Environmental Interactions of *Lactobacillus reuteri*

Signal Transduction, Gene Expression and Extracellular Proteins of a Lactic Acid Bacterium

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Till mamma och pappa

Abstract

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The commensal bacterium *Lactobacillus reuteri* inhabits the human gastrointestinal tract and possesses putative probiotic, i.e. health-promoting, properties. In this thesis, features important for the ecological performance of *L. reuteri* and for interactions between the bacterium, its host and the environment were characterised. Extracellular proteins were identified in two *L. reuteri* strains. Firstly, fifty-three proteins were revealed in strain DSM 20016 using signal sequence phage display. Secondly, a draft genome sequence of strain ATCC 55730 was screened with bioinformatics tools and 126 genes encoding extracellular proteins were identified. Few obvious adhesion or colonisation factors were found, although an R28-like proteins putatively involved in adherence to mucosal surfaces was detected.

The early response to sudden acid shock was studied with gene expression analyses using microarrays. When *L. reuteri* was exposed to an acidic pH, similar to the conditions in the human stomach, the chaperone-encoding *clpA* and genes putatively involved in cell envelope biogenesis were induced. Inactivation of *clpA* resulted in an acidic-sensitive phenotype.

Seven complete two-component systems consisting of a histidine kinase and a response regulator were identified in the genome of *L. reuteri* ATCC 55730. Sequence analysis indicated that all seven systems belonged to the OmpR family. In order to expose the function of one of these systems, the response regulator was inactivated. The resulting mutant displayed increased sensitivity to NaCl and the antibiotic ampicillin. Results from gene expression analyses indicate that this two-component system regulates genes involved in cell envelope alterations in relation to stress. Therefore, this system was designated Lea (*L. reuteri* Envelope Altering).

Taken together, these studies demonstrate the importance of the cell envelope for interactions between *L. reuteri* and its environment. The findings provide a basic insight in the adaptation mechanisms and the life strategies of this species. In addition, this work provides a foundation for further investigations and characterisation of the commensal bacterium *L. reuteri*.

Keywords: *Lactobacillus reuteri*, signal transduction, two-component system, stress, acid, extracellular proteins, lactic acid bacteria

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Contents

Appendix

List of papers

This thesis is based on the following papers, which are referred to by their Roman numerals:

I Wall, T., Roos, S., Jacobsson, K., Rosander, A. & Jonsson, H. 2003. Phage display reveals 52 novel extracellular and transmembrane proteins from *Lactobacillus reuteri* DSM 20016^{T}. *Microbiology* 149: 3493-3505.

II Båth, K., Roos, S., Wall, T. & Jonsson, H. 2005. The cell surface of *Lactobacillus reuteri* ATCC55730 highlighted by identification of 126 extracellular proteins from the genome sequence. *FEMS Microbiology Letters* (In press).

III Wall, T., Båth, K., Britton, RA., Jonsson, H., Versalovic, J. & Roos, S. The early response to acid shock in *Lactobacillus reuteri* involves the ClpA chaperone and cell surface alteration. *Manuscript.*

IV Wall, T., Hüfner, E., Jonsson, H., Hertel, C. & Roos, S. Two-component signal transduction in *Lactobacillus reuteri*. *Manuscript*.

Paper I and II are reprinted with permission from the respective publisher.

My contribution to these papers was as follows:

I Performed the construction and panning of the phage display library. Performed the major part of sequencing and data analysis. Main author of the paper.

II Participated in the bioinformatic analysis of the extracellular proteins. Shared first author of the paper.

III Major part of planning the experiment. Performed all laboratory work and data analysis of the microarray experiments. The microarray slides were constructed by Robert A Britton and James Versalovic. The disruption mutant was constructed by Stefan Roos. Main author of the paper.

IV Initiated the studies of two-component systems in *Lactobacillus reuteri*. Performed the planning of the experiments together with Stefan Roos and Hans Jonsson. Performed the major part of the laboratory work and data analysis with the exception of creating the disruption mutant of RR2, which was constructed by Eric Hüfner. Main author of the paper.

Life in the microbial world is no picnic.
John S. Parkinson

Introduction

Life is full of interactions. In nature, no living being is ever alone. Furthermore, all organisms are affected by the surrounding world. This is true also for bacteria.

