-3'
TM-ery-FAM101	5'-FAM-TGCACACTCAAGTCTCGATTCAGCA-TAMRA-3'
qPCR-MM	5'-FAM-CTAGAACAATTACGGCGCGATATGC-TAMRA-3'

Table 4: Overview of the constructed *L. plantarum* mutant strains, the functions of the replaced ORFs and their maximal growth rate on MRS and CDM media.

Strain	Replaced ORF(s) (genes)	Removed function(s)	μ_{max} (h^{-1}) on MRS	μ_{max} (h^{-1}) on CDM
L. plantarum WCFS1			0.80 ± 0.02	0.19 ± 0.02
L. plantarum Δlp_0190	lp_0190	conserved hypothetical	0.83 ± 0.17	0.17 ± 0.01
L. plantarum Δlp_0237[a]	lp_0237	integral membrane protein	0.82 ± 0.06	0.18 ± 0.02
L. plantarum Δlp_0682[b]	lp_0682 and lp_0683	prophage P1 protein 59 and 60	0.96 ± 0.10	0.22 ± 0.02
L. plantarum Δlp_1019	lp_1019 (*clpC*)	ATP-dependent Clp protease	0.76 ± 0.11	0.18 ± 0.01
L. plantarum Δlp_1164	lp_1164 (*pts14C*)	cellobiose PTS, EIIC	0.87 ± 0.03	0.21 ± 0.01
L. plantarum Δlp_1403	lp_1403	cell surface protein	0.87 ± 0.14	0.22 ± 0.02
L. plantarum Δlp_2940	lp_2940	cell surface protein precursor	0.91 ± 0.04	0.23 ± 0.01
L. plantarum Δlp_3055[a]	lp_3055 (*copA*), lp_3057 and lp_3058	copper transporting ATPase and conserved hypothetical (2x)	0.84 ± 0.08	0.22 ± 0.01
L. plantarum Δlp_3281	lp_3281	transport protein	0.90 ± 0.16	0.19 ± 0.02
L. plantarum Δlp_3514	lp_3514 (*bglG4*)	transcription antitermination	0.85 ± 0.05	0.21 ± 0.01
L. plantarum Δlp_3646	lp_3646	transcription regulator	0.91 ± 0.12	0.21 ± 0.02
L. plantarum Δlp_3659	lp_3659 (*rbsD*) and lp_3660 (*rbsK3*)	ribose transport protein and ribokinase	0.81 ± 0.04	0.21 ± 0.02

[a] In these strains the chloramphenicol gene is transcribed in the opposite orientation as compared to the original gene

[b] The corresponding clone identified as *in vivo* induced in the R-IVET procedure harbored lp_0682 and lp_0683 in the wrong orientation to drive expression of the reporter gene (3)

DNA techniques

Plasmid DNA was isolated from *E. coli* on a small scale using the alkaline lysis method (2, 25). Large scale plasmid DNA isolations were performed using Jetstar columns according to the manufacturer's instructions (Genomed GmbH, Bad Oberhausen, Germany). DNA isolation and transformation in *L. plantarum* were performed as described previously (7, 15). Standard procedures were applied for DNA manipulations in *E. coli* (25). Restriction endonucleases, *Taq* and *Pfx* polymerase, and high-concentration T4 DNA ligase were used following the recommendations of the manufacturer (Promega, Leiden, the Netherlands and Boehringer, Mannheim, Germany). Primers were purchased from Pharmacia Biotech (Roosendaal, the Netherlands).

Plasmid construction and gene replacement in *L. plantarum*

An efficient method was developed for the construction of relatively large numbers of gene replacement mutants in *L. plantarum*. For this purpose the vector pNZ7101 was constructed that allows single-step selection of *L. plantarum* gene replacement mutants. The pACYC184-derived origin of replication (24) was PCR amplified as a 2.0 kb fragment using *Pfx* polymerase, with the primers pNZ84F and pNZ84R (Table 1), and pNZ84 (26) as a template. The obtained amplicon was ligated with the 1.5 kb *Nae*I fragment of pGIZ850 (Philippe Goffin and Pascal Hols, UCL laboratory collection, Louvain, Belgium, unpublished data) that contains the *cat*-194 gene (14) under control of the lactococcal P32 promoter (27). The resulting plasmid was digested with *Sma*I and *Sph*I, and ligated to a 1.1 kb *Sma*I-*Sph*I fragment of pUC18Ery (28), which contains the erythromycin-resistance encoding *ery* gene. The resulting vector was designated pNZ7101 and is a suitable mutagenesis vector that harbors two selectable markers (*ery* and *cat*) that can both be selected at single-copy expression level in the *L. plantarum* chromosome (Fig. 1).

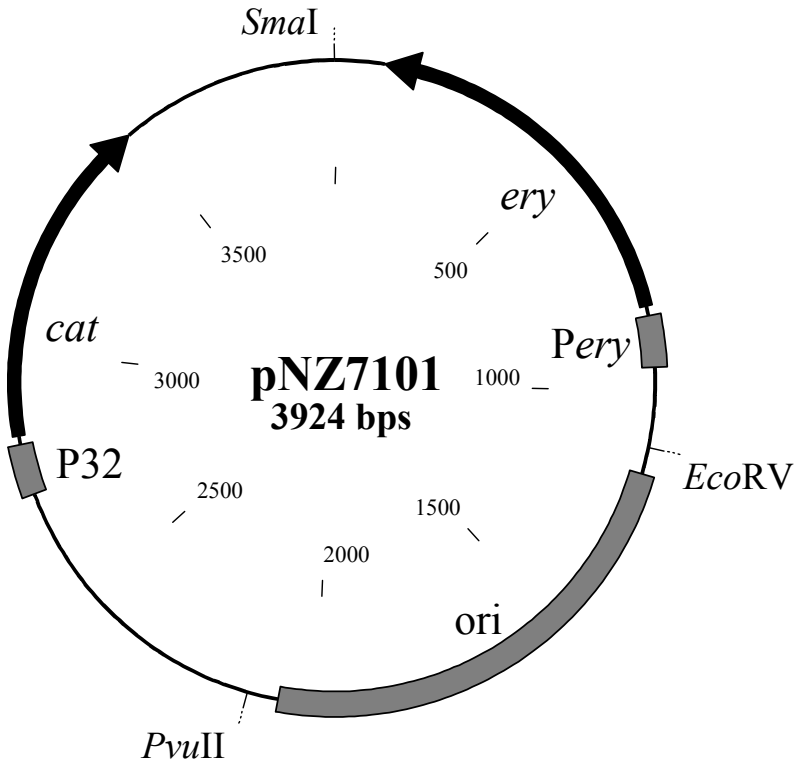


Fig. 1: Schematic representation of the mutagenesis vector pNZ7101 that allows single-step selection of *cat*-gene replacement mutants in *L. plantarum*.

pNZ7101 was utilized for the construction of 12 *cat*-replacement mutants in *L. plantarum* WCFS1. As a first step, the 24 5'- and 3'- flanking regions of the 12 target genes were amplified using *Pfx* polymerase, locus specific primers (Table 2), and chromosomal DNA of *L. plantarum* WCFS1 as template. The resulting amplicons were glass matrix purified (Amersham Pharmacia Biotech Inc) and the 5'-flanking regions were cloned in *Pvu*II digested pNZ7101. The ligation mixtures were digested with *Pvu*II prior to transformation to *E. coli* MC1061 (6) to reduce the number of transformants harboring backligated vector DNA. After 48 h, full-grown colonies were used directly as templates in a PCR reaction using primer PvuII-ori and the corresponding mutant specific 5'R primer (Table 3 and 2, respectively). This PCR reaction only generates a product when an insert is cloned in the appropriate orientation. Using this approach, 12 clones were selected that harbor a PNZ7101 derivative containing the gene-specific 5'-flanking region. Plasmid DNA derived from these clones was digested with *Sma*I and ligated to the PCR-amplicon of the corresponding 3'-flanking region. The procedure used was essentially the same as that described for the 5'-flanking regions, including *Sma*I digestion of the ligation mixtures prior to transformation, and orientation-specific PCR-based clone selection using primers SmaI-ori and the locus-specific 3'R primer (Table 3 and 2, respectively). This approach resulted in the construction of 10 plasmids that harbor the chloramphenicol resistance gene flanked by the 5'- and 3'-flanking region of 10 *ivi* target genes. For 2 target genes (lp_0237 and lp_3055) cloning of the 3' flanking region was unsuccessful. Therefore, the 5'- and 3'-flanking regions of these genes were cloned in pNZ7101 in the opposite orientation (5'- and 3'-flanking regions cloned in *Sma*I and *Pvu*II, respectively). Using this approach the 2 *cat*-gene replacement vectors for lp_0237 and lp_3055 could be obtained. Notably, all 12 plasmids constructed were subjected to extensive restriction and PCR analyses, confirming their anticipated organization (data not shown).

A double cross-over chloramphenicol replacement strategy was used for the stable *cat*-gene replacement of the 12 selected *ivi* genes. The mutagenesis plasmids were transformed to *L. plantarum* WCFS1 and integrants were selected on MRS plates containing 5 µg/ml chloramphenicol. After 48 h, full-grown colonies were replica-plated to MRS plates containing 5 µg/ml chloramphenicol, with or without 30 µg/ml erythromycin. Colonies displaying an erythromycin sensitive phenotype represent candidate *cat*-gene replacements that result from a simultaneous double cross-over event in both the 5'- and the 3'-flanking regions of the *ivi* gene. The anticipated *cat*-replacement genotype of these candidate mutants was verified by five PCR reactions using chromosomal DNA of the mutants as template. These reactions include a multiplex PCR using the primer pairs HTPcmF plus HTPcmR, and HTPeryF plus HTPeryR to confirm the presence of the *cat* gene and the absence of the *ery* gene in the chromosomes of the mutants, respectively (Table 3). Moreover, the absence of

plasmid-derived DNA sequences was investigated using the primer combinations SmaI-ori and eryF, and PvuII-ori and PvuII-SCO (Table 3). Finally, the conformation of the *cat*-integration loci was checked using the primer pairs PvuII-ori combined with the corresponding mutant specific 5'R primer, and SmaI-ori combined with the corresponding 3'R primer (Table 2 and 3). Using these PCR reactions the anticipated genotype of the 12 *cat*-replacement mutant strains could be confirmed (Table 4).

Maximal growth rate determination

The maximal growth rates of *L. plantarum* WCFS1 and its *cat*-replacement mutant derivatives on MRS and chemically defined medium (CDM) medium (23) were determined. Full-grown cultures were diluted 100-fold in fresh media in a 96-well plate and growth was monitored by automatic OD_{600} measurements using an SPECTRAmax® PLUS^{384} microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, USA). All experiments were performed in triplicate and the obtained data were used to calculate the maximal logarithmic growth rate of all strains in MRS and CDM.

Animal experiments

The animal experiments were performed at the Institute Pasteur in Lille in an accredited establishment (N° A59107) according to guidelines N°86/609/CEE of the French government. Seven weeks-old female Balb/c mice were purchased from Iffa Credo (St Germain sur l'Arbresle, France) and had free access to tap water and standard mice chow during the experiments. To compare the *in vivo* persistence of the *L. plantarum* NZ7109 control strain (3) and the 12 *ivi* gene mutants in a competitive model, equal amounts of overnight cultures of all bacterial strains were mixed, pelleted by centrifugation, and resuspended at approximately 10^{10} CFU (total population) per ml in MRS. Three mice received a 100 µl oral dose of the bacterial suspension by intragastric administration at 2 consecutive days (day –1 and 0). Individual faecal samples were collected daily during 6 days following the last administration (day 1 to 6) and the samples were stored at –80 ^{o}C until they were further processed.

DNA isolation from faecal samples

DNA was isolated from the mixed input populations administered to the mice, and from faecal samples essentially as described before (31). Approximately 10^{8} CFU from the mixed input population or 0.1 g of faecal sample were resuspended in 0.4 ml TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0) containing 10 µg/ml RNase. After thorough homogenization, 50 µl 10% SDS and 10 µl proteinase K solution (20 mg/ml) were added, and the resulting suspensions were incubated for 1 h at 65 ^{o}C. Subsequently, 0.5 g of zirconium glass beads, 0.5 ml phenol and 200 µl chloroform

were added and the cells were disrupted using two treatments of 40 seconds in a Fastprep™ (Qbiogene Inc, Cedex, France) interspaced by 1 minute on ice. After centrifugation, the aqueous phase was used for DNA isolation using several phenol-chloroform extractions, followed by precipitation of the DNA using 1 volume of isopropanol. Subsequently, the pellets were washed with 70 % (v/v) ethanol, and dissolved in 200 µl TE buffer by overnight incubation at 4 ^{o}C.

Quantitative PCR analysis

To compare the relative population dynamics of the erythromycin-resistant control strain *L. plantarum* NZ7109 (3) and the group of chloramphenicol resistant *L. plantarum* replacement mutants, a quantitative PCR (qPCR) was performed to determine the *cat:ery* gene ratio in DNA derived from mixed input populations and faecal samples at different time points. Each 50 µl amplification reaction contained 5 µl of the isolated total DNA, 1x Taqman Universal PCR master mix (Applied Biosystems), 200 nM of primers TM-cat-F96, TM-cat-R97, TM-ery-F99, TM-ery-R100, and 200nM of the Taqman probes TM-cat-VIC98 and TM-ery-FAM101 (Table 3 and Fig. 2). Reactions were performed using an ABI Prism 7700 PCR machine (Applied Biosystems, Nieuwekerk a/d IJsel, the Netherlands). Reactions were initiated at 95 °C for 10 minutes, followed by 40 amplification cycles of 95 °C for 15s and 55 °C for 60s. Cycle threshold (C_t) values were obtained upon manual setting of the baseline at a threshold value at which fluorescence was appreciably above background fluorescence for each reaction and within the exponential phase of amplification for all reactions. Reactions were performed in triplicate and the reciprocal of the 2-power of the C_t values was used as a measure for the absolute amount of *cat* and *ery* templates. The ratios between these two absolute values for *cat* and *ery* were taken as the mutant:wild-type ratio. To compare the relative *in vivo* abundance of all constructed *L. plantarum cat*-replacement mutants, essentially the same qPCR conditions were used. However, each reaction contained 200 nM of uniF primer, mutant specific qPCR primer and the Taqman qPCR-MM probe (Table 3 and Fig. 2). The mutant-specific C_t values were used to calculate the relative abundance per mutant compared to the overall mutant population size in the DNA samples, which were calculated from the C_t values obtained by the *cat*-specific PCR on the same faecal samples, as described above.

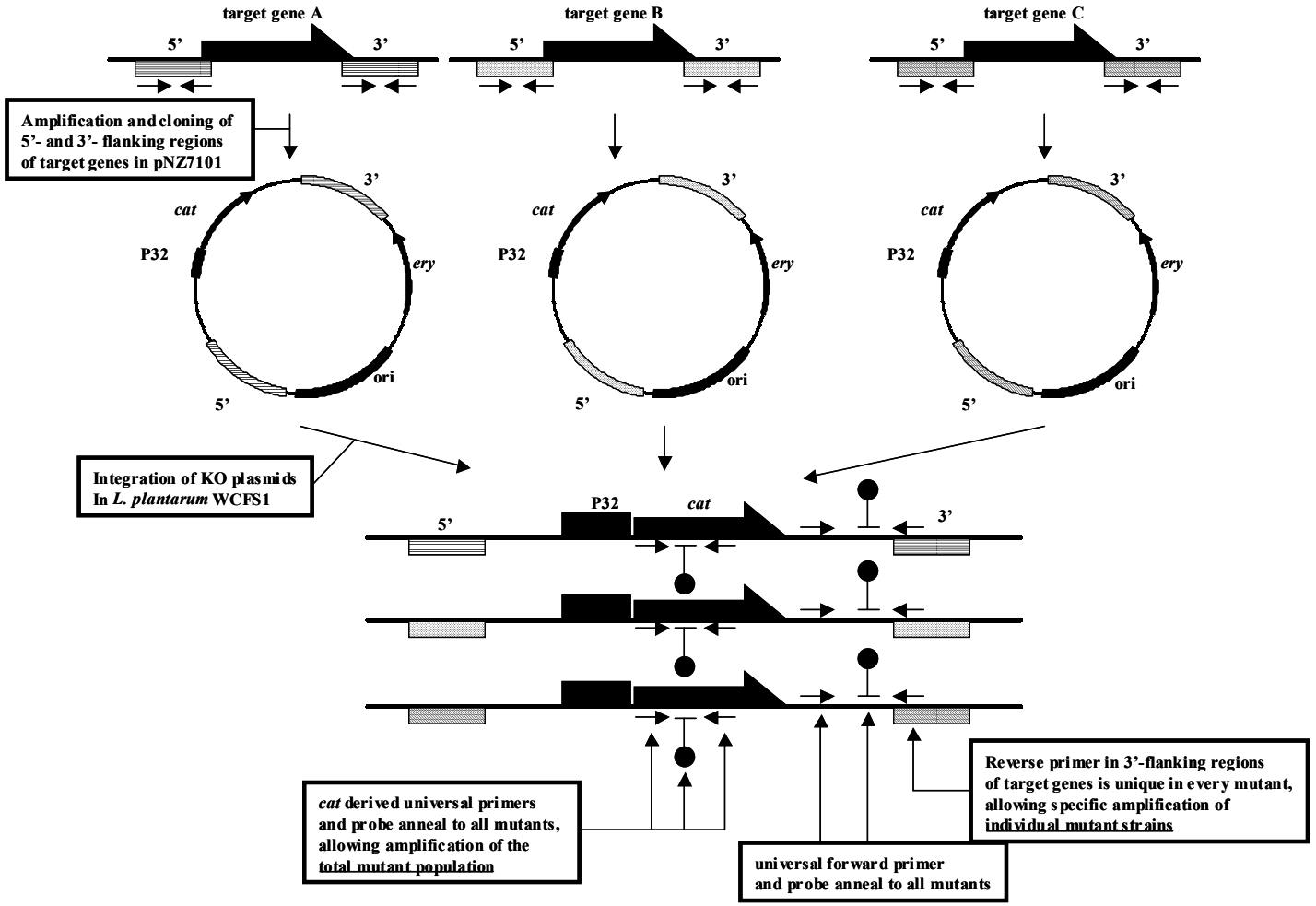


Fig. 2: Schematic overview of the strategy that was employed to construct multiple *cat*-replacement mutants in *L. plantarum*, and the design of two qPCR approaches that allows quantitative amplification of the total mutant population or quantitative amplification of individual mutants strains.

Results

Gene replacements and *in vitro* growth characteristics

Previously we have described the identification of 72 *L. plantarum* genes that are induced during gastrointestinal tract passage in mice as compared to laboratory conditions (3). 12 of these *ivi* genes were selected, of which the majority encodes proteins with a predicted role in cell envelope functionality (lp_0237, lp_1164, lp_1403, lp_2940, lp_3281, and lp_3659), stress response (lp_1019, lp_3055), or regulation (lp_3514 and lp_3646). Moreover, two genes were selected that encode proteins for which no function in GI-tract functionality of *L. plantarum* could be anticipated (lp_0190 and lp_0682). In order to establish a role for these genes in the observed persistence of *L. plantarum* in this complex niche, the 12 *ivi* genes were mutated by *cat*-gene replacement. Initially, the growth characteristics of the 12 *L. plantarum cat*-replacement mutants were investigated *in vitro* on MRS medium (Table 4), revealing that the maximal growth rate of all mutants appeared to be highly similar to that observed for the wild-type strain. Moreover, the final OD_{600} reached by all strains was virtually identical (data not shown). The fact that the genes mutated in these *L. plantarum* WCFS1 derivatives were selected by R-IVET screening implies that these genes are not expressed under laboratory conditions (3). Therefore, the finding that mutation of these genes did not result in dramatic effects on growth under laboratory conditions was not unexpected and is in agreement with the R-IVET results. Nevertheless, these genes could still play an important role *in vitro* when *L. plantarum* is grown on poorer media as compared to the rich MRS medium. Therefore, the maximal growth rates of the mutants and the wild-type strain were compared on CDM. All mutants appeared to grow in this medium at a rate that is highly similar to that observed for the wild-type strain (Table 4). These data could suggest that these genes have a role under more specific conditions encountered in the GI-tract.