The connecting link between bacteria and the outside world is the cell envelope, which consists of the cell membrane covered by the cell wall. The cell membrane contains a large number of inserted proteins, while other proteins are attached to the external surface of the membrane or to the cell wall. These proteins have diverse functions and are of major importance for the characteristics of the bacterium.

Bacteria have to be dynamic and adapt to the external conditions in order to survive. Therefore, they have to make informed decisions on how to behave under the various environmental conditions they experience. Several systems for sensing and adapting to the environment are present in bacteria, usually involving signal transduction mechanisms. In two-component signal transduction, a cell membrane-anchored sensor protein monitors the environment and on encountering changed conditions transmits a signal to the inside of the cell. This signal activates an intracellular regulator, which initiates a response to the new environmental situation. The response usually consists of changes in the genes expressed by the cell. This modified gene expression, followed by changes in protein production, results in new features of the bacterium and improved possibilities to persist and survive.

Lactobacillus reuteri is a non-pathogenic bacterium that colonies the gastrointestinal tract of humans and other animals. Due to its properties, such as production of the antimicrobial substance reuterin, which inhibits the growth of pathogenic bacteria, *L. reuteri* has the potential to function as a probiotic. Probiotics are living microorganisms with a beneficial influence on their host, meaning that they either promote health or prevent and treat diseases. However, the majority of the interactions between *L. reuteri* and its environment are currently uncharacterised and remain to be revealed. The focus of the presented work is on how *L. reuteri* senses and adapts to different environmental conditions and the strategies this bacterium has to interact with its surrounding.

Aims

The aim of this thesis was to characterise properties of the lactic acid bacterium *Lactobacillus reuteri* important for its ecological performance and for interactions with its host and the environment. The main approaches were to identify and describe extracellular proteins, components in signal transduction and responses to environmental conditions (Fig. 1). To identify extracellular proteins, the genome of *L. reuteri* was screened experimentally using signal sequence phage display and with bioinformatical tools. Two-component signal transduction systems in *L. reuteri* were examined with mutation and expression analysis in order to detect pathways for external stimuli leading to cellular responses. To establish how *L. reuteri* is affected by external factors, e.g. an acidic environment, and to connect these factors to changes in phenotype, the survival and gene expression after a sudden decrease in pH were studied.

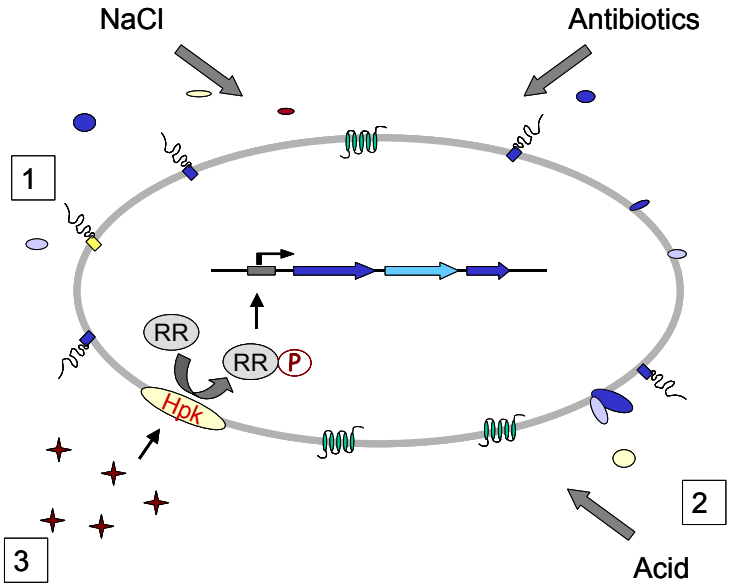

Fig. 1. Overview of the approaches to study *L. reuteri* in this thesis. 1. Extracellular proteins were searched for experimentally and with bioinformatics tools. 2. The response of acid shock was studied with microarray analyses. 3. A two-component signal transduction systems was mutated and the phenotype of the mutant was tested.

Background

The commensal bacterium *Lactobacillus reuteri*

Since genetic properties and environmental factors shape all organisms, studies of the interactions between them increase the understanding of life. The genetics and ecology of *Lactobacillus reuteri* are so far only partially characterised. *L. reuteri* was described as a distinctive species in 1980 (Kandler, Stetter & Köhl, 1980) and belongs to the group of lactic acid bacteria. The lactic acid bacteria consist of gram-positive, relatively closely related, facultative anaerobic rods or cocci. In addition, the species in this group generate lactic acid during hetero- or homofermentative metabolism (Axelsson, 2004). Lactic acid bacteria are often found in, and utilised to produce, fermented food, such as yoghurt, sourdough and cheese. The lactic acid and other substances produced in fermentation contribute to the preservation, the texture and flavour of the food (Ross, Morgan & Hill, 2002). Outside the genera *Streptococcus* and *Enterococcus*, lactic acid bacteria rarely possess any pathogenic properties (Axelsson, 2004). Since lactic acid bacteria have a relatively simple metabolism, they require growth media with a high content of nutrients and are often found in nutrient-rich environments. Consequently, the genomes of lactic acid bacteria are generally relatively small (Klaenhammer *et al.*, 2002).

The ecological habitats of *L. reuteri* are mainly the gastrointestinal tract and other mucosal surfaces of humans and animals, but it is also found in sourdough and other fermented foods (Hammes & Hertel, 2005; Vogel *et al.*, 1999). The microbial community in the gastrointestinal tract is complex and consists of several hundred species, of which lactic acid bacteria constitute a minor proportion (Guarner & Malagelada, 2003; Reuter, 2001). *L. reuteri*, like many other lactic acid bacteria, is a non-pathogenic, commensal species that does not harm the eukaryotic host. Nevertheless, non-pathogenic colonisers of the intestine and their hosts communicate and the outcome of this cross-talk is likely to affect the physiology of both (Falk *et al.*, 1998; Hooper *et al.*, 2001). Several lactic acid bacteria have the potential to promote the health of the host or to prevent and treat diseases. Such bacteria are referred to as probiotic, meaning ‘for life’ (Latin *pro*=for and *biotic*=life). The effects of probiotic microorganisms on the host are discussed extensively. However, beneficial influence of probiotics have been demonstrated in clinical studies (reviewed by Ouwehand, Salminen & Isolauri, 2002). *L. reuteri* also has the potential to function as a probiotic (Casas & Dobrogosz, 2000). Studies of rotavirus infection in children showed positive effects of administration of *L. reuteri* such as shortened duration of the infection (Shornikova *et al.*, 1997a; Shornikova *et al.*, 1997b). In addition, administration of *L. reuteri* resulted in fewer febrile episodes and fewer gastrointestinal infections in infants in day care (Weizman, Asli & Alsheikh, 2005).

The interactions between the host and commensal bacteria are far from fully comprehended. Likewise, the properties required for a probiotic function are mainly unknown. Although not proven, a number of characteristics are considered important for probiotic activity, for example adherence to intestinal surfaces and

colonisation of the host, reduction of colonisation of pathogenic bacteria; production of antimicrobial substances; and interaction with the host and its immune system (reviewed by Dunne, 2001; Reid & Burton, 2002). *L. reuteri* colonises the human gastrointestinal tract (Valeur *et al.*, 2004), produces the antimicrobial substance reuterin (Axelsson *et al.*, 1989), binds to mucus (Jonsson, Ström & Roos, 2001; Roos & Jonsson, 2002) and interact with the immune system for example by modulating the expression of cytokines (Christensen, Frokiaer & Pestka 2002; Maassen *et al.*, 2000). Taken together this suggests that *L. reuteri* is suitable for use in probiotic products.

The environment in the gastrointestinal tract

The gastrointestinal tract is the organ in the human body with the largest surface area, in an adult between 150 and 200 m^2 (Holzapfel *et al.*, 1998). Interesting in this context is the fact that a huge number of microorganisms live and interact with the host in the stomach and gut. Subsequently, the human gastrointestinal provides a gigantic niche and 10^{14} viable bacteria representing several hundred species are normally found in this environment (Hentschel, Dobrindt & Steinert, 2003). The bacteria are affected both by the physiological conditions and by other microorganisms. Because of the presence of enzymes, salts and acids in the gastric juice, the environmental conditions in the stomach are destructive to a number of microorganisms, (Holzapfel*, et al.*, 1998). The gastric juice contains hydrochloric acid, which creates an extremely low pH. The fasting pH in the stomach is approximately 1.5, while the pH increases to between 3.0 and 5.0 when food is eaten (Cotter & Hill, 2003). In the intestine the conditions are less extreme, but the bacteria still have to endure bile and pancreatic juices. Other factors affecting microbial life in this ecosystem are immunoglobulins, defensins, a continuously regenerating epithelium, peristaltic movement of intestinal content and a viscous mucus layer. The environmental conditions are also strongly dependent on the host. For example, diet, stress and drugs are important for the composition of the microflora and the host-microbial interactions. The microbes also affect each other, which results in both antagonism and mutualistic effects. Antagonism is seen as decreased nutrient availability and production of antimicrobial compounds, while mutualistic effects can involve metabolic cooperation and vitamin excretion (Holzapfel*, et al.*, 1998). Bacteria, living in this environment, have to adapt to these conditions. Several species have evolved strategies to colonise this habitat and to tolerate these stressful conditions.