Relative population dynamics

A method was developed to monitor the relative population dynamics of the group of *L. plantarum cat*-replacement mutants during passage through the GI-tract in competition with each other and a wild-type control strain NZ7109. Notably, NZ7109 harbors a unique erythromycin-resistance marker at a neutral locus in its chromosome (3), allowing its incorporation in these experiments as a wild-type control strain. This experimental set-up allows the use of Taqman qPCR for the simultaneous marker-gene based quantitative determination of the *cat*-marked total mutant population versus the *ery*-marked wild-type control population. The *cat*- and *ery*-derived amplicons were generated in a single, 4-primer PCR reaction and were differentially detected and quantified using amplicon-specific Taqman probes with different fluorescent labels. To investigate differences in PCR efficiency in the two reactions, a

dilution range of pNZ7101 was initially used as template for the amplification of the *cat* and *ery* PCR products. The reciprocal of the 2-power of the obtained C_t values was used as a measure for the absolute amount of template. The average ratio of these absolute amounts calculated for the *cat* and *ery* reactions was 0.88 ± 0.05. Although this ratio is quite close to the actual template ratio of 1, the *cat* and *ery* template quantifications and their ratios presented below were corrected for this difference in amplification and/or detection efficiency.

To assess their relative persistence in a competitive intestinal model, the 12 *cat* replacement mutants and the control strain NZ7109 were intragastrically administered in equal amounts to three mice on two consecutive days (day –1 and 0) and faecal samples were collected during 6 days (day 1-6). The qPCR method described above was employed to determine *cat*- and *ery*-derived C_t values that were used to calculate the abundance of the collective mutant group relative to NZ7109 in total DNA derived from the bacterial mixtures administered to mice and the faecal samples collected (Fig. 2 and 3). The input samples on day –1 and 0 appeared to contain approximately 15-fold more *cat*-marked mutants than *ery*-marked NZ7109, which is relatively close to the ratio in which the mutant strains were mixed with the control strain prior to the intragastric intubation (12:1). These results clearly confirm the suitability of this qPCR approach for the quantitative population determination of the strains used in this *in vivo* experiment. In contrast to the input bacterial mixtures, the faecal samples from day 1-6 appeared to contain approximately equal amounts of the collective mutant group and the control strain NZ7109, indicating that after GI-tract passage the relative abundance of the mutant group is drastically decreased (Fig. 3A). Moreover, virtually identical *cat* and *ery* quantification curves were obtained for the three mice. Therefore, these individual abundance numbers per mouse were used to calculate the average *cat:ery* ratio (Fig. 3B). These data clearly illustrate the significantly reduced survival or persistence in the GI-tract of the *ivi* gene mutants as a group compared to the control strain NZ7109.

A second qPCR strategy was developed that allows the determination of the relative population dynamics within the group of the *cat*-replacement mutants. The procedure employed generates a specific amplicon for every mutant by using one mutant-specific primer in combination with a universal primer and probe (Fig. 2). In control experiments it could be shown that a primer combination designed to specifically amplify a single *ivi* mutant did not generate a PCR product when chromosomal DNA of any of the other mutants was used as template (data not shown). This complete lack of cross-amplification establishes the high specificity of this mutant-specific qPCR approach. Subsequently, mutant-specific qPCR determinations were used to assess the relative population of individual mutants as compared to the total group of mutants, as was determined using the *cat*-gene specific qPCR described above. The data obtained clearly demonstrated that the input

population size of individual mutants present in the mixtures administered to the mice on day –1 and 0 are not significantly different (data not shown). Moreover, the relative abundance of some of the mutants appeared to be consistently and significantly lower as compared to other mutant strains in all 3 mice. This finding is clearly exemplified by the finding that in one mouse the relative abundance of the mutant strains Δlp_1164, Δlp_2940, and Δlp_3055 appeared to be 100- to 1000-fold lower as compared to the mutant strains Δlp_1403, Δlp_3281 and Δlp_3659, in which the latter mutants are representatives of the group of strains that displayed the highest relative abundance (Fig. 4). The measurements performed in the other 2 mice clearly confirmed the distinction between these strains, although the abundance reduction factor for *L. plantarum* Δlp_1164, Δlp_2940, and Δlp_3055 appeared to be variable between mice. Several other mutant strains, including Δlp_1019 and Δlp_3514, appeared to consistently display intermediary abundance in the faecal samples analyzed (data not shown). However, significant assessment of their relative persistence requires expansion of the number of measurements and mice. Finally, the individual measurements per time point for strain Δlp_0190 appeared to be relatively variable (data not shown). In conclusion, these experiments demonstrate the suitability of the qPCR strategy to assess and compare the *in vivo* persistence of multiple mutants in a single host animal, and clearly establish the importance of lp_1164, lp_2940 and lp_3055 for *L. plantarum* during passage of the GI-tract.

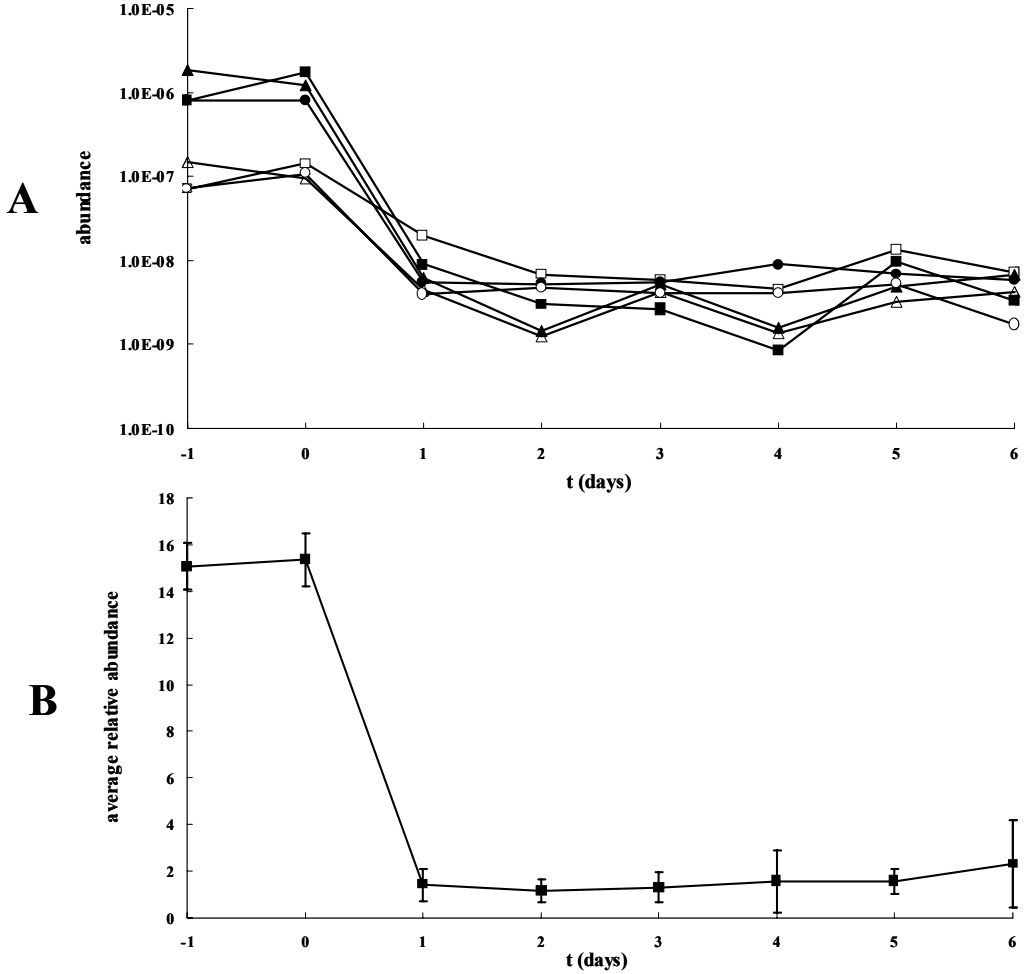


Fig. 3: Assessment by qPCR of the population dynamics of the *cat*-replacement mutants as a group, relative to the control strain NZ7109. panel 3A: the population abundance was calculated as the reciprocal of the 2-power of the obtained average C_t values, and is plotted per mouse (squares, triangles and circles represent the 3 individual mice) for the mutant group (closed symbols) and the control strain NZ7109 (open symbols). Panel 3B: the average of the abundance ratio for the mutant group and the control strain NZ7109 (*cat:ery*) is presented, and was calculated on basis of the data obtained from all 3 mice.

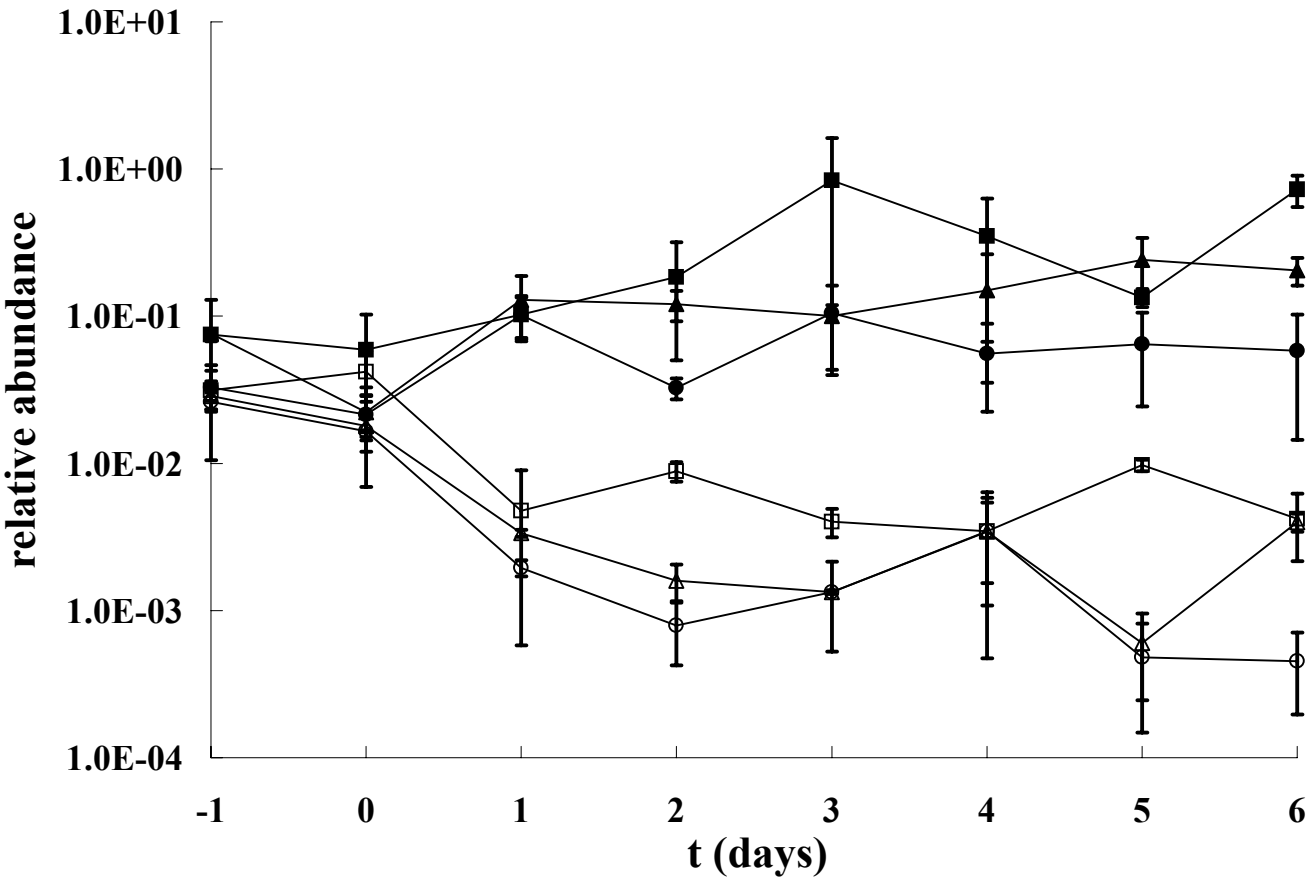


Fig. 4: Determination of the relative abundance of individual mutants as compared to the group of mutants. The relative abundance is calculated as the reciprocal 2-power of the obtained C_t values, normalized by the abundance score obtained for the total mutant population (Fig. 3). The open circles, triangles and squares represent the low-abundance mutant strain Δlp_2940, Δlp_1164 and Δlp_3055, whereas the closed circles, triangles and squares represent the high-abundance mutant strains Δlp_1403, Δlp_3659 and Δlp_3281, respectively.

Discussion

This paper describes the construction of 12 isogenic *L. plantarum* WCFS1 gene-replacement mutants. These mutations were introduced in genes that were previously demonstrated to be induced during passage of the mouse GI-tract. The growth characteristics of all mutants on two different laboratory media (MRS and CDM) were highly similar to that of the wild-type strain. Furthermore, a qPCR approach demonstrated that the abundance of the mutant group in the GI-tract of mice is drastically reduced relative to a wild-type control strain, indicating that most, if not all, *ivi* gene mutants have a reduced persistence or survival rate in the GI-tract. In addition, a second mutant-specific qPCR experiment focused on the relative population dynamics within the group of mutants. It would be interesting to validate whether the group-specific qPCR data (representing DNA template abundance) can be confirmed by selective viable count determinations (representing living cell abundance). Subsequently, the relative abundance of individual *cat*-replacement mutants could in its turn be validated by mutant specific qPCR on the collective chloramphenicol resistant, viable bacterial population retrieved from faecal samples.

The mutant-specific qPCR experiments revealed that in particular the abundance of *L. plantarum* Δlp_1164, *L. plantarum* Δlp_2940 and *L. plantarum* Δlp_3055 was decreased relative to the other mutants. These data indicate that these genes play a role in the functionality of *L. plantarum* during passage of the GI-tract. The IIC transport component of the cellobiose PTS system predicted to be encoded by lp_1164 is not located in a typical PTS-operon structure. Notably, the best homologs of lp_1164 in the Genbank and ERGO databases (http://www.ncbi.nlm.nih.gov/ and http://ergo.integratedgenomics.com/ERGO/, respectively) include several orphan PTS-IIC transport components in other bacteria. Moreover, in *Streptococcus thermophilus*, *Pediococcus pentosaceus*, *L. johnsonii,* and *L. casei* the genomic organization of these homologs appears to be similar to the lp_1164 locus in *L. plantarum*, since the downstream gene encodes an acetyltransferase/hydrolase with a typical αβ-hydrolase fold in all these organisms. In *Listeria monocytogenes* orphan cellobiose-PTS-IIC components have an established role in host-specific signaling, leading to modulation of virulence gene expression (18). Notably, many of these PTS systems are absent in *Listeria innocua* (11). These findings together with the experiments presented here suggest that host-factor mediated gene regulation in bacteria could involve analogous bacterial signaling factors (orphan cellobiose PTS-IIC components) in different bacteria, including *L. plantarum*.

The lp_2940 gene is predicted to encode an extracellular protein that contains an LPQTNE motif involved in anchoring of this protein to the bacterial cell wall (5, 22). The bacterial surface is the primary site of interaction with the host and the protein encoded by lp_2940 might represent a factor that mediates interaction with a (host-derived) GI-tract specific factor, or with host epithelial cells. Notably, the protein encoded by lp_2940 has no homologues in the Genbank and ERGO databases. Moreover, no recognizable sequence motifs or domains could be identified in this protein (5). Therefore, it remains to be established what the exact role of this protein in *L. plantarum* is.

lp_3055 is predicted to encode a copper transporting ATPase and could be involved in copper acquisition. Alternatively, this transporter could act as an exporter, thereby preventing accumulation of copper in the cytoplasm. These possibilities could be analyzed *in vitro* by comparison of growth characteristics under copper limitation of the wild-type and lp_3055 mutant, or by determination of their relative LD50 for copper. Heavy metal transport is a common theme that arose from several IVET screens in pathogens (20), and copper homeostasis has been recognized in an IVET screen as important during the infection process of *Salmonella typhimurium* (13). Furthermore, among the best homologs of *L. plantarum* CopA is the CtpA protein of *Listeria monocytogenes* (56% identity), which has an established role in copper transport. Gene expression studies suggest that CtpA functions both as detoxification and acquisition mechanism, since the expression of *ctpA* is induced under copper limitation and high copper concentrations. Growth of *Listeria monocytogenes ctpA* insertion mutants was restricted by copper-chelating agents (8). Moreover, recovery of *ctpA* mutants from tissue of infected mice was dramatically reduced as compared to the wild-type strain, and a significant impairment in terms of *in vivo* persistence in mixed-infection competition experiments was observed (9). Our results establish a similar role in *in vivo* persistence for CopA in *L. plantarum*, which is likely related to the same *in situ* function displayed by *copA* and *ctpA* in the two species. Whether the *in situ* importance of these proteins relates to copper detoxification or copper acquisition remains to be established, but *in vitro* functional analysis of *copA* in *L. plantarum* might suggest which of the two functions is required in the GI-tract.

Several papers describe the construction of *ivi* gene deletion mutants in pathogenic bacteria, followed by assessment of the abundance of individual mutants compared to the wild-type strain. Notably, these experiments involve selective plate counts of mutant and wild-type strains and are usually performed with bacterial cells that are in the post-invasion stage, e.g. isolated from infected tissues or organs (10, 12, 19). These plate counts generally do not allow the differential enumeration of more than one mutant in a single experiment and, hence, can only be performed in binary experiments comparing an individual mutant with the wild-type strain. In contrast, the qPCR approach described here is a culture independent approach and allows for

assessment of groups of mutants in complex competition experiments, generating a large amount of relative persistence data for individual mutants. In addition, it can be anticipated that the relative abundance of individual mutants is strongly influenced by the number of competitors it encounters in its niche. Therefore, relatively minor persistence reduction scored for specific mutants in a binary comparison with its corresponding wild-type strain, could be amplified in a more complex and thus more competitive system. In contrast, a drawback of large and complex mixed-mutant populations could be that the phenotype of a specific mutant is masked or compensated by other members of the mixed population. Examples of such inter-bacterial compensation have been reported for the simultaneous analysis of larger numbers of STM mutants in a single animal model (20). However, it is difficult to envision how elimination of the predicted functions of the genes targeted here could be compensated in a mixed population. Nevertheless, following these competitive population dynamics experiments, selected mutant strains could be analyzed in binary experiments with the wild-type *L. plantarum* to generate a more detailed view of these mutants. Such experiments could include qPCR using total DNA derived from different locations in the GI-tract, e.g. stomach, small intestine or colon, combined with selective enumeration of mutant and wild-type viable cells, and might reveal the molecular trigger responsible for the relatively low abundance of specific mutants.

Acknowledgements

The authors gratefully acknowledge Annick Mercenier, Corinne Grangette, Catherine Desseyn-Daniel and Benoît Foligné from the Pasteur Institute (Lille, France) for their expertise and technical support during the animal experiments. We thank Alexander Lindenbergh for construction of pNZ7101 and Douwe Molenaar for help with the database searches.

References

1. **Angelichio, M. J., and A. Camilli.** 2002. *In vivo* expression technology. Infect. Immun. **70:**6518-23.

2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513-1523.

3. **Bron, P. A., C. Grangette, A. Mercenier, W. M. de Vos, and M. Kleerebezem.** 2003. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice (Chapter 6).