Stress responses and acid tolerance

Although the gastrointestinal tract is a harsh environment for the bacteria inhabiting it, microbes possess several defence systems and abilities to adapt to various stresses. Some of these are large coordinated regulation systems that drastically change the gene expression in the cell and the cellular processes, like the general stress response (Hengge-Aronis, 2002). This system is activated by limited growth and regulates the synthesis of general stress proteins, which

provide increased tolerance to factors such as acid, alkali, ethanol, heat and osmotic- and oxidative stress.

Other mechanisms are specific responses to certain stress conditions. In the gastrointestinal tract, the responses to acidic conditions are crucial for survival and acid stress has been extensively studied in lactic acid bacteria. The intracellular pH (pH_i) of bacteria is usually higher than the external environmental pH (pH_{ex}). However, the pH_i of lactic acid bacteria varies with changes in pH_{ex}. After a rapid decrease in pH_{ex} from 7 to 5, pH_i of the three lactic acid bacterial strains *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus* and *Lactococcus lactis* is reduced to 5.5, while the pH_i of *Listeria innocua* remain neutral (Siegumfeldt, Rechinger & Jakobsen, 2000). This indicates that the cellular processes of lactic acid bacteria are functioning in a wide range of pH. The cell envelope permeability of protons is usually low, but in extremely acidic environments, such as the gastrointestinal tract, external protons leaks through the membrane (Foster, 2000). Intracellular acidification affects the pH gradient over the membrane and therefore also the proton motive force, an important source of energy for the cell (Konings, 2002). In addition, acid damages various components of the cell, for example it inactivates enzymes and destroys DNA and proteins. Gram-positive bacteria in general and lactic acid bacteria in particular have developed a number of strategies to survive low pH (reviewed by Cotter & Hill, 2003; De Angelis & Gobbetti, 2004; van de Guchte *et al.*, 2002). Interestingly, pre-exposure to mildly acidic pH increases the ability of bacteria to endure acids, due to the acid tolerance response (Lorca, Font de Valdez & Ljungh, 2002). One way to handle acidic stress is to remove protons from the cytoplasm. F_0F_1-ATPase is a pump that transports protons out of the cell using energy from ATP. Glutamate decarboxylase (GAD) combines glutamate with a proton and exports the product in exchange for a new amino acid from the external environment. Other systems with similar effects as GAD utilise the amino acids lysine and arginine instead of glutamate. An additional strategy is to increase the external pH of the environment. In the arginine deiminase pathway (ADI), arginine is converted to ornithine, ammonia and carbon dioxide and the alkaline ammonia molecule raises the pH (reviewed by Konings, 2002). Similarly, the enzyme urease produces ammonia from urea. Furthermore, the cell envelope can be modified by various mechanisms to decrease the permeability of protons. Systems to protect intact macromolecules and to repair damaged components, e.g. DNA or proteins, are also induced in acidic environments (reviewed by Cotter & Hill, 2003; De Angelis & Gobbetti, 2004; van de Guchte*, et al.*, 2002).

Cell surface-associated and secreted proteins

It is envisioned that bacterial extracellular proteins are important for adherence and eventually persitance in the gastro-intestinal tract. Extracellular proteins are either secreted or attached to the cell surface. They are involved in various, often essential, cellular functions such as nutrient up-take, cell division, transport and efflux, motility, and signal transduction. In pathogenic bacteria, extracellular proteins have been extensively studied as putative virulence factors, for example toxins, enzymes and colonisation factors (Navarre & Schneewind, 1999).

However, less is known about extracellular proteins and their importance in commensal bacteria.

Secretion and anchoring to the cell

Extracellular proteins are synthesised in the cytoplasm and directed to the plasma membrane, the cell wall or the outside of the cell. There are several different protein secretion pathways in bacteria, e.g. the general secretory (Sec) pathway, the twin-arginine translocation (Tat) pathway and the ATP-binding cassette (ABC) transporter system (reviewed in Tjalsma *et al.*, 2000). Of these, the Sec pathway is the most common in gram-positive bacteria. The transport of extracellular proteins is controlled by an amino- (N-) terminal signal peptide in the protein precursor. The cell sorting and translocation machinery recognises the signal peptide and exports the protein through the plasma membrane (Tjalsma*, et al.*, 2000; van Wely *et al.*, 2001). When the protein has reached its destination, the signal peptide is either cleaved off by signal peptidases or inserted into the plasma membrane as an N-terminal transmembrane signal anchor (Martoglio & Dobberstein, 1998). In the latter case, the protein is retained on the surface of the cell. Based on the amino acid sequence, it is sometimes difficult to distinguish between cleavable signal peptides and signal anchors. Components of the Sec pathway are also involved in translocation of proteins with multiple membrane-spanning domains (Dalbey & Chen, 2004). Membrane proteins often have functions similar to those of extracellular proteins, but less is known about their role in environmental and host interactions.

In addition to anchoring via N-terminal transmembrane mechanisms domains mentioned above, extracellular proteins can be attached to the surface by various other mechanisms. The anchoring mechanisms mainly described in lactic acid bacteria include C-terminal transmembrane anchors, LPXTG motifs, LysM domains and lipoboxes in lipoproteins. Similarly to proteins with N-terminal anchors, some proteins are anchored C-terminally in a similar fashion by an hydrophobic membrane spanning domain followed by positively charged residues serving as stop-transfer signals (Dalbey & Chen, 2004). Proteins with LPXTG motifs in the amino acid sequence have been extensively studied as virulence factors in pathogenic bacteria. The threonine residue convalently links the protein to the cell surface by binding to peptidoglycan in the cell wall (Ton-That, Marraffini & Schneewind, 2004). The widely spread LysM domains (Bateman & Bycroft, 2000) possess a general peptidoglycan-binding function (Joris *et al.*, 1992; Steen *et al.*, 2003) and are instead involved in anchoring proteins non-convalently to the cell wall. Lipoproteins are N-terminally anchored to the long chain fatty acid in the plasma membrane. The precursors of lipoproteins contain signal peptides with a conserved motif called lipobox and the peptides are cleaved in front of an invariable cysteine residue by SPase II (reviewed by Tjalsma *et al.*, 2000).

Extracellular proteins in L. reuteri

A number of extracellular proteins probably important for the ecological performance of *L. reuteri* have been characterised. The proteins Mub (mucus-binding protein), CnBP (collagen-binding protein) and Lsp (large surface protein) are putatively involved in colonisation of the gastrointestinal tract of the host. *In vitro*, Mub binds to mucus, which covers the epithelial cells in the intestine (Roos & Jonsson, 2002). Although Mub is lacking in many strains of *L reuteri* (Jonsson, Ström & Roos, 2001), homologues proteins are found in several lactic acid bacterial species, for example in *L. acidophilus*, *L. gasseri* and *L. plantarum*. Interestingly, inactivation of the gene encoding a Mub-homologue in *L. acidophilus* resulted in reduced adherence to Caco-2 cells (Klaenhammer *et al.*, 2005). This further supports the putative importance of Mub for colonisation of the gastrointestinal tract. The ABC transporter component CnBP has the ability to bind to the extracellular matrix-component collagen (Roos *et al.*, 1996). The biological importance CnBP have not been studied. However, the homologous protein BspA from *L. fermentum* is likely to be a cysteine-binding protein involved in oxidative defence (Hung *et al.*, 2005). Lsp is a large protein with a molecular mass of 300 kDa. Lsp is involved in binding to the gastrointestinal epithelium in mice, possibly by initiating adherence (Walter *et al.*, 2005). Furthermore, an aggregation promoting protein (AggH) has been identified in *L. reuteri* (Roos, Lindgren & Jonsson, 1999). Formation of multi-cellular aggregates is possibly important for the colonisation ability and ecological interactions of bacteria. An extracellular fructosyl transferase in *L. reuteri* has been reported to synthesise inulin fructooligosaccarides and high-molecular-weight inulin (van Hijum *et al.*, 2002). In addition, some species of *L. reuteri* possesses genes encoding glucansucrases (Kralj *et al.*, 2004; Kralj *et al.*, 2002). Since certain gluco- and fructo-oligosaccharides have antimicrobial properties, the enzymes producing them may have effects on the growth of other endogenic strains (Chung & Day, 2002) and thus also on the host.