4. **Bron, P. A., M. Marco, S. M. Hoffer, E. van Mullekom, W. M. de Vos, and M. Kleerebezem.** 2004. Genetic characterization of the bile salt response in *Lactobacillus plantarum* and analysis of responsive promoters *in vitro* and *in situ* in the gastrointestinal tract (Chapter 4).

5. **Bron, P. A., R. Siezen, A. Lindenbergh, E. Peters, G. Pretzer, W. M. de Vos, and M. Kleerebezem.** 2004. The role of sortase and its target proteins in *Lactobacillus plantarum* agglutination and persistence in the gastrointestinal tract of mice (Chapter 8).

6. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138:**179-207.

7. **Ferain, T., D. Garmyn, N. Bernard, P. Hols, and J. Delcour.** 1994. *Lactobacillus plantarum ldhL* gene: overexpression and deletion. J. Bacteriol. **176:**596-601.

8. **Francis, M. S., and C. J. Thomas.** 1997. The *Listeria monocytogenes* gene *ctpA* encodes a putative P-type ATPase involved in copper transport. Mol. Gen. Genet. **253:**484-91.

9. **Francis, M. S., and C. J. Thomas.** 1997. Mutants in the CtpA copper transporting P-type ATPase reduce virulence of *Listeria monocytogenes*. Microb. Pathog. **22:**67-78.

10. **Gahan, C. G., and C. Hill.** 2000. The use of listeriolysin to identify *in vivo* induced genes in the gram-positive intracellular pathogen *Listeria monocytogenes*. Mol. Microbiol. **36:**498-507.

11. **Glaser, P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend, F. Baquero, P. Berche, H. Bloecker, P. Brandt, T. Chakraborty, A. Charbit, F. Chetouani, E. Couve, A. de Daruvar, P. Dehoux, E. Domann, G. Dominguez-Bernal, E. Duchaud, L. Durant, O. Dussurget, K. D. Entian, H. Fsihi, F. Garcia-del Portillo, P. Garrido, L. Gautier, W. Goebel, N. Gomez-Lopez, T. Hain, J. Hauf, D. Jackson, L. M. Jones, U. Kaerst, J. Kreft, M. Kuhn, F. Kunst, G. Kurapkat, E. Madueno, A. Maitournam, J. M. Vicente, E. Ng, H. Nedjari, G. Nordsiek, S. Novella, B. de Pablos, J. C. Perez-Diaz, R. Purcell, B. Remmel, M. Rose, T. Schlueter, N. Simoes, A. Tierrez, J. A. Vazquez-Boland, H. Voss, J. Wehland, and P. Cossart.** 2001. Comparative genomics of *Listeria* species. Science **294:**849-52.

12. **Gort, A. S., and V. L. Miller.** 2000. Identification and characterization of *Yersinia enterocolitica* genes induced during systemic infection. Infect. Immun. **68:**6633-42.

13. **Heithoff, D. M., C. P. Conner, P. C. Hanna, S. M. Julio, U. Hentschel, and M. J. Mahan.** 1997. Bacterial infection as assessed by *in vivo* gene expression. Proc. Natl. Acad. Sci. U.S.A. **94:**934-9.

14. **Horinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pC194, a plasmid that specifies inducible chloramphenicol resistance. J Bacteriol **150:**815-25.

15. **Josson, K., T. Scheirlinck, F. Michiels, C. Platteeuw, P. Stanssens, H. Joos, P. Dhaese, M. Zabeau, and J. Mahillon.** 1989. Characterization of a gram-positive broad-host-range plasmid isolated from *Lactobacillus hilgardii*. Plasmid **21:**9-20.

16. **Khan, M. A., and R. E. Isaacson.** 2002. Identification of *Escherichia coli* genes that are specifically expressed in a murine model of septicemic infection. Infect. Immun. **70:**3404-12.

17. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U.S.A. **100:**1990-5.

18. **Kreft, J., and J. A. Vazquez-Boland.** 2001. Regulation of virulence genes in *Listeria*. Int. J. Med. Microbiol. **291:**145-57.

19. **Lowe, A. M., D. T. Beattie, and R. L. Deresiewicz.** 1998. Identification of novel staphylococcal virulence genes by *in vivo* expression technology. Mol. Microbiol. **27:**967-76.

20. **Mahan, M. J., D. M. Heithoff, R. L. Sinsheimer, and D. A. Low.** 2000. Assessment of bacterial pathogenesis by analysis of gene expression in the host. Annu. Rev. Genet. **34:**139-164.

21. **Mahan, M. J., J. M. Slauch, and J. J. Mekalanos.** 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259:**686-8.

22. **Mazmanian, S. K., G. Liu, H. Ton-That, and O. Schneewind.** 1999. *Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall. Science **285:**760-3.

23. **Poolman, B., and W. N. Konings.** 1988. Relation of growth of *Streptococcus lactis* and *Streptococcus cremoris* to amino acid transport. J. Bacteriol. **170:**700-7.

24. **Rose, R. E.** 1988. The nucleotide sequence of pACYC184. Nucleic Acids Res. **16:**355.

25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

26. **van Alen-Boerrigter, I. J., R. Baankreis, and W. M. de Vos.** 1991. Characterization and overexpression of the *Lactococcus lactis pepN* gene and localization of its product, aminopeptidase N. Appl. Environ. Microbiol. **57:**2555-61.

27. **van der Vossen, J. M., D. van der Lelie, and G. Venema.** 1987. Isolation and characterization of *Streptococcus cremoris* Wg2-specific promoters. Appl. Environ. Microbiol. **53:**2452-7.

28. **van Kranenburg, R., J. D. Marugg, S. van, II, N. J. Willem, and W. M. de Vos.** 1997. Molecular characterization of the plasmid-encoded *eps* gene cluster essential for exopolysaccharide biosynthesis in *Lactococcus lactis*. Mol. Microbiol. **24:**387-97.

29. **Vesa, T., P. Pochart, and P. Marteau.** 2000. Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. Aliment. Pharmacol. Ther. **14:**823-8.

30. **Wang, J., A. Mushegian, S. Lory, and S. Jin.** 1996. Large-scale isolation of candidate virulence genes of *Pseudomonas aeruginosa* by *in vivo* selection. Proc. Natl. Acad. Sci. U.S.A. **93:**10434-9.

31. **Zoetendal, E. G., K. Ben-Amor, A. D. Akkermans, T. Abee, and W. M. de Vos.** 2001. DNA isolation protocols affect the detection limit of PCR approaches of bacteria in samples from the human gastrointestinal tract. Syst. Appl. Microbiol. **24:**405-10.

Chapter 8

The Role of Sortase and its Target Proteins in *Lactobacillus plantarum* Agglutination and Persistence in the Gastrointestinal Tract of Mice

PETER A. BRON,[1,2] ROLAND SIEZEN,[1,2,3] ALEXANDER LINDENBERGH,[1,2] ERIK PETERS,[1,2] GABRIELE PRETZER,[2] WILLEM M. DE VOS,[1] AND MICHIEL KLEEREBEZEM.[1,2]

[1] Wageningen Centre for Food Sciences, [2] NIZO food research, P.O. Box 20, 6710 BA Ede, The Netherlands. [3] Centre for Molecular and Biomolecular Informatics, University of Nijmegen, Nijmegen, The Netherlands.

Manuscript in preparation.

Abstract

In gram-positive bacteria covalent attachment of proteins to the cell wall is catalyzed by an enzyme termed sortase. In several pathogenic bacteria the role of sortase and its target proteins in virulence has been established. Here we describe the functional study of a sortase homologue in the non-pathogenic bacterium *Lactobacillus plantarum* WCFS1. The complete genome sequence of this bacterium was analyzed and one sortase encoding gene (*srtA*) and 26 target proteins of SrtA could be identified. A *srtA::cat* gene replacement mutant (NZ7104) was constructed. Transcription analysis of *srtA* and its flanking genes in *L. plantarum* WCFS1 and NZ7104 revealed that the *srtA::cat* mutation did not cause detrimental polar effects. Furthermore, the mutation did not appear to affect growth under laboratory conditions. However, in contrast to the wild-type strain, the *srtA::cat* mutant strain had completely lost the capacity to agglutinate *Saccharomyces cerevisiae*, suggesting that at least one of the sortase target proteins plays a role in agglutination. Next to the *srtA* mutant, two strains were constructed that harbor a chloramphenicol or erythromycin resistance marker in different neutral chromosomal loci (NZ7102 and NZ7109, respectively). These strains were used in competition experiments to determine the persistence of the *srtA* mutant strain in the mouse intestine. Following administration of equal amounts of these strains to mice, the *srtA* mutant and the control strain were enumerated in faecal samples collected over a period of 6 days. this analysis revealed that the *srtA* mutant is not affected in its capacity to persist in the intestine of mice.

Introduction

Gram-positive organisms employ the cell wall peptidoglycan for the covalent attachment of proteins to the cell wall. This attachment is catalyzed by a transpeptidase termed sortase (SrtA) (31). Initially, the mechanism of action of SrtA, encoded by *srtA*, was elucidated using *Staphylococcus aureus* protein A as a model target protein (27, 28, 38, 39). All sortase anchored proteins have a typical N-terminal signal peptide involved in targeting and transport across the cytoplasmic membrane. In addition, these proteins possess a conserved LPXTG-like motif near their C-terminus, followed by a hydrophobic region and a positively charged tail (14). During the sorting reaction these target proteins are hydrolyzed between the threonine and glycine residue of their LPXTG motif. Subsequently, the carboxyl group of threonine is covalently linked to the free amino group of the pentaglycine cross-bridge in the cell-wall via a transpeptidation reaction that is catalyzed by sortase (30, 40). Importantly, the role of SrtA in the pathogenicity of *Staphylococcus aureus* has been established, since *srtA* mutants displayed reduced virulence (20, 26). Moreover, the 3D-structure of the staphylococcal SrtA was determined by NMR spectroscopy (17).

The sequence of SrtA from *Staphylococcus aureus* has been used to identify homologs in the genomes of several gram-positive bacteria, including *Listeria monocytogenes* (5, 15), *Corynebacterium diphteriae* (41), and several *Streptococcus* species (4, 25, 32). Notably, all sortase homologs identified to date possess a TLXTC motif, which has been shown to be the active site in the *Staphylococcus aureus* enzyme (38). The importance of SrtA in virulence has been implicated in all of the aforementioned pathogens (5, 15, 20, 25, 26, 41). Moreover, a second gene encoding a sortase homolog in *Staphylococcus aureus* was designated *srtB* and a deletion mutant did not cause a defect in the anchoring of the SrtA target proteins FnbA, FnbB, protein A or ClfA. These results demonstrated that SrtA and SrtB do not fulfill redundant function, suggesting that SrtB is involved in sorting of other surface proteins (28). In analogy, the function of the two sortase homologs encoded on the *Streptococcus pyogenes* genome was investigated and it could clearly be established that these proteins play a role in the non-redundant, differential recognition of surface proteins (4). Furthermore, intravenous inoculation of mice with staphylococcal *srtA*, *srtB* and *srtA-srtB* double mutants resulted in significantly reduced weight loss and increased survival of the mice, as compared to those that received the wild-type strain. Notably, mice that received the *srtA* mutant did not manifest severe arthritis, while arthritis in mice inoculated with the *srtB* mutant appeared comparable to that observed in mice that were infected with the wild-type strain. Overall, these results indicate SrtB merely plays a contributing role during pathogenesis of staphylococcal infections, while SrtA is an essential virulence factor for the establishment of septic arthritis (19). These conclusions were corroborated in a similar, independent study, using both mouse and rat animal models (44). Interestingly, recent *in silico* analysis of

complete genome sequences has demonstrated that most gram-positive bacterial genomes encode more than one sortase homolog. The same analysis also revealed that the number of LPXTG-motif containing potential target proteins of these sortase homologs is highly variable (33).

Traditionally lactic acid bacteria (LAB) are applied extensively in the production of a wide variety of fermented food and feed products. Based on this long history of application in products for human consumption, most LAB are generally regarded as safe (GRAS). More recent research has shown that certain LAB, in particular species from the genus *Lactobacillus*, are among the natural inhabitants of the human and animal gastrointestinal tract (GI-tract) (2, 16, 37). Moreover, several studies claim that specific *Lactobacillus* species have probiotic effects in man and animals (42). *Lactobacillus plantarum* is a representative of the *Lactobacillus* genus that is encountered in many environmental niches, including some dairy, meat and many vegetable fermentations as well as the human GI-tract (23). Moreover, it was demonstrated that several *L. plantarum* strains have the capacity to specifically adhere to human colonic cell lines (1). Recently, the complete 3.3 Mbp genome sequence of *L. plantarum* WCFS1 has been published (23). This strain is a single colony isolate of strain NCIMB8826, which effectively survives passage of the human stomach in an active form, reaches the ileum in high numbers as compared to other strains, and is detectable in the colon (43). A recombination-based *in vivo* expression technology (R-IVET) screen revealed 72 *L. plantarum* WCFS1 genes that are *in vivo* induced (*ivi*) in the GI-tract of mice as compared to laboratory conditions (8). These *ivi* genes include four genes that encode extracellular proteins. Notably, two of these proteins contain an LPXTG-like motif. As the bacterial surface is the primary site of interaction with the environment, these surface anchored proteins might represent factors that mediate interaction with specific (host-derived) GI-tract factors, and could be involved in the direct interaction with host epithelial cells. Moreover, the presence of an LPXTG-like motif in these proteins indicates a role for sortase in the functionality of *L. plantarum* in the GI-tract.

To date, functional analyses on sortase homologs have only been performed in pathogenic organisms. Although the functional role of sortase in food-grade organisms has not been established, a streptococcal nuclease tagged with a LPXTG sorting signal appeared to be properly sorted and covalently attached to the cell wall when expressed in *Lactococcus lactis* and several *Lactobacillus* strains. These experiments clearly suggest that endogenous sortase homologs are present in these LABs (11). Accordingly, *in silico* analysis using the available LAB genomic sequence information (http://www.jgi.doe.gov/) showed that all these LAB genomes harbor at least one sortase encoding gene. Here, we describe the functional analysis of a sortase homolog in the food-grade organism *L. plantarum* WCFS1. A single sortase encoding gene (*srtA*) and 26 potential target proteins were identified *in silico* in the *L.*

plantarum WCFS1 genome sequence. A *srtA* mutant was constructed by a gene replacement strategy. The *srtA::cat* mutation did not affect the growth characteristics of *L. plantarum* under laboratory conditions. Transcription analysis of the genes flanking *srtA* showed that the *srtA::cat* mutation did not cause detrimental polar effects. Nevertheless, in contrast to the wild-type strain, the *srtA* mutant completely lacked the capacity to cause *Saccharomyces cerevisiae* agglutination *in vitro*. Finally, *in vivo* competitive persistence analysis in the GI-tract, using a mouse model system, revealed that the *srtA* mutation had no significant effect on the persistence of *L. plantarum* in this niche.

Material and Methods

In silico analysis of sortases and its target proteins

To search for sortase enzymes encoded in the chromosome of *L. plantarum*, we first created Hidden Markov Models (http://hmmer.wustl.edu/)(12) based on known amino acid sequences of sortases. The NCBI PubMed database (http://www.ncbi.nih.gov/entrez/query.fcgi) was searched to find proteins which had been experimentally shown to have sortase activity. Sortase A proteins were found in *Listeria monocytogenes* (5), *Staphylococcus aureus* (27), *Streptococcus gordonii* (7), *Streptococcus mutans* (25), *Streptococcus pneumoniae* (22), *Streptococcus pyogenes* (4) and *Streptococcus suis* (32). Sortase B proteins were found in *Staphylococcus aureus* (29) and *Streptococcus pyogenes* (4). Hidden Markov Models (HMMs) were generated from these sequences with the program HMMER (version 2.2g), and subsequently used to search in 113 published microbial genomes in the NCBI database (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/), version May 21, 2003. In this way, over 80 sortases were identified, and these sequences were used in an iterative way to create improved HMMs for searching for sortases A and B (Boekhorst *et al*., unpublished data). The HMMs were created with both the complete sortase precursor sequences and with the core sequences of the sortase enzymes.

The program FindPatterns (GCG Wisconsin Package Version 9.0) was used to search for the presence of LPXTG-anchor motifs in proteins encoded in the genome of *L. plantarum* WCFS1 (Genbank accession number AL935263), using a modified version of the pattern described by Janulczyk and Rasmussen (18). Homology searches of the identified LPXTG proteins were performed with BLASTP version 2.2.4 (3) against the Swiss-Prot, spTrEMBL and the spTrEMBL (updates) databases at BioASP (https://gw1-prod.nbic.nl/http://ps1-prod.nbic.nl/portal/dt). Domain analysis was performed by searching against the Superfamily (http://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/), Pfam (http://www.sanger.ac.uk/Software/Pfam/), SMART (http://smart.embl-heidelberg.de/) and Interpro (http://www.ebi.ac.uk/interpro/) databases.

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

Material	Relevant features	Source or reference
Strains		
E. coli		
MC1061	cloning host	(10)
L. plantarum		
WCFS1	wild-type for which the genome sequence is available	(23)
WCFS1-R	Rif^R, derivative of *L. plantarum* WCFS1	(8)
NZ7102	Rif^R Cm^R, *L. plantarum* WCFS1 containing *cat* marker in intergenic region of lp_2681-lp_2683	this work
NZ7104	Rif^R Cm^R, *L. plantarum* WCFS1, *srtA:: cat*	this work
NZ7109	Rif^R Em^R, *L. plantarum* WCFS1 containing *ery* marker in intergenic region of lp_3503-lp_3504	(8)
Plasmids		
pCRblunt	Kan^R, cloning vector	Invitrogen
pUC18	Amp^R, cloning vector	(45)
pNZ7105	Kan^R, pCRblunt derivative containing 0.7 kb 5´-flanking region of *srtA*	this work
pNZ7106	Amp^R, pUC18 derivative containing 0.7 kb 3´-flanking region of *srtA*	this work
pNZ7101	Cm^R Em^R, vector for construction of *L. plantarum* gene replacement mutants	(9)
pNZ7104	Cm^R Em^R, *srtA::cat* replacement derivative of pNZ7101 containing pNZ7105 and pNZ7106 derived 5'- and 3'-flanking regions of *srtA*	this work
pGEMt	Amp^R, cloning vector	Promega
pNZ7111	Amp^R, pGEMt derivative containing 2.1 kb fragment of the *L. plantarum* lp_2681 and lp_2683 locus	this work
pNZ7102	Amp^R Cm^R, pNZ7111 derivative containing P32-*cat* originating from pNZ7101	this work
Primers		
lp_0513F	5'-GGGGTACCCCCAATGCTTCTGTCAGG-3'	
lp_0513R	5'-CGGGATCCTTGCTTGGACTTCATTAATCC-3'	
lp_0515F [a]	5'-CGGGATCCCATTTTAATAACAAATATTAAC-3'	
lp_0515R [a]	5'-ATGCTCTAGAACGTGTGCCGCTTGTTGC-3'	
HF	5'-ACNGCNACNTTYAAYGARAAYCARATHTAYCG-3'	
HR	5'-CGNCARACNGARCARCARCCNACNGCNGTNTAYCC-3'	
P32catF [a]	5'- ATGCTTAATTAAGATGAGTACGGTCAAGTATG -3'	
P32catR [a]	5'- ATGCTTAATTAAGGCAACAGTTTAACGATTAC -3'	
SCO_2681	5'- CTGATCAGGAAACGGTGACG - 3'	
catR	5'-CTCTTCCAATTGTCTAAATC-3'	
SCO_2683	5'-GCTTGGAAACGGGCTGTCCG-3'	
catF	5'- CAGATAGGCCTAATGACTGG -3'	
lp_0513SB-F	5'-CAACAACGTGAGGATGGCCC-3'	
lp_0513SB_R	5'-AGGTCACCGCCACGTTTTTC-3'	
lp_0514SB_F	5'-CGGGTTAAAGTGGTTAGGTC-3'	
lp_0514SB_R	5'-CTTATCACAGGTAATTAGGG-3'	
lp_0515SB_F	5'- TTTAATTATCGTTGCGGTCG -3'	
lp_0515SB_R	5'-CTGCTTCAGGAACAGTTAAG-3'	
lp_0516SB_F	5'-AGCAAATTGCCCGCAACAAG-3'	
lp_0516SB_R	5'-TAACAAACTGATGGCCGCTG-3'	
univHTP	5'-GCCGACTGTACTTTCGGATC-3'	
NZ7102HTP	5'-TTAGTTGTTCAGATTCCAGGC-3'	
NZ7104HTP	5'-GCTATCATCAATAGACCCCC-3'	

[a] underlined sequences indicate restriction sites subsequently used in cloning procedures

[b] Ap^R, ampicillin resistant; Em^R, erythromycin resistant; Cm^R, chloramphenicol resistant; Rif^R, rifampicin resistant; Kan^R, kanamycin resistant

Bacterial strains and plasmids

The bacterial strains and plasmids that were used in this study are listed in Table 1. *Escherichia coli* strain MC1061 (10) was used as cloning hosts during construction of pNZ7102 and pNZ7104. *E. coli* was grown aerobically in TY medium (35). *L. plantarum* WCFS1 (23), and its derivatives WCFS1-R (8), NZ7102 (see below), NZ7104, (*srtA::cat*, see below) and NZ7109 (8) were grown at 37 ^{o}C in MRS (Difco, Surrey, U.K.) without aeration. When appropriate, antibiotics were added to the media; for *E. coli* ampicillin (50 μg/ml), kanamycin (50 μg/ml) and chloramphenicol (10 μg/ml); for *L. plantarum* chloramphenicol (5 μg/ml), erythromycin (5 or 30 μg/ml for selection or replica-plating, respectively) or rifampicin (50 μg/ml).

DNA techniques

Plasmid DNA was isolated from *E. coli* on a small scale using the alkaline lysis method (6, 35). Large scale plasmid DNA isolations were performed using Jetstar columns according to the manufacturer's instructions (Genomed GmbH, Bad Oberhausen, Germany). DNA isolation and transformation in *L. plantarum* were performed as described previously (13, 21). Standard procedures were applied for DNA manipulations in *E. coli* (35). Restriction endonucleases, *Taq* and *Pfx* polymerase, and T4 DNA ligase were used following the recommendations of the manufacturer (Promega, Leiden, the Netherlands and Boehringer, Mannheim, Germany). Primers were purchased from Pharmacia Biotech (Roosendaal, the Netherlands).

Construction of the integration vectors pNZ7102 and pNZ7104

The mutagenesis vector pNZ7101 was previously developed in our laboratory (9) and is suitable for the single step selection of *cat* replacement mutants (Fig. 1A). This vector was used for the construction of a *L. plantarum srtA::cat* replacement mutant (Fig. 1B). The chromosomal 5'- and 3'-flanking regions of *srtA* were amplified by *Pfx*-PCR using *L. plantarum* WCFS1 chromosomal DNA as template and the primer combinations lp_0513F and lp_0513R, or lp_0515F and lp_0515R, respectively (Table 1). The 0.7 kb 5'-PCR product was ligated into pCRblunt (Invitrogen, Breda, the Netherlands) and the 0.7 kb 3'-PCR product was digested with *Xba*I and *Bam*HI (sites introduced in the primers) and ligated into similarly digested pUC18 (45), yielding the plasmids pNZ7105 and pNZ7106, respectively. The sequence of both *srtA* flanking regions was verified by automatic double strand sequence analysis using an ALF*red* DNA sequencer. Sequence reactions were performed with an Autoread kit using Cy5-labeled universal and reverse primers, following the instructions of the manufacturer (Pharmacia Biotech, Roosendaal, the Netherlands). Subsequently, the 3'-flanking region of *srtA* was retrieved from pNZ7106 as a *Pvu*II fragment and cloned into *Sma*I digested pNZ7101. The 5'-flanking region of *srtA* was cloned into

the *Pvu*II site of the resulting plasmid as a *Ecl*136II-*Eco*RV fragment obtained from pNZ7105. The resulting plasmid was designated pNZ7104 and harbors the chloramphenicol resistance cassette flanked by the 5'- and 3'-flanking regions of *L. plantarum srtA*. This plasmid was used for *srtA::cat* replacement in the chromosome of *L. plantarum* WCFS1 (see below).

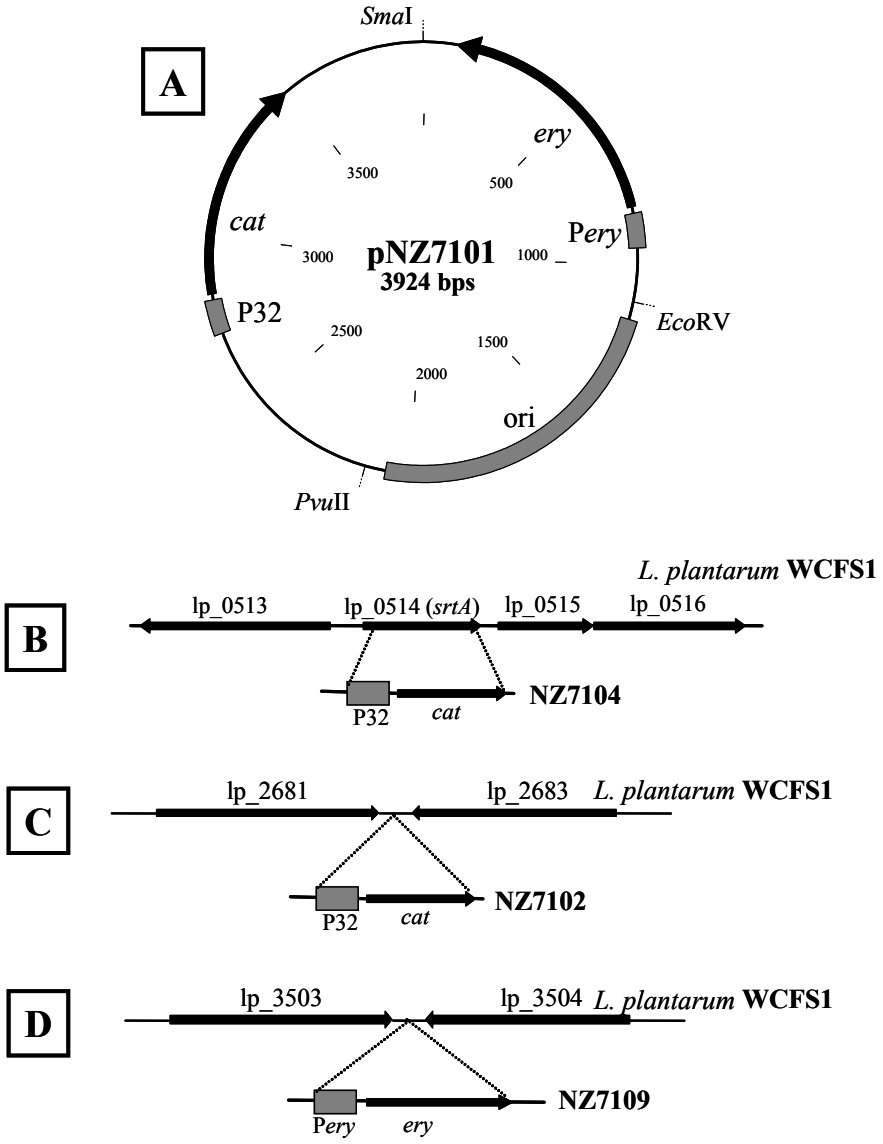


Fig. 1: Schematic overview of the constructed *L. plantarum* mutants. Mutagenesis vector pNZ7101 was used for the cloning of *L. plantarum* chromosomal regions (Fig. 1A). The chromosomal modifications introduced in *L. plantarum* strains NZ7104, NZ7102 and NZ7109 are schematically depicted in Fig. 1B, C and D, respectively.

To construct a chloramphenicol resistant control derivative of *L. plantarum* WCFS1, the pNZ7101 derived chloramphenicol cassette was integrated in the intergenic region of the convergently oriented genes lp_2681 and lp_2683 (Fig. 1C). To this end, a 2.1 kb fragment of the lp_2681-2683 locus was amplified by PCR, using primers HF and HR and chromosomal DNA of *L. plantarum* WCFS1 as template, and cloned into pGEMt (Promega, Madison, USA), yielding pNZ7111. The chloramphenicol resistance cassette of pNZ7101 was amplified by *Pfx* PCR using the primers P32catF and P32catR. The resulting 1.1 kb amplicon was digested with *Pac*I and cloned into similarly digested pNZ71111. The resulting plasmid pNZ7102 was used to introduce the pNZ7101 derived *cat*-cassette in the lp_2681-lp_2683 intergenic region of *L. plantarum* WCFS1 (see below).

Construction of NZ7102 and NZ7104

Stable integration of pNZ7102 and pNZ7104 into the chromosome of *L. plantarum* was achieved by double cross-over replacement (Fig. 1). The plasmids were transformed to *L. plantarum* WCFS1-R (8) and the cells were plated on MRS containing 5 μg/ml chloramphenicol. After 48 h, pNZ7102 derived double cross over mutants were directly selected by colony-PCR using the primer pairs SCO_2681 plus catR, and SCO_2683 plus catF (Table 1). A colony generating both flanking-PCR products, resulting from a double cross-over event at the correct locus, was selected and designated NZ7102. The pNZ7104 integrants were replica-plated to MRS containing 5 μg/ml chloramphenicol, with or without 30 μg/ml erythromycin. A colony displaying the anticipated erythromycin sensitive phenotype was selected and designated NZ7104. The genotypes of NZ7102 and NZ7104 were verified by Southern blot analysis (data not shown). NZ7102 and NZ7104 as well as the previously constructed NZ7109 strain (8)(Fig. 1D), were used for an *in vivo* competitive persistence analysis (see below).

RNA isolation and transcription analysis

L. plantarum WCFS1 and NZ7104 strains were grown in liquid MRS medium to an optical density at 600nm (OD_{600}) of 0.85. The cells were pelleted by centrifugation (4500 rpm for 10 min at 4^{o}C) and resuspended in 0.4 ml ice-cold MRS. Cell suspensions were transferred to ice-cold tubes containing 1 g of zirconium glass beads, 0.4 ml phenol, 100 μl chloroform, 30 μl 10% SDS and 30 μl 3M NaAc pH 5.2. The cells were disrupted using two treatments of 40 seconds in a Fastprep^{TM} (Qbiogene Inc, Cedex, France) interspaced by 1 min on ice. After centrifugation, 200 μl of the aqueous phase was used for RNA isolation using the High Pure kit, including a 1 h treatment with DNaseI, according to the manufacturer's recommendations (Roche Diagnostics, Mannheim, Germany).

9 µg of total RNA or 1 µg of *L. plantarum* WCFS1 chromosomal DNA were spotted directly on Gene Screen Plus membranes (Dupont) according to the protocols provided by the manufacturer. Internal fragments of lp_0513, lp_0514, lp_0515 and lp_0516 were generated by PCR, using *L. plantarum* WCFS1 chromosomal DNA as the template and the four primer pairs presented in Table 1. The amplicons were glass matrix purified (Amersham Pharmacia Biotech Inc), labeled by nick translation (35) with [α-32P] dATP (Amersham International plc), and subsequently used as probes. Hybridization, washing and autoradiography procedures were performed as described previously (35).

L. plantarum* mediated agglutination of *S. cerevisiae

The capacity of the *L. plantarum* WCFS1 and NZ7104 strains to agglutinate *S. cerevisiae* was tested essentially as described previously (1). Briefly, full-grown cultures of *L. plantarum* and baker's yeast (*S. cerevisiae*) were pelleted, washed and resuspend in PBS (pH 7.4). Subsequently, 50 µl bacterial suspension (5-fold concentrated as compared to full-grown culture), 50 µl PBS, and 100 µl of 1 % (w/v) *S. cerevisiae* suspension were mixed in a 96-well microtiter (Greiner bio-one, Alphen a/d Rijn, The Netherlands). The microtiterplates were incubated at room temperature for 10 min and examined by bright-light microscopy at 200-fold magnification (Nikon Eclipse TS100).

***In vivo* persistence analysis**

Animal experiments were performed at the Institute Pasteur in Lille in an accredited establishment (N° A59107) according to guidelines N°86/609/CEE of the French government. Seven weeks-old female Balb/c mice were purchased from Iffa Credo (St Germain sur l'Arbresle, France) and had free access to tap water and standard mice chow during the experiments. To compare the *in vivo* persistence of the *srtA* mutant strain NZ7104 and the control strain NZ7102 (Fig. 1B and C, respectively), overnight cultures of the 2 bacterial strains were mixed, pelleted by centrifugation, and resuspended at 10^{10} CFU per ml in MRS. Two mice received a 100 µl dose of the NZ7104-NZ7102 bacterial suspension by intragastric administration at 2 consecutive days (day -1 and day 0). Individual faecal samples were collected daily during 6 days following the last administration (day 1 to 6) and the samples were resuspended in MRS medium. After extensive homogenization, appropriate dilutions of the suspensions were plated on MRS plates containing 5 µg/ml chloramphenicol and 50 µg/ml rifampicin. After 72 h of growth at 37 ^{o}C, an average of 36 full-grown colonies per time-point per mouse were identified as NZ7104 or NZ7102 using a 3-primer (univHTP, NZ7102HTP and NZ7104HTP) colony-PCR procedure, designed to result in a 0.5 or 0.8 kb amplicon using NZ7102 or NZ7104 cells, respectively. The ratio of the NZ7102 and NZ7104 population per time-point was used as an indication for the

in vivo persistence of the two strains. Similarly, a second experiment was performed, using NZ7104 and an alternative control strain (NZ7109, see Fig. 1D) that was previously constructed (8) and is erythromycin and rifampicin resistant. NZ7104 and NZ7109 population ratios were directly determined in faecal samples by differential enumeration of erythromycin (NZ7109) and chloramphenicol (NZ7104) resistant colonies.

Results and Discussion

In silico identification of sortase and its target proteins in *L. plantarum*

Only a single sortase A encoding gene (lp_0514; *srtA*) was identified in the *L. plantarum* WCFS1 genome by searching with Hidden Markov Models, while no sortase B homologue could be identified. The *L. plantarum* WCFS1 SrtA homologue consists of 234 amino acid residues and has all the features of a typical SrtA protein. Firstly, a TLXTC motif containing the active site cysteine residue (Cys-195) is present in the protein. Secondly, a conserved active site histidine residue (His-132) was identified. Finally, an N-terminal hydrophobic transmembrane sequence for anchoring of this protein to the cytoplasmic membrane was found. The best homologs of *srtA* were found in *Enterococcus faecalis* (42% identity over the entire protein sequence), *Lactococcus lactis* (39%) and *Streptococcus agalactiae* (37%).

Twenty-six *L. plantarum* proteins containing LPXTG-type motifs for processing by sortase were identified (Table 2). All these proteins contain typical signal peptides for secretion by the Sec-dependent pathway. The majority (22 of 26) of the sortase-target motifs contain a distinct variation relative to the canonical LPXTG, i.e. LPQTXE, which might reflect the specificity of the sortase A encoded in the genome (23). Twenty of the sortase-target proteins can be divided into 6 families based on sequence homology, domain composition or other shared characteristics (Table 2A). All sortase-target proteins are large multi-domain proteins with sizes ranging from 419 to 2219 amino acid residues, with exception of the 6 family F members, which are less than 150 amino acids residues. The latter family may be part of extracellular protein complexes, since their genes are always clustered with several genes encoding extracellular proteins of unknown function (Siezen *et al*., unpublished data). Genes for other sortase target proteins are generally not part of large operons in *L. plantarum*, although some do occur adjacent to other genes for extracellular proteins (Table 2A). Only one of the target proteins (lp_1124) has a predicted enzymatic function, since it has the consensus signature GXSXG (Ser being the catalytic residue), typical of αβ-hydrolases, such as lipases and esterases. Most of the other sortase-target proteins contain one or more conserved domains (Table 2B), either with an unknown function (DUF285, DUF1085, Big_3 domains), or with a predicted binding function to either proteins (Collagen_bind, Cna_B domains) or carbohydrates (Cellulose_bind, ConA-like, Mub1 domains). In addition, many of

these sortase-target proteins also contain amino acid sequence repeats of 3-40 residues, which are often found in extracellular proteins, and function as spacers to position the enzymatic or binding domains outside the cellular envelope. The LPQTXE-surface-anchored proteins of *L. plantarum* are likely to play a role in interaction with their environment, possibly including adhesion to host-derived components.

Tabel 2A: *In silico* identification of sortase target proteins in *L. plantarum* WCFS1, and their characteristics.