Two-component signal transduction

Two-component systems, or Histidine-Asparagine Phosphorelay (HAP) systems, represent a mechanism for signal transduction that is widely spread among bacteria. In the classical examples these systems consist of two components: a histidine protein kinase (Hpk) and a response regulator (RR). The Hpk has an N-terminal extracellular sensor domain, while the C-terminal part of the protein is located in the cytoplasm. The RR is cytoplasmatic and consists of a receiver domain and a DNA-binding domain (Bijlsma & Groisman, 2003). The extracellular part of the Hpk monitors the external conditions. On encountering a change in the environmental conditions the Hpk transmits a signal in the form of a phosphorylation, via a conserved histidine residue in the Hpk, to an asparagine residue in receiver domain of the RR (Robinson, Buckler & Stock, 2000). Upon phosphorylation, the RR is activated and the DNA-binding domain binds to the DNA, as a transcriptional activator or repressor, hence changing the gene expression of the cell (Fig. 2).

Two-component signal transduction is involved in regulation of a number of processes. These systems regulates for example: adaptation to various stresses (Mizuno & Mizushima, 1990; Morel-Deville, Fauvel & Morel, 1998) genetic competence (Tortosa & Dubnau, 1999); virulence and interactions between pathogens and their host (Bijlsma & Groisman, 2005; Lamy *et al.*, 2004; McCluskey *et al.*, 2004; Novick, 2003); biofilm formation (Kulasekara *et al.*, 2005; Li *et al.*, 2002); and antibiotic resistance (Stephenson & Hoch, 2002). Some two-component systems are regulated by a quorum sensing mechanism, for example systems involved in lantibiotic production (Kleerebezem, 2004).

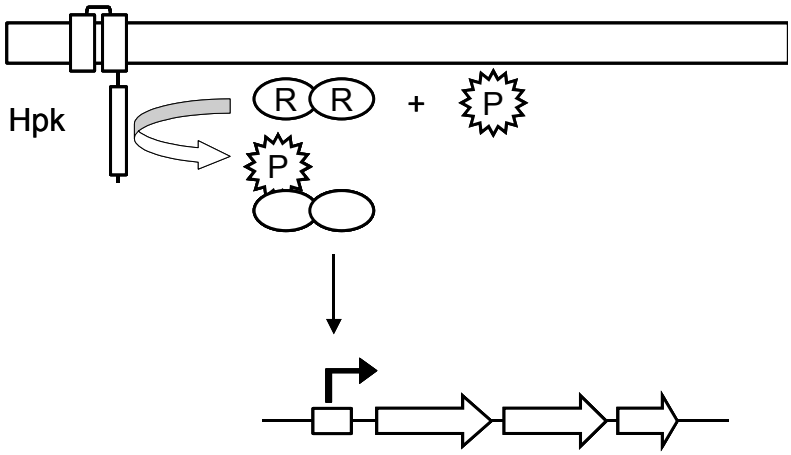

Fig. 2. The mode of action of two-component signal transduction. Upon a change in the external conditions the Hpk activates the RR via transfer of a phophoryl group. The RR then modifies the gene expression of the cell.

The number of two-component systems varies greatly between different species. *Pseudomonas aeruginosa* harbours 72 Hpks and 76 RRs, while *E. coli* has 33 Hpks and 33 RRs. Other species encode notably fewer two-component proteins or as in the case of *Mycoplsma genitalium* and *Mycoplasma pneumoniae* none at all (Koretke *et al.*, 2003). In general, lactic acid bacteria have relatively few two-component systems. *L. acidophilus* encodes 9 systems (Altermann *et al.*, 2005) and *L. plantarum* 13 (Kleerebezem *et al.*, 2003).

In many situations, two-component signal transduction is more complex than described above. The structure of Hpks and RRs vary (Foussard *et al.*, 2001). Furthermore, some systems contain more than two components. For example, some systems consist of one Hpk and two RR. In addition, multiple sensor can act on a single response regulator (Bijlsma & Groisman, 2003). The different two-component system can also interact with each other, resulting in 'cross-talk' and neural networks (Hellingwerf, 2005; Hellingwerf *et al.*, 1998).