ORF nr	size (AA)	annotation	domains	repeats	best paralogs in other genomes	context
Family A						
lp_0946	1189	cell surface protein precursor	DUF1085 (8)	no	*Listeria* *Lactococcus*	alone in operon; adjacent to restriction-modification genes
lp_2486	917	cell surface protein precursor	DUF1085 (5)	yes	*Listeria* *Lactococcus*	alone in operon or with large hypothetical protein and MarR-type regulator
lp_3127	1189	cell surface protein precursor	DUF1085 (5)	no	*Listeria* *Lactococcus*	alone in operon
Family B						
lp_0197	1006	cell surface protein precursor	Collagen_bind (1) Cna_B (2 ?)	yes	*L. brevis* *Listeria*	alone in operon
lp_2578	705	cell surface protein precursor	Collagen_bind (1) Cna_B (2)	yes	*L. brevis* *Listeria* *Lactococcus*	alone in operon
lp_2588	570	cell surface protein precursor	Collagen_bind (1)	yes	*L. brevis*	adjacent to cell surface hydrolase, membrane-bound
Family C						
lp_0923	806	cell surface protein precursor		?	no hits	alone in operon
lp_2940	419	cell surface protein precursor		?	no hits	alone in operon
Family D						
lp_0800	2139	cell surface protein precursor	DUF285 (1) Big_3 (16?)	no	*Listeria* *L. johnsonii* *Enterococcus*	alone in operon
lp_2795	1039	cell surface protein precursor	DUF285 (1) Big_3 (4)	no	*Listeria* *L. johnsonii* *Enterococcus*	2795-2796 adjacent genes
lp_2796	1038	cell surface protein precursor	DUF285 (1) Big_3 (3)	no	*Listeria* *L. johnsonii* *Enterococcus*	2795-2796 adjacent genes

Family E						
lp_1643	2219	cell surface protein precursor	Mub (6)	no	*L. brevis* *L. gasseri* *L. johnsonii* *Lactococcus lactis* *Pediococcus*	in operon with lp_1645 (encoding hypothetical protein)
lp_3114	2032	cell surface protein precursor	Mub (4)	no	*L. brevis* *L. gasseri* *L. johnsonii* *Lactococcus lactis* *Pediococcus*	in gene cluster with lp_3115-3117 encoding extracellular proteins
lp_3059	1356	cell surface protein precursor	Mub (1)	yes	*L. brevis* *L. gasseri* *L. johnsonii* *Lactococcus lactis* *Pediococcus*	in operon with lp_3060 transcriptional regulator
Family F						
lp_1447	123	cell surface protein precursor		no	no hits	in gene cluster of extracellular proteins
lp_2976	125	cell surface protein precursor (putative)		no	no hits	in gene cluster of extracellular proteins
lp_3065	141	cell surface protein precursor		no	no hits	in gene cluster of extracellular proteins
lp_3074	123	cell surface protein precursor		no	no hits	in gene cluster of extracellular proteins
lp_3454	91	cell surface protein precursor		no	no hits	in gene cluster of extracellular proteins)
lp_3677	113	cell surface protein precursor		no	no hits	in gene cluster of extracellular proteins
Singles						
lp_0373	1231	cell surface protein precursor		no	*Enterococcus* *Listeria*	in operon with lp_0374 (extracellular protein), lp_0375-0376 (hypotheticals)
lp_1124	901	cell surface hydrolase	CSH2 (1)	no	many Gram+	alone in operon
lp_1229	1010	cell surface protein precursor	ConA-like (1) Mub (1-2)	yes	*Staphylococcus* (ConA domain) *Lactobacillus* (Mub domain) *Lactococcus* (Mub domain)	at end of large polysaccharide biosynthesis cluster; linked to lp_1230 transcriptional regulator
lp_2925	824	cell surface protein precursor		yes	no hits	alone in operon
lp_2958	617	cell surface protein precursor	KXGD (1) Collagen_bind (1)	yes	*Streptococcus mutans* *Pediococcus* *Lactococcus lactis*	in operon with transport protein lp_2959
lp_3001	1074	cell surface protein precursor	ConA-like (1) Cellulose_bind (1)	yes	*Bacteroides* *Pseudomonas*	alone in operon

Table 2B: Domain descriptions.

domain name	domain code[a]	Size (AA)	found in *L.plantarum* (nr domains)	found in other bacteria	putative function
DUF1085	PF06458	~60	lp_0946 (8), lp_2486 (5), lp_3127 (5)	*Listeria,* *Lactococcus,* *Staphylococcus*	unknown
Collagen_bind	PF05737	~180	lp_0197 (1) lp_2578 (1) lp_2588 (1) lp_2958 (1)	*L. brevis* *Listeria* *Bacillus anthracis* *Lactococcus*	adhesion to proteins (like collagen, receptors)
Cna_B	PF05738	~90	lp_0197 (2) lp_2578 (2)	*Listeria innocua* *Listeria monocytogenes* *Lactococcus* *Bacillus halodurans*	repeated domain in collagen-binding surface proteins ; occurs together with Collagen_bind domain; repeats could form stalk
DUF285	PF03382	~160	lp_0800 (1) lp_2795 (1) lp_2796 (1)	*Mycoplasma*	unknown
Big_3	PF07523	~80	lp_0800 (16?) lp_2795 (1) lp_2796 (1)	*Listeria* *Bifidobacterium* *Enterococcus* *Bacillus halodurans*	unknown; Bacteral Ig-like (group 3); domain with Ig-like fold, found in a variety of bacterial surface proteins
Cellulose_bind	SSF49384	~130	lp_3001 (1)	*Clostridium*	carbohydrate binding
ConA-like	SSF49899	~250	lp_1229 (1) lp_3001 (1)	many	common property of reversibly binding to specific complex carbohydrates; bacterial and fungal beta-glucanases carry out the acid catalysis of beta-glucans found in micro-organisms and plants; many Con A-like domains found in proteins involved in cell recognition and adhesion
Abhydrolase_1	PF00561	~220	lp_1124 (1)	*Enterococcus* *Listeria* *Streptococcus pyogenes*	cell-surface hydrolase; consensus GXSXG typical of lipase/esterase serine active site residue
Mub1		~175-200	lp_1643 (6) lp_3114 (4) lp_1229 (1-2) lp_3059 (1)	*L. reuteri* *L. johnsonii* *Lactococcus lactis*	experimentally determined role in mucin binding in *L.reuteri* (34)
KXGD		~150	lp_2958 (1)	*Listeria* *Enterococcus* *Streptococcus mutans* *Bacillus cereus*	unknown; in N-terminus between signal peptide and Collagen_bind domain

[a]PF: Pfam codes (http://www.sanger.ac.uk/Software/Pfam/)

SSF: Superfamily database of known 3-D structures (http://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/)

Construction and determination of growth characteristics of *L. plantarum* NZ7102, NZ7104 and NZ7109

To study the role of sortase in *L. plantarum* WCFS1, strain NZ7104 was constructed (Fig. 1B). In this strain the sortase encoding gene *srtA* (lp_0514) is replaced by a chloramphenicol resistance marker. Moreover, two control *L. plantarum* strains were constructed that carry a chromosomal copy of the chloramphenicol or erythromycin resistance gene in two different regions of convergently transcribed genes (NZ7102 and NZ7109, respectively). The maximal growth rate of the three strains appeared to be highly similar under laboratory conditions (Table 3). Moreover, the final OD_{600} reached by the three strains was identical (data not shown). Apparently, the absence of a functional copy of *srtA* in NZ7104 does not significantly influence the growth characteristics of *L. plantarum* under the conditions tested. Similar observations have been made in other bacteria, including *Staphylococcus aureus* and *Listeria monocytogenes* (15, 26). The unaffected growth characteristics of the control strains NZ7102 and NZ7109 justifies the application of these strains as selectable wild-type control strains in the *in vivo* competition experiment described below.

Table 3: Maximal growth rate determination of *L. plantarum* NZ7102, NZ7104 and NZ7109. Growth was monitored by automatic OD_{600} measurements in a 96-well plate and used to calculate the maximal logarithmic growth rate.

strain	μ_{max} (h^{-1})
L. plantarum NZ7102	0.70 ± 0.02
L. plantarum NZ7104	0.66 ± 0.02
L. plantarum NZ7109	0.65 ± 0.02

The effects of the *srtA::cat* mutation in NZ7104 on the transcription levels of the *srtA* flanking genes were investigated. RNA was isolated from exponentially growing *L. plantarum* WCFS1 and NZ7104 and the transcription levels of lp_0513, *srtA* (lp_0514), lp_0515 and lp_0516 were determined using individual gene-specific probes in an RNA spot-blot experiment. Transcripts for all genes studied could be detected using *L. plantarum* WCFS1 derived RNA (Fig 2, row 3). In contrast, using RNA obtained from NZ7104, no transcript could be detected for *srtA* (Fig. 2, row 4). These results demonstrate the anticipated absence of a *srtA* transcript in strain NZ7104, and confirm the correct deletion of the encoding gene (Fig 2, row 1 and 2). The *srtA::cat* mutation in NZ7104 did not appear to affect the transcription level of the divergently oriented gene lp_0513, while the transcription of the tandemly oriented genes lp_0515 and lp_0516 appeared to be slightly enhanced as compared to the wild-type levels (Fig 2, row 3 and 4). The latter effect probably results from read-

through transcription from the chloramphenicol gene in NZ7104. Overall, the data indicate that the *srtA* flanking genes are properly transcribed in NZ7104, and exclude deleterious polar effects of the *srtA::cat* mutation.

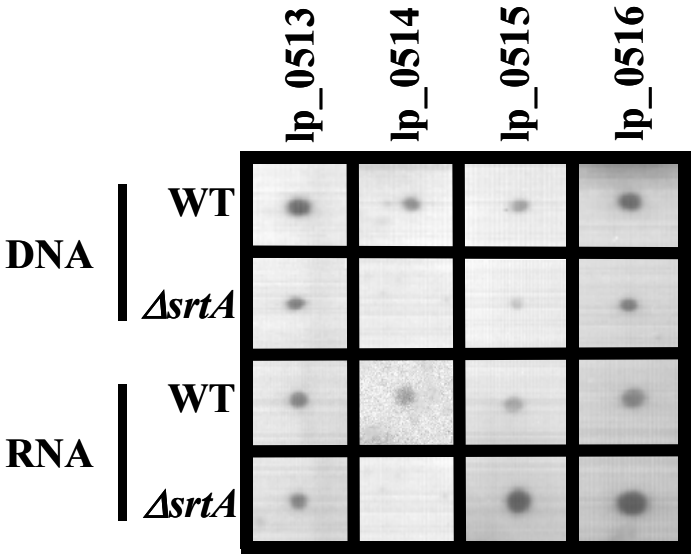


Fig. 2: Transcription and chromosomal DNA analysis of sortase and its flanking genes lp_0513, lp_0514 (*srtA*), lp_0515 and lp_0516 in *L. plantarum* WCFS1 and its *srtA::cat* derivative NZ7104.

In vitro agglutination properties of NZ7104 and *L. plantarum* WCFS1

Research on the adhesive capacities of *L. plantarum* 299 and 299v revealed that the ability of these strains to adhere to HT-29 cells correlates with an ability to agglutinate *S. cerevisiae* (1), suggesting that the result of such an agglutination test can be extrapolated to the *in vivo* adhesion capacities of a strain in the GI-tract. Therefore, the effect of the *srtA::cat* mutation on the agglutinating capacities of *L. plantarum* was investigated (Fig. 3). The results clearly demonstrated that the wild-type *L. plantarum* WCFS1 strain is capable to agglutinate the yeast cells. Notably, this agglutination phenotype could be observed using up to 16-fold diluted suspensions of *L. plantarum* WCFS1. These results indicate that the ability to agglutinate cells of *S. cerevisiae* is a conserved property shared among several *L. plantarum* strains, including *L. plantarum* WCFS1. In contrast, undiluted suspensions of NZ7104 completely lacked the ability to agglutinate *S. cerevisiae*. The impaired sorting of LPQTXE-containing surface proteins in the *srtA* mutant apparently disturbs the interaction that normally leads to agglutination of *S. cerevisiae* by *L. plantarum*. This observation strongly suggests that at least one of the 26 *L. plantarum* WCFS1 target proteins of sortase plays a role in the agglutination of *S. cerevisiae*. Moreover, it is likely that these observations can be extrapolated to other *L. plantarum* strains for which agglutination has been observed (1).

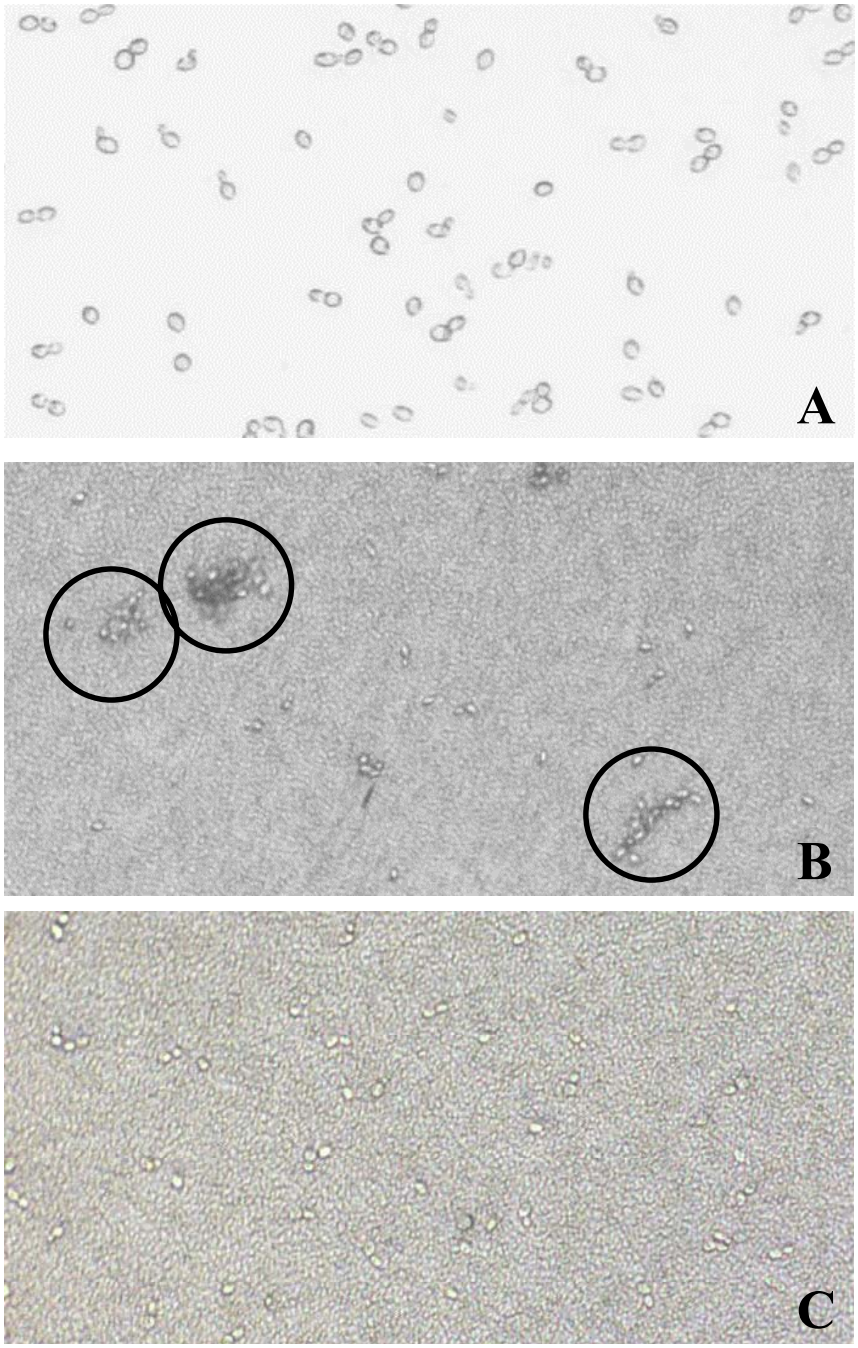


Fig. 3: *L. plantarum* mediated agglutination of *S. cerevisiae*. Figure 3A shows *S. cerevisiae* cells without bacteria, 3B and 3C show *S. cerevisiae* incubated with *L. plantarum* wild-type and its *srtA::cat* derivative, respectively. Clear agglutination can only be observed upon incubation with wild-type *L. plantarum* (3B).

Competitive *in vivo* persistence analysis of NZ7104

As the *in vitro* agglutination phenotype was disturbed in NZ7104, the *in vivo* persistence of this strain was analyzed in a mouse model system. Equal amounts of NZ7104 and a selectable WCFS1-derivative that is used as a wild-type control strain (either NZ7102 or NZ7109) were administered to mice on two consecutive days. Mutant and wild-type population ratios were determined at each time point (prior and post administration) by either three primer differential PCR (NZ7104 and NZ7102) or selective plating (NZ7104 and NZ7109). These analyses confirmed that the *srtA::cat* mutant (NZ7104) and control strains NZ7102 or NZ7109 were administered to the mice in similar quantities at days –1 and 0 (Fig. 4). The NZ7102:NZ7104 population ratio remained approximately 1 in the faecal samples collected at all time points (up to 6 days after the last intragastric administration), indicating the presence of equal amounts of NZ7102 and NZ7104. Although the relative error in individual measurements appeared to be higher as compared to the experiments described above, essentially the same results were obtained when NZ7109 was used as a competitive control strain. Also in these experiments, the *srtA* mutant could be detected efficiently up to 6 days after the last administration, and was not significantly under or overrepresented compared to the erythromycin resistant control strain NZ7109 (data not shown). In conclusion, these two competition experiments, using two control strains (NZ7102 and NZ7109) that harbor different resistance markers in different chromosomal locations, both indicate that the *srtA::cat* mutation in NZ7104 does not have a significant effect on the persistence of this strain during residence in the GI-tract of mice. These observations are in clear contrast to animal experiments using *srtA* mutants of several pathogens, including *Staphylococcus aureus*, *Listeria monocytogenes*, *Corynebacterium diphteriae* and *Streptococcus mutants*, which revealed reduced pathogenicity of these mutants relative to the wild-type strains (5, 15, 20, 25, 26, 41).

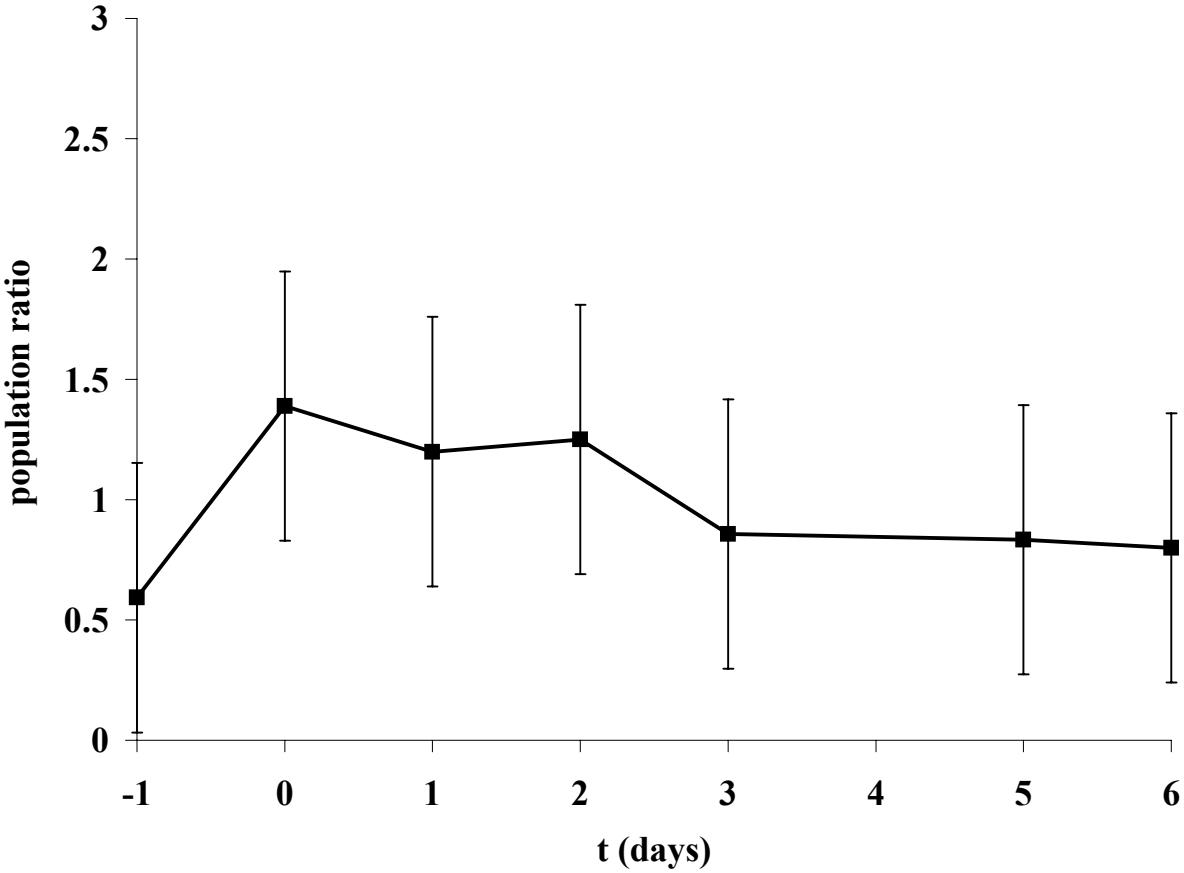


Fig. 4: *In vivo* persistence of *L. plantarum* NZ7102 and NZ7104, represented as the population ratio NZ7102:NZ7104. Error bars represent the average error margins calculated from the duplicate experiments

Concluding remarks

This paper describes the identification and characterization of the sortase encoding *srtA* gene of *L. plantarum*. To our knowledge, this is the first time that a functional analysis was performed on sortase in a non-pathogenic bacterium. A single sortase A homologue, no sortase B, and 26 potential target proteins could be identified in the genome of *L. plantarum* WCFS1. Although several motifs and/or repeats could be detected in these target-proteins, none of these proteins has a clear functional annotation. A sortase gene replacement mutant did not display altered growth characteristics as compared to the wild-type strain under laboratory conditions. In contrast to the wild-type strain, the *srtA* mutant could not agglutinate *S. cerevisiae* cells *in vitro*. Remarkably, the *in vivo* persistence of the *srtA* mutant in the mouse GI-tract was not altered as compared to the wild-type strain. This finding seems to contrast with the observation that *srtA* mutants of pathogenic bacteria show decreased virulence. However, experiments to determine the persistence of these pathogens in terms of viable counts rather than virulence were usually not performed, which could

explain the contrasting conclusion drawn in *L. plantarum*. Only recently, the recovery of reduced viable counts for a *Staphylococcus aureus srtA* mutant as compared to the wild type from an infected animal model were reported (44). However, these counts were performed on *Staphylococcus aureus* cells that were in a post-invasion stage of infection, i.e., inside infected organs. Hence, it is difficult to seperate the effects caused by reduced persistence and/or virulence in this experiment. Therefore, these results do not exclude the possibility that sortase mutants of pathogenic bacteria can still adhere, but are impaired in an invasion specific factor. However, in *Listeria monocytogenes* the protein internalin (InlA) mediates bacterial adhesion and invasion of epithelial cells in the human intestine through interaction with its host cell receptor E-cadherin (24, 36). Notably, InlA contains an LPXTG-like motif near its C-terminus, suggesting that in this species the sortase function is likely to play a key role during very early stages of *Listeria monocytogenes* virulence. The results described here, indicate that persistence and adherence of *L. plantarum* in the mouse GI-tract are not correlated to LPQTXE-dependent functions. This is in apparent contrast with our observation that a *L. plantarum* gene disruption mutant in lp_2940, which encodes an LPQTXE-motif containing protein (table 2A), showed a significantly reduced GI-tract persistence in a mouse model system (9). Although it can be assumed that the lp_2940 gene product is not covalently linked to peptidoglycan in the *srtA* mutant, it is possible that this protein is still located at the cellular surface through C-terminal anchoring. This could explain the contradicting observations on GI-tract persistence for the lp_2940 and *srtA* mutants. At present experiments are performed in our laboratory to verify this hypothesis. These experiments include comparison of the fate of the LPQTXE-motif containing proteins in the wild-type and *srtA* mutant strain by proteome analysis of the extracellular proteins. In addition, current experiments using the nuclease-based model protein that was previously employed to establish LPXTG-dependent cell-wall sorting in *Lactococcus lactis* (11), should provide direct evidence for the complete loss of sortase activity in NZ7104. Therefore, the latter experiment should also exclude the possible existence of a second, unrecognized sortase encoding gene in *L. plantarum* WCFS1.

Acknowledgements

The authors gratefully acknowledge Corinne Grangette, Catherine Desseyn-Daniel and Benoît Foligné from the Pasteur Institute (Lille, France) for help during the animal experiments. We thank Roger Bongers for the construction of pNZ1111 and Mieke Meijer for growth characteristic determination and performance of 3-primer PCR. We appreciate the help of Mark de Been and Quinta Helmer during bioinformatics searches for genes encoding sortases and its target proteins, and domain analysis of these proteins.

References

1. **Adlerberth, I., S. Ahrne, M. L. Johansson, G. Molin, L. A. Hanson, and A. E. Wold.** 1996. A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29. Appl. Environ. Microbiol. **62:**2244-51.

2. **Ahrne, S., S. Nobaek, B. Jeppsson, I. Adlerberth, A. E. Wold, and G. Molin.** 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. J. Appl. Microbiol. **85:**88-94.

3. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389-402.

4. **Barnett, T. C., and J. R. Scott.** 2002. Differential recognition of surface proteins in *Streptococcus pyogenes* by two sortase gene homologs. J. Bacteriol. **184:**2181-91.

5. **Bierne, H., S. K. Mazmanian, M. Trost, M. G. Pucciarelli, G. Liu, P. Dehoux, L. Jansch, F. Garcia-del Portillo, O. Schneewind, and P. Cossart.** 2002. Inactivation of the *srtA* gene in *Listeria monocytogenes* inhibits anchoring of surface proteins and affects virulence. Mol. Microbiol. **43:**869-81.

6. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513-1523.

7. **Bolken, T. C., C. A. Franke, K. F. Jones, G. O. Zeller, C. H. Jones, E. K. Dutton, and D. E. Hruby.** 2001. Inactivation of the *srtA* gene in *Streptococcus gordonii* inhibits cell wall anchoring of surface proteins and decreases *in vitro* and *in vivo* adhesion. Infect. Immun. **69:**75-80.

8. **Bron, P. A., C. Grangette, A. Mercenier, W. M. de Vos, and M. Kleerebezem.** 2004. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice (Chapter 6).

9. **Bron, P. A., M. Meijer, R. Bongers, W. M. de Vos, and M. Kleerebezem.** Competitive population dynamics of *ivi*-gene mutants of *Lactobacillus plantarum* in the gastrointestinal tract of mice (Chapter 7).

10. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138:**179-207.

11. **Dieye, Y., S. Usai, F. Clier, A. Gruss, and J. C. Piard.** 2001. Design of a protein-targeting system for lactic acid bacteria. J. Bacteriol. **183:**4157-66.

12. **Durbin, R., S. Eddy, A. Krogh, and G. Mitchison.** 1998. Biological Sequence Analysis: Probabilistic Models of Proteins and Nucleic Acids. Cambridge University Press.

13. **Ferain, T., D. Garmyn, N. Bernard, P. Hols, and J. Delcour.** 1994. *Lactobacillus plantarum ldhL* gene: overexpression and deletion. J. Bacteriol. **176:**596-601.

14. **Fischetti, V. A., V. Pancholi, and O. Schneewind.** 1990. Conservation of a hexapeptide sequence in the anchor region of surface proteins from gram-positive cocci. Mol. Microbiol. **4:**1603-5.

15. **Garandeau, C., H. Reglier-Poupet, I. Dubail, J. L. Beretti, P. Berche, and A. Charbit.** 2002. The sortase SrtA of *Listeria monocytogenes* is involved in processing of internalin and in virulence. Infect. Immun. **70:**1382-90.

16. **Heilig, H. G., E. G. Zoetendal, E. E. Vaughan, P. Marteau, A. D. Akkermans, and W. M. de Vos.** 2002. Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl. Environ. Microbiol. **68:**114-23.

17. **Ilangovan, U., H. Ton-That, J. Iwahara, O. Schneewind, and R. T. Clubb.** 2001. Structure of sortase, the transpeptidase that anchors proteins to the cell wall of *Staphylococcus aureus*. Proc. Natl. Acad. Sci. U.S.A. **98:**6056-61.

18. **Janulczyk, R., and M. Rasmussen.** 2001. Improved pattern for genome-based screening identifies novel cell wall-attached proteins in gram-positive bacteria. Infect Immun **69:**4019-26.

19. **Jonsson, I. M., S. K. Mazmanian, O. Schneewind, T. Bremell, and A. Tarkowski.** 2003. The role of *Staphylococcus aureus* sortase A and sortase B in murine arthritis. Microbes. Infect. **5:**775-80.

20. **Jonsson, I. M., S. K. Mazmanian, O. Schneewind, M. Verdrengh, T. Bremell, and A. Tarkowski.** 2002. On the role of *Staphylococcus aureus* sortase and sortase-catalyzed surface protein anchoring in murine septic arthritis. J. Infect. Dis. **185:**1417-24.

21. **Josson, K., T. Scheirlinck, F. Michiels, C. Platteeuw, P. Stanssens, H. Joos, P. Dhaese, M. Zabeau, and J. Mahillon.** 1989. Characterization of a gram-positive broad-host-range plasmid isolated from *Lactobacillus hilgardii*. Plasmid **21:**9-20.

22. **Kharat, A. S., and A. Tomasz.** 2003. Inactivation of the *srtA* gene affects localization of surface proteins and decreases adhesion of *Streptococcus pneumoniae* to human pharyngeal cells *in vitro*. Infect. Immun. **71:**2758-65.

23. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U.S.A. **100:**1990-5.

24. **Lecuit, M., S. Vandormael-Pournin, J. Lefort, M. Huerre, P. Gounon, C. Dupuy, C. Babinet, and P. Cossart.** 2001. A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. Science **292:**1722-5.

25. **Lee, S. F., and T. L. Boran.** 2003. Roles of sortase in surface expression of the major protein adhesin P1, saliva-induced aggregation and adherence, and cariogenicity of *Streptococcus mutans*. Infect. Immun. **71:**676-81.

26. **Mazmanian, S. K., G. Liu, E. R. Jensen, E. Lenoy, and O. Schneewind.** 2000. *Staphylococcus aureus* sortase mutants defective in the display of surface proteins and in the pathogenesis of animal infections. Proc. Natl. Acad. Sci. U.S.A. **97:**5510-5.

27. **Mazmanian, S. K., G. Liu, H. Ton-That, and O. Schneewind.** 1999. *Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall. Science **285:**760-3.

28. **Mazmanian, S. K., H. Ton-That, and O. Schneewind.** 2001. Sortase-catalysed anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. Mol. Microbiol. **40:**1049-57.

29. **Mazmanian, S. K., H. Ton-That, K. Su, and O. Schneewind.** 2002. An iron-regulated sortase anchors a class of surface protein during *Staphylococcus aureus* pathogenesis. Proc. Natl. Acad. Sci. U.S.A. **99:**2293-8.

30. **Navarre, W. W., and O. Schneewind.** 1994. Proteolytic cleavage and cell wall anchoring at the LPXTG motif of surface proteins in gram-positive bacteria. Mol. Microbiol. **14:**115-21.

31. **Navarre, W. W., and O. Schneewind.** 1999. Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol. Mol. Biol. Rev. **63:**174-229.

32. **Osaki, M., D. Takamatsu, Y. Shimoji, and T. Sekizaki.** 2002. Characterization of *Streptococcus suis* genes encoding proteins homologous to sortase of gram-positive bacteria. J. Bacteriol. **184:**971-82.

33. **Pallen, M. J., A. C. Lam, M. Antonio, and K. Dunbar.** 2001. An embarrassment of sortases - a richness of substrates? Trends Microbiol. **9:**97-102.

34. **Roos, S., and H. Jonsson.** 2002. A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. Microbiology **148:**433-42.

35. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

36. **Schubert, W. D., C. Urbanke, T. Ziehm, V. Beier, M. P. Machner, E. Domann, J. Wehland, T. Chakraborty, and D. W. Heinz.** 2002. Structure of internalin, a major invasion protein of *Listeria monocytogenes*, in complex with its human receptor E-cadherin. Cell **111:**825-36.

37. **Tannock, G. W., A. Tilsala-Timisjarvi, S. Rodtong, J. Ng, K. Munro, and T. Alatossava.** 1999. Identification of *Lactobacillus* isolates from the gastrointestinal tract, silage, and yoghurt by 16S-23S rRNA gene intergenic spacer region sequence comparisons. Appl. Environ. Microbiol. **65:**4264-7.

38. **Ton-That, H., G. Liu, S. K. Mazmanian, K. F. Faull, and O. Schneewind.** 1999. Purification and characterization of sortase, the transpeptidase that cleaves surface proteins of *Staphylococcus aureus* at the LPXTG motif. Proc. Natl. Acad. Sci. U.S.A. **96:**12424-9.

39. **Ton-That, H., S. K. Mazmanian, K. F. Faull, and O. Schneewind.** 2000. Anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. Sortase catalyzed in vitro transpeptidation reaction using LPXTG peptide and NH(2)-Gly(3) substrates. J. Biol. Chem. **275:**9876-81.

40. **Ton-That, H., and O. Schneewind.** 1999. Anchor structure of staphylococcal surface proteins. IV. Inhibitors of the cell wall sorting reaction. J. Biol. Chem. **274:**24316-20.

41. **Ton-That, H., and O. Schneewind.** 2003. Assembly of pili on the surface of *Corynebacterium diphtheriae*. Mol. Microbiol. **50:**1429-38.

42. **Vaughan, E. E., M. C. de Vries, E. G. Zoetendal, K. Ben-Amor, A. D. Akkermans, and W. M. de Vos.** 2002. The intestinal LABs. Antonie Van Leeuwenhoek **82:**341-52.

43. **Vesa, T., P. Pochart, and P. Marteau.** 2000. Pharmacokinetics of *Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* KLD, and *Lactococcus lactis* MG 1363 in the human gastrointestinal tract. Aliment. Pharmacol. Ther. **14:**823-8.

44. **Weiss, W. J., E. Lenoy, T. Murphy, L. Tardio, P. Burgio, S. J. Projan, O. Schneewind, and L. Alksne.** 2004. Effect of *srtA* and *srtB* gene expression on the virulence of *Staphylococcus aureus* in animal models of infection. J. Antimicrob. Chemother.

45. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved m13 phage cloning vectors and host strains; nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103-119.

Chapter 9

Summary, Concluding Remarks and Future Perspectives

PETER A. BRON

This thesis describes the identification and functional analysis of *Lactobacillus plantarum* promoters and genes that play a role during intestinal passage of this lactic acid bacterium (LAB). During these studies the mouse gastrointestinal tract (GI-tract) and *in vitro* studies were utilized as models for the human situation. The molecular tools developed and the most important results obtained during this PhD project, as well as the application potential of the identified promoters, and the future direction in the research field of intestinal microbiology is discussed below.

Tool Development

At the start of the research described in this thesis, relatively few molecular tools were available for *L. plantarum.* Therefore, our initial efforts focused on expansion of the molecular toolbox available for this bacterium. Notably, the number of selectable antibiotic resistance markers in this organism is limited, with only examples of the effective use of chloramphenicol and erythromycin resistance markers available in literature (7, 11). Therefore, a strategy aiming at expansion of this number of selectable markers was initiated and focused on implementation of *alr*, encoding alanine racemase, as a complementation marker in *L. plantarum*. Since an isogenic mutant of *L. plantarum* carrying an *alr* deletion (Δ*alr*) showed auxotrophy for D-alanine (11), plasmids carrying a heterologous *alr* gene were constructed and could be selected, based on complementation of the D-alanine auxotrophy in the *L. plantarumΔalr* strain. Moreover, the *alr* complementation marker appeared to be selectable at single copy expression levels, allowing its use as a chromosomal marker in *L. plantarum* (Chapter 2).

A second molecular tool developed was a promoter-probe for the detection of conditionally active promoters in *L. plantarum*. The β-glucuronidase encoding *gusA* gene of *Escherichia coli*, has previously been used to study promoters originating from *Lactococcus lactis* (18). Since β-glucuronidase encoding genes were not found in the *L. plantarum* WCFS1 genome (14), the potential of the *E. coli gusA* gene as a promoter-probe in *L. plantarum* was initially investigated. However, *L. plantarum* harboring a plasmid carrying *gusA* under control of a constitutive promoter, displayed severe fluctuation of blue-coloring on X-gluc containing plates, preventing the efficient application of this gene as a promoter-probe vector in *L. plantarum*. Since we already exploited the *alr* gene as selection marker, the applicability of this gene as an alternative promoter probe for the selection of conditionally active promoters was investigated. An *alr* complementation library was constructed in the *L. plantarumΔalr* strain and an exemplary promoter screen demonstrated the effectiveness of the alanine racemase complementation screening method for the identification of conditional, high-salt inducible promoters (Chapter 3).

A third molecular tool that was developed during the course of this PhD project allowed the implementation of recombination-based *in vivo* expression technology (R-IVET) in *L. plantarum*. Previously, the *tnpR-res* resolvase system (19) has been applied to trap promoter activities in R-IVET experiments in several pathogenic bacteria (1). Therefore, initial attempts aimed at implementation of this system in *L. plantarum*. A *res-ery-res* cassette was successfully integrated into the chromosome of this bacterium and a promoterless copy of the *tnpR* gene was cloned on a low-copy plasmid. Despite the successful cloning of the endogenous, highly active *ldhL1* promoter upstream of *tnpR*, excision of the *res-ery-res* cassette from the chromosome of *L. plantarum* was never observed. These experiments indicate that the *tnpR* resolvase is not functionally expressed in *L. plantarum* under the conditions applied during the experiments. Therefore, an alternative system was chosen to implement R-IVET in *L. plantarum*, which involved the *cre-loxP* system (2). This system was previously demonstrated to be functional in another LAB, *Lactococcus lactis* (5). Hence, a *loxP-ery-loxP* cassette was integrated into the chromosome of *L. plantarum* and a promoterless copy of *cre* was cloned on a low-copy vector. This system appeared to be functional in *L. plantarum*, as *ldhL*-promoter driven expression of the *cre* gene led to the irreversible excision of the *loxP-ery-loxP* cassette from the chromosome (Chapter 6). Notably, the R-IVET system described here for *L. plantarum* is expected to allow the *in vivo* identification of promoters in related organisms, during residence in their complex niches. To this end, a project has recently been initiated that aims at the identification of *Lactococcus lactis* genes that are induced during cheese ripening (van Hylckama Vlieg, personal communication).

A fourth tool that was developed during this project is the mutagenesis vector pNZ7101 that allows single-step selection of *L. plantarum* gene replacement mutants (Chapter 7). Especially the effective blunt-cloning of PCR products in this vector simplifies construction design and improves the rate of successful construction of gene replacement vectors. The two markers available on this vector allow either cat or *ery* gene replacement strategies, which can be selected directly by the differential evaluation of the corresponding chloramphenicol- and erythromycin-resistance or sensitivity. However, using this vector the *cat* or *ery* gene remains integrated in the chromosome after a double cross-over event, hampering the construction of double mutants. To overcome this drawback, the functional implementation of the *cre-loxP* system in *L. plantarum* has recently been exploited to construct a pNZ7101 derived mutagenesis vector that can be used for the sequential mutation of several loci in the same genetic background, without the loss of selectable antibiotic resistance markers in the resulting strain. To this end, 2 different mutant *loxP* sites (*loxP*66 and *loxP*71), carrying a single nucleotide mutation relative to the native *loxP* site (17, 23), were introduced into pNZ7101 at positions flanking the *cat* gene. Using this pNZ7101 derivative, a double cross-over event on the chromosome of *L. plantarum* results in

replacement of the target gene by the *cat* gene flanked by the *loxP*66 and *loxP*71 sequences. Subsequently, an unstable plasmid expressing the *cre* gene can be introduced in these mutant strains, resulting in excision of the *cat* marker from the chromosome by recombination of the two mutant *loxP* sequences. Finally, overnight culturing of these transformants without selection for the unstable plasmid results in the effective curing of this *cre*-encoding plasmid. The recombination of the *loxP*66 and *loxP*71 sites results in a chromosomal *loxP* site that carries two single nucleotide mutations and as a consequence is not recognized anymore by the *cre* encoded resolvase (17, 23). In the resulting strain the complete target gene is deleted from the chromosome and replaced by a single *loxP*6671 double mutant sequence, allowing the construction of double gene deletion mutant by performing a second mutagenesis round following the same strategy (Lambert *et al.*, unpublished data). Overall, the tools presented in this thesis have contributed significantly to the development of a basic molecular tool-box for *L. plantarum*, and these basic tools are currently exploited for the development of more sophisticated tools.

Characteristics of the promoter and gene identification approaches

Three different approaches have been employed in the identification of genes and promoters that are important during intestinal passage and conditions. Two of these approaches, namely the *alr* complementation screen (Chapter 3) and DNA micro-array technology (Chapter 5), focus on the genetic response of *L. plantarum* to bile during growth on solid media. The *alr* complementation approach is a genetic screen that identifies bile-inducible promoters by monitoring the conditional colony forming capability on plates containing bile. Since the alanine racemase encoded by *alr* is essential for growth, expression of *alr* is required continuously during growth under the condition evaluated. Therefore, the promoters and corresponding genes identified by such a screening procedure are a reflection of the bacterial response over an extended time-range. In contrast, the transcript levels assessed with DNA micro-arrays represent the abundance of mRNAs at one specific time point and thus reflect the response to the physico-chemical conditions present at that time point. Consequently, these analyses provide a snap-shot of the bacterial expression profile, namely at the time of RNA isolation. Hence, within the experimental set-up used here, it could very well be that the *alr* complementation screen has identified genes involved in the initial stress response of *L. plantarum* upon bile treatment, while DNA micro-array analyses have unraveled the later, bile adaptive response. These subtle differences could explain the low level of similarity found in the outcome of these two screens. Nevertheless, these complementary screens both clearly demonstrated the major impact of bile on the architecture of the *L. plantarum* cell envelope. Additional DNA micro-array experiments that aim at elucidation of both the transient, initial bile stress response as well as the subsequent adaptive response in *L. plantarum* could

provide more detailed insight in the proposed possibilities. Such experiments could simply be performed in batch cultures to which at a chosen moment bile is added and transcript profiles are determined for samples taken at various time points following bile addition. A common characteristic between the *alr* complementation (Chapter 3) and R-IVET screen (Chapter 6) lies within the fact that both screens allow the identification of promoters of which activation has occurred in the past. Nevertheless, a major difference is that R-IVET can be employed for the identification of promoters of which the expression is only temporarily induced in a specific compartment of the host's GI-tract, while continuous expression is required for positive selection in the *alr* complementation screen.