Results and discussion

Identification of extracellular proteins from *L. reuteri* (I and II)

Screening for extracellular proteins in *L. reuteri* was performed with two different methods. Firstly, before the genome sequence of *L. reuteri* was available, extracellular proteins of the strain DSM 20016 were sought using signal peptide phage display (Paper I). Secondly, a draft genome of *L. reuteri* strain ATCC 55730 was screened with bioinformatical tools for genes encoding a signal peptide or an N-terminal transmembrane domain orientating the protein to the outside of the cell (Paper II). Both DSM 20016 and ATCC 55730 are of human origin. However, the strain DSM 20016 was isolated from faeces, while ATCC 55730 is an isolate from mother's milk. Although not fully investigated, the properties of the two strains partially differ. For example, the two strains exhibit different antibiotic sensitivity profiles and strain ATCC 55730 possesses several, possibly up to six plasmids that are lacking in strain DSM 20016 (Klein *et al.*, 2000). In addition, DSM 20016 adheres to mucus to a notably higher extent than ATCC 55730 (Jonsson, Ström & Roos, 2001).

Signal sequence phage display

For twenty years, phage display has been used to study interactions between proteins. With this method, foreign peptides or proteins are expressed in fusion with coat proteins on the surface of a filamentous phage (Smith, 1985). The fusion protein is usually expressed from a phagemid vector, i.e. a plasmid containing a filamentous phage intergenic region and sequences required for packing into the phage particle, while all other proteins are provided by a helper phage (Bass, Greene & Wells, 1990). A genetic library is constructed by insertion of randomly fragmented chromosomal DNA from the target organism into the phagemid vector. The vector is transformed into *E. coli* cells, which are then infected by the helper phages in order to produce a stock of phages displaying fusion proteins. Phages with fusion proteins that can bind to other molecules are selected by affinity panning against a ligand of choice. The vector normally encodes the signal peptide required for direction of the fusion protein to the cell membrane, where phage assembly occurs (Jacobsson & Frykberg, 2001). Signal sequence phage display is a development of this technique (Rosander *et al.*, 2002) used to screen bacterial genomes for genes encoding extracellular proteins. The vector pG3DSS constructed for signal sequence phage display lacks a signal peptide (Fig. 3). In order to be transported to the cell surface and incorporated into the phage coat, the foreign DNA inserted into the vector has to encode a signal sequence. Consequently, only proteins containing a signal peptide, i.e. extra-cellular proteins or membrane proteins, can be displayed as fusion proteins. To allow a simple affinity selection, the pG3DSS also encodes an E-tag epitope, i.e. a short peptide recognised by monoclonal antibodies. Since panning against anti-E-tag antibodies isolates phages displaying fusion proteins, the foreign inserts of such phages can be sequenced and the genes encoding extracellular proteins revealed.