The results of the R-IVET screen (Chapter 6) indicated that the distribution of the *in vivo* induced (*ivi*) genes over the generally recognized classes of main biological functions appeared to be random, which is in agreement with the outcome of (R-)IVET screens in pathogens (1, 16). A slight overrepresentation of R-IVET genes is observed around the origin of replication (50' to 10') as compared to the rest of the genome (Fig. 1). These results corroborate earlier suggestions that the proteins encoded in the so-called sugar island, which is located in this region, could provide important functional characteristics for the interaction of *L. plantarum* with its environment (14). A large number of the functions and pathways identified by R-IVET in *L. plantarum* have previously been identified in pathogens as being important *in vivo*, during infection (Chapter 6). The striking amount of parallels between the pathogenic and non-pathogenic *in vivo* response to contact with the host suggests that survival or persistence rather than virulence is the explanation for the importance of these genes during host residence.

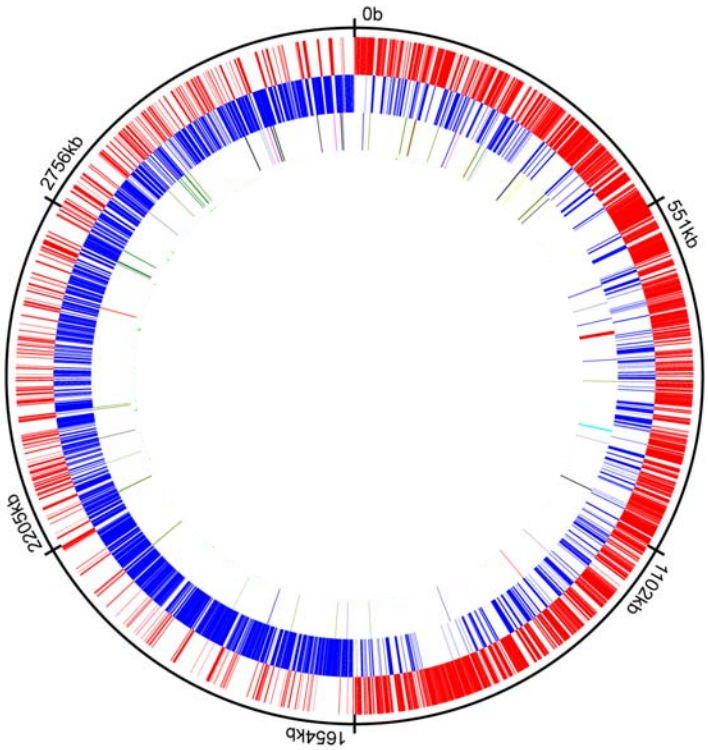


Fig. 1: Using R-IVET 72 *L. plantarum* genes could be identified as *in vivo* induced (*ivi*) during passage of the mouse GI-tract. The chromosomal localization of these *ivi* genes is represented in the inner circle, while the outer two circles represent the ORFs on the positive (outer circle) and negative (middle circle) chromosomal DNA strand.

Most important scientific achievements during the project

Three main strategies that allow the identification of conditionally active genes have been implemented in *L. plantarum*, namely an *in vitro* screen to trap bile-inducible promoters (Chapter 2 and 3), DNA micro-array technology to identify mRNAs that are up-regulated in the presence of bile (Chapter 5), and a R-IVET screen to select promoters that are induced in the GI-tract of a mouse model system (Chapter 6). Matching of the genes identified with these three strategies (Chapters 4, 5 and 6) revealed two genes, encoding an integral membrane protein and an argininosuccinate synthase, which appeared to be induced by bile *in vitro* as well as *in vivo* in the mouse GI-tract. As the duodenum is the intestinal site of bile release, expression of these two genes at this specific location in the host's GI-tract was investigated using quantitative reverse transcription PCR (qRT-PCR). The results demonstrated that the expression levels of these two genes were dramatically increased in *L. plantarum* cells isolated from the mouse duodenum relative to cells grown on standard laboratory medium (Chapter 4). Furthermore, gene disruption mutants were constructed in 12 of the *ivi* genes identified by the R-IVET screen. The effect of the mutations on the survival and persistence of *L. plantarum* were investigated using quantitative PCR (qPCR) to monitor the relative population dynamics of the group of mutants in faecal samples

after passage through the GI-tract of mice. This qPCR approach allows the assessment of groups of mutants in complex competition experiments, generating a large amount of relative persistence data for individual mutants. Notably, the number of animal models required for significant assessment of the relative persistence in such competition experiments is drastically lower than for binary experiments comparing only a single mutant with the wild-type strain. In addition, it can be anticipated that the relative abundance of individual mutants is strongly influenced by the number of competitors it encounters in its niche. Therefore, relatively minor persistence reduction scored for specific mutants in a binary comparison with its corresponding wild-type strain, could be amplified in a more complex and thus more competitive system. In contrast, a drawback of large and complex mixed-mutant populations could be that the phenotype of a specific mutant is masked or compensated by other members of the mixed population. Examples of such inter-bacterial compensation have been reported for the simultaneous analysis of larger numbers of STM mutants in a single animal model (16). Nevertheless, our competition experiments led to the identification of three *ivi* gene mutants that displayed 100- to 1000-fold reduced abundance in the faecal samples as compared to other mutant strains. These data indicate that the products of the disrupted genes (an orphan IIC transport component of a cellobiose PTS system, an extracellular protein that contains an LPQTNE motif involved in anchoring of this protein to the bacterial cell wall, and a copper transporting ATPase) play a key-role in the survival and/or persistence of *L. plantarum* during passage of the GI-tract (Chapter 7). Especially remarkable was the identification of *copA*, since the encoded protein (CopA) has high homology with a protein in *Listeria monocytogenes* (CtpA, 56% identity) that has an established role in virulence and persistence of this pathogen in the GI-tract (8, 9). Finally, a *L. plantarum* gene replacement mutant was constructed that lacks a functional copy of the *srtA* gene, which encodes the transpeptidase named sortase that catalyzes the covalent attachment of LPQTNE-motif-containing proteins to the cell wall. Although the *srtA* mutant strain had completely lost the capacity to agglutinate *Saccharomyces cerevisiae*, the *in vivo* persistence and/or survival characteristics were virtually identical to those of the wild-type strain (Chapter 8). This finding seems to contrast with the observation that *srtA* mutants of pathogenic bacteria show decreased virulence (3, 13, 15, 21). However, experiments to determine the persistence of these pathogens in terms of viable counts rather than virulence were usually not performed, which could explain the contrasting conclusion drawn in *L. plantarum*.

Potential applications

The obtained knowledge on promoter elements regulating gene expression of the food-grade bacterium *L. plantarum* in the GI-tract (Chapter 6) could have application possibilities, especially since these bacteria have great potential to serve as delivery vehicles of health-promoting or therapeutic compounds to the human GI-tract (10, 20). Recent efforts have resulted in the identification of a functional bacteriophage-derived lytic cassette for *L. plantarum*. Following introduction of the plasmid-based, *nisA* promoter controlled lytic cassette in *L. plantarum*, nisin-mediated induction of expression of the *hol-lys* genes resulted in complete lysis (Hols *et al*., unpublished data and Fig. 2A). Our R-IVET approach has provided the required promoters that allow the construction of *L. plantarum* based dedicated GI-tract delivery vehicles that only express certain desired functions *in situ*. Such a function could be virtually any molecule of interest, e.g. enzymes, antigens or proteins involved in mannose-specific adhesion. Moreover, combination of the *in situ* activated R-IVET promoters with the lytic system identified, might generate LAB strains that specifically release their cytoplasmic content in the GI-tract (Fig. 2B). Complete *in vivo* lysis using such a delivery vehicle could generate highly effective and tightly controlled release of a desired factor, but could also intrinsically provide the biological-containment control that would facilitate application of health-promoting genetically modified organisms in foods. The latter aspect is directly related to the fact that this strategy would prevent the release of living genetically modified bacteria into the environment. For this reason, the R-IVET results and their potential application possibilities have been protected by a WCFS-patent application (4).

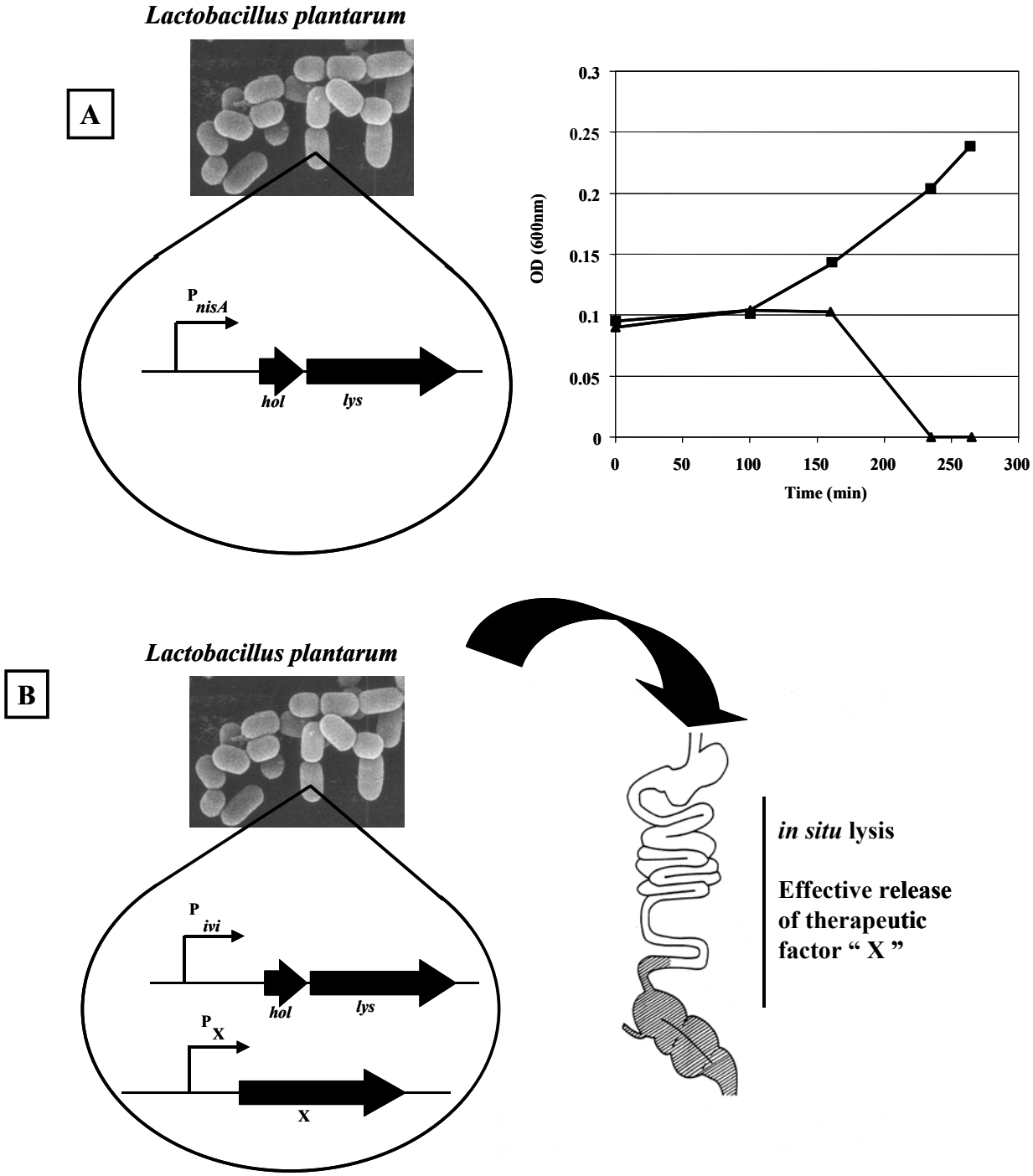


Fig. 2: Application potential of R-IVET promoters identified in *L. plantarum*. Panel A: Expression of a *hol-lys* cassette in *L. plantarum* under control of the nisin-inducible promoter P_{nisA}. In the absence of the inducer molecule nisin, this strain is capable of normal growth (squares), while the strain displays lytic behavior after the addition of nisin (triangles). Panel B: Expression of the *hol-lys* cassette under control of a R-IVET promoter (P_{ivi}) could lead to *in situ* lysis in the GI-tract. Constitutive expression of a therapeutic factor from a second plasmid would then result in effective delivery of this factor in the GI-tract. The authors gratefully acknowledge Pascal Hols for construction of Fig. 2A and the opportunity to use this figure prior to publication.

Future directions in intestinal microbiology

Current follow-up qRT-PCR studies aim at the confirmation of gene-induction of several *L. plantarum* genes initially identified with our R-IVET screen (Marco *et al*., unpublished data). Analogous to the match identified between the *alr*-based *in vitro* bile response screen (Chapter 4) and the R-IVET screen (Chapter 6), current and future micro-array based transcription profiling under a variety of *in vitro* conditions could reveal the conditions responsible for the *in situ* induction of specific genes in the complex environment of the mouse GI-tract. The *in vitro* and *in vivo* expression of these genes could subsequently be analyzed in more detail by gene-specific qRT-PCR. This approach would potentially reveal the specific environmental cue involved in *in situ* induction in the GI-tract and could eventually elucidate the regulatory mechanism(s) involved. In analogy, the application of gene-specific qRT-PCR on samples derived from various parts of the GI-tract could unravel the geographical differentiation of *L. plantarum* gene expression along the GI-tract, i.e. specific induction in the stomach, regions of the small intestine or colon. More detailed insight on the exact spatial distribution of *in situ* gene activation in the GI-tract might allow the construction of highly site specific delivery vehicles (Fig. 2). Another promising development is the optimization of bacterial RNA isolation protocols from GI-tract samples from conventional mice fed with *L. plantarum* (Marco *et al*., unpublished data) or human cancer patients who volunteered to consume an oatmeal-based drink containing high numbers of *L. plantarum* prior to surgery (de Vries *et al*. unpublished data). Although it is technically difficult to isolate high quality bacterial mRNAs from these samples, such RNA samples originating from an animal tissue could be analyzed using DNA micro-array technology, providing extremely valuable information on the *in situ* expression levels of thousands of bacterial genes. Recent results indicate that this approach is feasible in human subjects who have consumed *L. plantarum* (de Vries *et al*., unpublished data). Moreover, comparison of bacterial responses in samples from the GI-tract from animal models and of human origin could provide insight in the anticipated partial overlap in the *L. plantarum* response towards residence in the GI-tract of different hosts. In analogy, interesting possibilities to assess the overlap in the response of *L. plantarum* towards contact with an animal model and the human GI-tract, could come from the 12 *L. plantarum ivi* gene mutants constructed (Chapter 7). Several of these mutants displayed reduced abundance in faecal samples of mice. A similar experimental set-up could reveal whether the reduction in abundance of these mutants can also be demonstrated in a human trial. For ethical reasons this would probably require the construction of clean deletion mutants prior to human consumption. However, since a vector for the high-through-put construction of clean deletion mutants in *L. plantarum* has recently been developed (Lambert *et al*., unpublished data), these constructions are anticipated to be relatively straight-forward.

(R-)IVET has been employed for the assessment of the genetic response of several bacteria towards contact with their host's GI-tract, including many (food-associated) pathogens (1, 16) and the food-grade microbes *L. reuteri* (22) and *L. plantarum* (Chapter 6). Moreover, DNA micro-array technology has been applied by Hooper and co-workers on the host side using germ-free (gnotobiotic) mice as a host to elucidate host-responses to microbe interactions in the GI-tract. Several elegant studies have revealed the molecular response of gastrointestinal epithelial cells of germ-free mice to colonization with the commensal bacterium *Bacteroides thetaiotaomicron* (12). An important question that still remains to be answered is to what extend the data obtained on host and bacterial gene expression using gnotobiotic animal models can be extrapolated to the situations in conventionally raised animals and, ultimately, humans. Notably, no (R-)IVET screens have been performed in commensal bacteria such as *B. thetaiotaomicron*, *L. johnsonii* or *L. gasseri*. It would be interesting to compare the *ivi* genes identified in a commensal with those previously found in food-grade and pathogenic bacteria. Such an approach would reveal whether the parallels in the bacterial responses towards contact with the host GI-tract found between transient food-grade and pathogenic bacteria (Chapter 6), can be extrapolated to residential, commensal bacteria.

In conclusion, the genome-wide transcript profiling approaches that have been performed to date have provided us with clues of the possible role of individual host and bacterial genes during host-microbe interactions (6). Combination of bacterium and host transcriptomes could generate the knowledge required for the construction of first generation molecular models of host-microbe interactions. These models would allow more pinpointed future experiments, designed on basis of a molecular interaction hypothesis. Since GI-tract bacteria like *L. plantarum* and *B. thetaiotaomicron* are genetically accessible, gene deletion and overexpression mutants can be constructed conveniently, and can be employed to study the effect of a single bacterial gene on host gene expression. After identification of responsive host genes, knock-out hosts and/or RNA interference approaches might allow gene silencing on the host side of the spectrum, thereby allowing the study of the effect of single host gene mutations on the colonization of microbes. Ultimately, such studies may provide a detailed molecular knowledgebase that is required to understand the selective GI-tract colonization of commensals or symbionts, and could help to explain probiotic effects associated with LAB and related species at the molecular level. This level of insight would at the same time allow the rational selection or construction of improved probiotic strains, which would be designed to generate a specific, premeditated health-promoting effect in their consumer.

References

1. **Angelichio, M. J., and A. Camilli.** 2002. *In vivo* expression technology. Infect. Immun. **70:**6518-23.

2. **Austin, S., M. Ziese, and N. Sternberg.** 1981. A novel role for site-specific recombination in maintenance of bacterial replicons. Cell **25:**729-36.

3. **Bierne, H., S. K. Mazmanian, M. Trost, M. G. Pucciarelli, G. Liu, P. Dehoux, L. Jansch, F. Garcia-del Portillo, O. Schneewind, and P. Cossart.** 2002. Inactivation of the *srtA* gene in *Listeria monocytogenes* inhibits anchoring of surface proteins and affects virulence. Mol. Microbiol. **43:**869-81.

4. **Bron, P. A., W. M. de Vos, and M. Kleerebezem.** 2004. Site-specific intestinal delivery and/or production of biologically active substances. **Patent file number PCT/NL03/00733**.

5. **Campo, N., M. L. Daveran-Mingot, K. Leenhouts, P. Ritzenthaler, and P. Le Bourgeois.** 2002. Cre-*loxP* recombination system for large genome rearrangements in *Lactococcus lactis*. Appl. Environ. Microbiol. **68:**2359-67.

6. **de Vos, W. M., P. A. Bron, and M. Kleerebezem.** 2004. Post-Genomics of Lactic Acid and Other Bacteria to Discover Gut Functionality. in press (Current Opinion in Biotechnology).

7. **Ferain, T., J. N. Hobbs, Jr., J. Richardson, N. Bernard, D. Garmyn, P. Hols, N. E. Allen, and J. Delcour.** 1996. Knockout of the two *ldh* genes has a major impact on peptidoglycan precursor synthesis in *Lactobacillus plantarum*. J. Bacteriol. **178:**5431-7.

8. **Francis, M. S., and C. J. Thomas.** 1997. The *Listeria monocytogenes* gene *ctpA* encodes a putative P-type ATPase involved in copper transport. Mol. Gen. Genet. **253:**484-91.

9. **Francis, M. S., and C. J. Thomas.** 1997. Mutants in the CtpA copper transporting P-type ATPase reduce virulence of *Listeria monocytogenes*. Microb. Pathog. **22:**67-78.

10. **Grangette, C., H. Muller-Alouf, M. Geoffroy, D. Goudercourt, M. Turneer, and A. Mercenier.** 2002. Protection against tetanus toxin after intragastric administration of two recombinant lactic acid bacteria: impact of strain viability and *in vivo* persistence. Vaccine **20:**3304-9.

11. **Hols, P., C. Defrenne, T. Ferain, S. Derzelle, B. Delplace, and J. Delcour.** 1997. The alanine racemase gene is essential for growth of *Lactobacillus plantarum*. J. Bacteriol. **179:**3804-7.

12. **Hooper, L. V., M. H. Wong, A. Thelin, L. Hansson, P. G. Falk, and J. I. Gordon.** 2001. Molecular analysis of commensal host-microbial relationships in the intestine. Science **291:**881-4.

13. **Jonsson, I. M., S. K. Mazmanian, O. Schneewind, M. Verdrengh, T. Bremell, and A. Tarkowski.** 2002. On the role of *Staphylococcus aureus* sortase and sortase-catalyzed surface protein anchoring in murine septic arthritis. J. Infect. Dis. **185:**1417-24.

14. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of Lactobacillus plantarum WCFS1. Proc. Natl. Acad. Sci. U.S.A. **100:**1990-5.

15. **Lee, S. F., and T. L. Boran.** 2003. Roles of sortase in surface expression of the major protein adhesin P1, saliva-induced aggregation and adherence, and cariogenicity of *Streptococcus mutans*. Infect. Immun. **71:**676-81.

16. **Mahan, M. J., D. M. Heithoff, R. L. Sinsheimer, and D. A. Low.** 2000. Assessment of bacterial pathogenesis by analysis of gene expression in the host. Annu. Rev. Genet. **34:**139-164.

17. **Oberdoerffer, P., K. L. Otipoby, M. Maruyama, and K. Rajewsky.** 2003. Unidirectional Cre-mediated genetic inversion in mice using the mutant *loxP* pair lox66/lox71. Nucleic Acids Res. **31:**e140.

18. **Platteeuw, C., G. Simons, and W. M. de Vos.** 1994. Use of the *Escherichia coli* beta-glucuronidase (*gusA*) gene as a reporter gene for analyzing promoters in lactic acid bacteria. Appl. Environ. Microbiol. **60:**587-93.

19. **Reed, R. R., and N. D. Grindley.** 1981. Transposon-mediated site-specific recombination *in vitro*: DNA cleavage and protein-DNA linkage at the recombination site. Cell **25:**721-8.

20. **Steidler, L., W. Hans, L. Schotte, S. Neirynck, F. Obermeier, W. Falk, W. Fiers, and E. Remaut.** 2000. Treatment of murine colitis by *Lactococcus lactis* secreting interleukin- 10. Science **289:**1352-1355.

21. **Ton-That, H., and O. Schneewind.** 2003. Assembly of pili on the surface of *Corynebacterium diphtheriae*. Mol. Microbiol. **50:**1429-38.

22. **Walter, J., N. C. Heng, W. P. Hammes, D. M. Loach, G. W. Tannock, and C. Hertel.** 2003. Identification of *Lactobacillus reuteri* genes specifically induced in the mouse gastrointestinal tract. Appl. Environ. Microbiol. **69:**2044-51.

23. **Zhang, Z., and B. Lutz.** 2002. Cre recombinase-mediated inversion using lox66 and lox71: method to introduce conditional point mutations into the CREB-binding protein. Nucleic Acids Res. **30:**e90.

Samenvatting

Melkzuurbacteriën worden wereldwijd toegepast in de industrie tijdens de productie van gefermenteerde voedselproducten. Vanwege de frequente consumptie van zuivel, olijven en worst, worden dagelijks grote hoeveelheden melkzuurbacteriën geconsumeerd door mensen. Vanwege deze lange historie van toepassing in producten bedoeld voor humane consumptie worden de meeste melkzuurbacteriën gezien als voedselveilig. Bovendien kunnen melkzuurbacteriën gebruikt worden voor het afleveren van gezondheidsbevorderende of therapeutische componenten in het humane maagdarmkanaal. *Lactobacillus plantarum* is een melkzuurbacterie die wordt gebruikt tijdens voedselfermentaties van onder andere zuivel, vlees en groenten. Naast de aanwezigheid van *L. plantarum* in ons dieet wordt deze melkzuurbacterie vaak aangetroffen als natuurlijke bewoner van het maagdarmkanaal. De complete genoomsequentie van de *L. plantarum* stam WCFS1 is recentelijk bepaald. Deze stam is nauw verwant aan de *L. plantarum* stam NCIMB 8826. Deze laatstgenoemde stam overleeft de passage van de humane maag zeer effectief, bereikt vervolgens eenvoudig de dunne darm en is zelfs detecteerbaar in de dikke darm. Dit proefschrift beschrijft de identificatie en functionele analyse van *L. plantarum* WCFS1 promoters en genen die een rol spelen tijdens de passage van het maagdarmkanaal. Tijdens deze studies is gebruikt gemaakt van de muis en *in vitro* studies als modellen voor de situatie in de humane darm.

Er zijn drie strategieën geïmplementeerd in *L. plantarum* voor de identificatie van conditioneel actieve promoters, namelijk een *in vitro* selectie van promoters waarvan de activiteit is verhoogd in aanwezigheid van galzouten (Hoofdstuk 2 en 3), DNA micro-array technologie ter identificatie van omhoog gereguleerde mRNAs in de aanwezigheid van galzouten (Hoofdstuk 5), en een resolvase gebaseerde *in vivo* expressie technologie (R-IVET) aanpak om promoters te selecteren die aangeschakeld worden in het maagdarmkanaal van een muis (Hoofdstuk 6). De uitkomsten van de *in vitro* screen en de DNA micro-array experimenten reflecteerden duidelijk de grote impact van galzout op de celwand en membraan (Hoofdstuk 5). Verdere vergelijking van de uitkomsten van de drie strategieën onthulde 2 genen, coderend voor een integraal membraan eiwit en een argininosuccinate synthase, die zowel *in vitro* geïnduceerd worden door galzout als *in vivo* in het maagdarmkanaal van de muis. Het expressieniveau van deze 2 genen in het duodenum (de locatie in de darm waar galzouten vrijkomen) werd onderzocht met behulp van kwantitatieve reverse transcriptie PCR. De resultaten toonden aan dat de expressieniveaus van deze 2 genen veel hoger waren in *L. plantarum* cellen geïsoleerd uit het duodenum van een muis, vergeleken met cellen gekweekt op standaard laboratorium medium (Hoofdstuk 4). Vervolgens werden er deletiemutanten geconstrueerd in 12 van de genen die geïdentificeerd waren met R-IVET als geïnduceerd in de darm. Het effect van deze mutaties werd onderzocht gebruikmakend van kwantitatieve PCR waarmee de

relatieve populatie dynamiek van de *L. plantarum* mutanten gevolgd kon worden in fecale monsters na passage door het maagdarmkanaal van muizen. Deze PCR aanpak maakte het mogelijk om in een competitie experiment de persistentie en/of overleving van alle mutanten onderling te vergelijken in 1 muis model systeem. Deze competitie experimenten onthulden 3 mutanten die 100- tot 1000-voudig minder dominant aanwezig waren vergeleken met andere mutanten. Deze resultaten duiden erop dat de producten van de gedeleteerde genen (een IIC-transport component van een incompleet cellobiose PTS systeem, een extracellulair eiwit dat een LPQTNE motief bevat voor verankering van dit eiwit aan de bacteriële celwand en een koper transporterende ATPase) een belangrijke rol spelen in de maagdarmkanaal functionaliteit van *L. plantarum*. Vooral de identificatie van *copA* is opmerkelijk, daar het gecodeerde eiwit (CopA) een hoge homologie vertoont met een eiwit in *Listeria monocytogenes* (CtpA, 56% identiteit) met een bewezen rol in virulentie en persistentie van deze pathogeen in het maagdarmkanaal (Hoofdstuk 7). Als laatste is er een *L. plantarum* stam geconstrueerd waarin geen functionele kopie van het *srtA* gen meer aanwezig is. Dit gen codeert voor sortase dat de covalente koppeling van LPQTNE-motief bevattende eiwitten aan de bacteriële celwand katalyseert. Hoewel de *srtA* mutant de capaciteit om gistcellen (*Saccharomyces cerevisiae*) te agglutineren volledig verloren had, werd er geen verschil gevonden in de overlevings-karakteristieken van deze mutant in vergelijking met een controle stam tijdens passage van de muizendarm.

Concluderend kan gesteld worden dat het onderzoek in dit proefschrift een eerste blik werpt op het gedrag van *L. plantarum* in een complexe, ontoegankelijke niche zoals de darm. Dit soort studies zal leiden tot een gedetailleerd, moleculair inzicht in de overlevingsstrategieën van melkzuurbacteriën in de darm en kan helpen om hun gezondheidsbevorderende (probiotische) effecten te verklaren. Uiteindelijk kan dit soort mechanistisch inzicht zelfs gebruikt worden voor de rationele selectie of constructie van probiotische stammen.

Nawoord

Op de voorkant van dit proefschrift staat alleen mijn naam, maar geen van de hoofdstukken was geworden zoals ze nu zijn zonder de hulp van anderen. Daarom ben ik blij dat dit nawoord mij in staat stelt om hen hiervoor te bedanken. In de eerste plaats mijn begeleiders Michiel Kleerebezem en Willem de Vos. Michiel, jouw vermogen om mensen te motiveren, je kritische houding en je gedrevenheid hebben mij als wetenschapper, en daarmee ook dit boekje, voor een belangrijk deel gevormd. Vanaf het begin zaten we op één lijn en ik heb onze samenwerking dan ook als zeer prettig ervaren. Daarnaast denk ik met veel plezier terug aan enkele buitenlandse trips die we samen hebben mogen maken. Willem, je sturende rol en je kritische analyses van de resultaten hebben een grote bijdrage geleverd aan dit boekje. Bedankt voor het in mij gestelde vertrouwen!

Ondanks dat ik als WCFSer gestationeerd was op het NIZO, was er in de kelder nooit sprake van discriminatie. Ik had vanaf de eerste dag het gevoel in een team te werken. Ik zal niet proberen om iedereen bij naam te noemen vanwege het grote risico dat ik daarbij mensen vergeet, maar de vele co-auteurs boven de diverse hoofdstukken van dit boekje geven wel aan hoe belangrijk de rol van menig collega is geweest. Als je meer op je werk bent dan thuis, zijn ook de sociale aspecten belangrijk, zoals de borrels, Substitute-bezoekjes, whiskyfestivals, vakanties en avondjes stappen met collega's. Deze hebben veelvuldig, in de afgelopen vier en een half jaar, plaats gevonden en ik hoop dat de afronding van dit proefschrift en mijn vertrek naar Ierland geen definitief einde betekent voor deze contacten. De zes studenten die ik in de afgelopen 4 jaar heb mogen begeleiden hebben samen ongeveer 4 manjaren bijgedragen aan dit boekje, waarmee hun inbreng van grote waarde was. Saskia, Armand, Jolanda, Esther, Alexander en Mieke, hierbij wil ik jullie (nogmaals) bedanken voor jullie inzet. Ook het contact met mijn virtuele WCFS collega's was zeer positief. Tijdens de maandelijkse projectbijeenkomsten heerste altijd een informele sfeer en aan de discussies over onze projecten heb ik veel gehad.

I cordially thank Annick Mercenier for the opportunity to work in her group at the Pasteur Institute in Lille (France). Furthermore, I would like to acknowledge Catherine, Corinne and the rest of the IPL group for their help with the animal experiments, which dramatically increased the quality of this thesis! I also appreciate the fact that you introduced me to the social life in Lille, which made my three visits to France really enjoyable.

De keuze van mijn beide paranimfen dwingt mij om terug te denken aan vervlogen tijden. Patrick, nog zo kort geleden zaten we met zijn tweeën te ranzen op het wissellab. We hadden een perfecte balans tussen humor en hard werken gevonden en het was nog geoorloofd om een grap uit te halen waar het lab een beetje smerig van werd. Tegenwoordig is dit wel anders, met als dieptepunten de wekelijkse, vaste opruimdag, het feit dat de eerste plantarum-SOP is geschreven (niet door mij!) en de

(geënsceneerde?) diefstal van de Huub Hangop CD. Je begrijpt, ik denk nog vaak terug aan de prettige chaos waarin wij werkten. Ik ben blij dat je mij de dienst van paranimf terug bewijst! Jeroen, onze geschiedenis reikt nog verder terug en begint al op de middelbare school toen we elkaar bijna dagelijks zagen. Onze levens zijn sinds die tijd drastisch veranderd, maar toch hebben we elkaar nooit uit het oog verloren. Ik hoop dan ook dat we onze vriendschap nog lang in stand kunnen houden en ben blij dat je mijn paranimf wilt zijn.

Ook mijn ouders verdienen een plaats in dit dankwoord. Pa en Ma, dankzij jullie heb ik van een onbezorgde jeugd kunnen genieten en ben ik altijd gestimuleerd om me verder te ontwikkelen. Het was fijn te merken dat jullie altijd achter mijn keuzes stonden, ook toen alle beroepstesten “Journalist” aangaven en ik Moleculaire Wetenschappen wilde gaan studeren.

Als laatste wil ik Mieke bedanken. Lieve Mieke, zonder jouw geduld en liefde hadden mijn leven en dit boekje er heel anders uitgezien. Met je verwoede pogingen om alles te relativeren heb je me af en toe toch nog een dagje achter de computer weg gekregen voor wat ontspanning. Bedankt dat je me door deze, soms moeilijke, tijd hebt gesleept. Het boek is nu af, het is tijd om nog meer te gaan genieten van het leven. Op naar ons volgende avontuur, op naar Ierland!

Peter

Curriculum vitae

Peter Allard Bron werd geboren op 3 augustus 1976 in Delft. Via de basisschool in Nijkerk en Drachten, behaalde hij in 1994 het VWO diploma aan het Christelijk Lyceum te Veenendaal. In de herfst van 1994 begon hij, na alweer een verhuizing, aan de studie Moleculaire Wetenschappen aan de Universiteit van Wageningen. Tijdens deze studie deed hij afstudeeronderzoeken aan het hyperthermostabiele β-glucosidase van *Pyrococcus furiosus* bij de vakgroep Microbiologie en het prolidase van *Aspergillus nidulans* bij de vakgroep Moleculaire Genetica van Industriële Micro-organismen. Vervolgens liep hij stage bij Fort Dodge Animal Health in Weesp waar hij werkte aan de ontwikkeling van een recombinant adenovirus vaccin. In september 1999 ronde hij zijn universitaire studie af en in dezelfde maand begon hij aan zijn promotieonderzoek bij het Wageningen Centre for Food Sciences (WCFS) op werklocatie NIZO Food Research te Ede. Dit promotieonderzoek werd uitgevoerd onder begeleiding van Prof. Dr. Willem M. de Vos en Dr. Michiel Kleerebezem en heeft geresulteerd in dit proefschrift. Nadat zijn AIO contract eind september 2003 afliep was hij 8 maanden werkzaam als onderzoeker voor het WCFS. Vanaf september 2004 zal hij werkzaam zijn als postdoctoraal onderzoeker bij het Alimentary Pharmabiotic Centre (APC) in Cork (Ierland) in de onderzoeksgroep van Prof. Dr. Colin Hill.

List of Publications and Patents

1. **Bron, P. A., M. G. Benchimol, J. Lambert, E. Palumbo, M. Deghorain, J. Delcour, W. M. de Vos, M. Kleerebezem, and P. Hols.** 2002. Use of the *alr* gene as a food-grade selection marker in lactic acid bacteria. Appl. Environ. Microbiol. **68:**5663-70.

2. **Bron, P. A., W. M. de Vos, and M. Kleerebezem.** 2004. Molecular analysis of host-microbe interactions in the gastrointestinal tract. In press: Gastrointestinal Microbiology (edited by Arthur Ouwehand and Elaine Vaughan, published by Marcel Dekker).

3. **Bron, P. A., W. M. de Vos, and M. Kleerebezem.** 2004. Site-specific intestinal delivery and/or production of biologically active substances. **Patent file number PCT/NL03/00733.**

4. **Bron, P. A., C. Grangette, A. Mercenier, W. M. de Vos, and M. Kleerebezem.** 2004. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. Submitted to J. Bacteriol.

5. **Bron, P. A., S. M. Hoffer, I. van Swam, W. M. de Vos, and M. Kleerebezem.** 2004. Selection and characterization of conditionally active promoters in *Lactobacillus plantarum*, using alanine racemase as a promoter probe. Appl. Environ. Microbiol. **70:**310-7.

6. **Bron, P. A., M. Marco, S. M. Hoffer, E. van Mullekom, W. M. de Vos, and M. Kleerebezem.** 2004. Genetic characterization of the bile salt response in *Lactobacillus plantarum* and analysis of responsive promoters *in vitro* and *in situ* in the gastrointestinal tract. Submitted to J. Bacteriol.

7. **Bron, P. A., D. Molenaar, W. M. de Vos, and M. Kleerebezem.** 2004. DNA micro-array based identification of bile-responsive genes in *Lactobacillus plantarum*. Manuscript in preparation.

8. **Bron, P. A., M. Meijer, R. Bongers, W. M. de Vos, and M. Kleerebezem.** 2004. Competitive population dynamics of *ivi*-gene mutants of *Lactobacillus plantarum* in the gastrointestinal tract of mice. Manuscript in preparation.

9. **Bron, P. A., R. Siezen, A. Lindenbergh, E. Peters, G. Pretzer, W. M. de Vos, and M. Kleerebezem.** 2004. The role of sortase and its target proteins in *Lactobacillus plantarum* agglutination and persistence in the gastrointestinal tract of mice. Manuscript in preparation.

10. d**e Vos, W. M., P. A. Bron, and M. Kleerebezem.** 2004. Post-genomics of lactic acid and other bacteria to discover gut functionality. In press: Curr. Opin. Biotechnol. **15/2**.

11. **Jalving, R., P. Bron, H. C. Kester, J. Visser, and P. J. Schaap.** 2002. Cloning of a prolidase gene from *Aspergillus nidulans* and characterisation of its product. Mol. Genet. Genomics **267:**218-22.

12. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. Proc. Natl. Acad. Sci. U.S.A. **100:**1990-5.

13. **Lebbink, J. H., T. Kaper, P. Bron, J. van der Oost, and W. M. de Vos.** 2000. Improving low-temperature catalysis in the hyperthermostable *Pyrococcus furiosus* beta-glucosidase CelB by directed evolution. Biochemistry **39:**3656-65.

This work was carried out at NIZO Food Research (Ede, The Netherlands) and was part of the scientific programme “Microbial Functionality and Safety” of the Wageningen Centre for Food Sciences (Wageningen, The Netherlands). Part of this work was supported by the EU project LABDEL (EU-QLRT-2000-00340).

The research described in this thesis was part of the research programme of the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

Lay-out: Mieke Prins

Cover design: Peter Bron

Printed by: Ponsen & Looijen