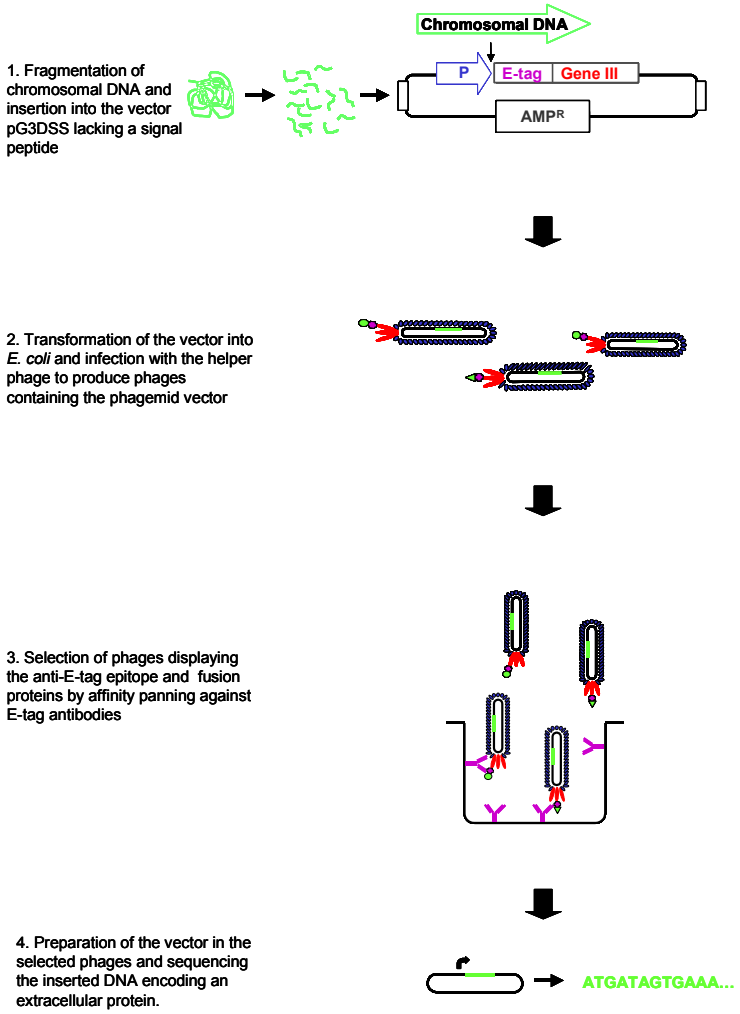

Fig. 3. Schematic description of signal sequence phage display.

Signal sequence phage display has been used to identify extracellular proteins in *Staphylococcus aureus* (Rosander *et al.*, 2002), *Bradyrhizobium japonicum* (Rosander *et al.*, 2003) and *Streptococcus equi* (Karlström *et al.*, 2004), as well as in *L. reuteri*.

Searching for extracellular proteins with signal sequence phage display has the advantages of providing experimental proof on secretion of the protein. To be identified with this method the signal sequence has to be functioning and at least be recognised by the translocation machinery in *E. coli*. This method is also relatively cheap and fast, and no genome data are required. However, the genes identified are seldom complete and not all genes encoded by the organisms studied are likely to be found. Rosander and co-workers (Rosander *et al.*, 2002) suggest that signal sequence phage display is more efficient for gram-negative bacteria than for gram-positive, since it is based on a system in *E. coli*. In addition, the efficiency of the method may depend on the size of the phage library.

Extracellular proteins identified in L. reuteri *DSM 20016 (I)*

Screening of a phage display library with chromosomal DNA from *L. reuteri* strain DSM 20016 identified 53 genes encoding proteins with putative signal peptide domains. Only one protein, the collagen-binding protein CnBP (Roos *et al.*, 1996), was previously known in *L. reuteri*. However, not all these genes encoded extracellular proteins. Instead, 23 genes[1] were classified as membrane proteins, since they contained several transmembrane domains. Some of the inserts did not encode the N-terminal signal peptide. Since the same translocation machinery recognizes transmembrane and extracellular proteins (Dalbey & Chen, 2004), the hydrofobic domains of the transmembrane proteins were sufficient to direct the fusion proteins to the cell membrane of *E. coli* for incorporation into the phage coat. However, 30[1] genes encoding extracellular proteins were identified. Since only a few extracellular proteins of *L. reuteri* were known at the time, this was a substantial contribution to knowledge of the cell surface of this organism.

The predicted functions of the extracellular and transmembrane proteins encoded by the genes identified with signal sequence phage display are presented in Fig. 4. The proteins were classified as: transport proteins, enzymes, sensors-regulators, host/microbial interaction proteins, conserved hypothetical proteins and unconserved hypothetical proteins. One of these proteins, Lre0020, was a histidine kinase in a two-component signal transduction system, probably involved in sensing environmental conditions. The two-component systems in *L. reuteri* are discussed in detail later. Besides CnBP (Roos *et al.*, 1996), two proteins (Lre0019, Lre0018) could be important for interactions with the host. Lre0019 displayed similarities to PspC, a cell surface adhesin from *S. pneumoniae*. In addition to participating in colonisation of mucosal surfaces in the nasopharynx (Balachandran *et al.*, 2002; Rosenow *et al.*, 1997), PspC interferes with immuno

[1] *The original numbers of extracellular and transmembrane proteins presented in Paper I have been modified on the bases of the characteristics of the complete amino acid sequence obtained from the genome of* L. reuteri *ATCC 55730 presented in PaperII.*

response components of its host (Cheng, Finkel & Hostetter, 2000; Dave *et al.*, 2001; Dave *et al.*, 2004a; Dave *et al.*, 2004b). However, sequencing the complete gene encoding Lre0019 revealed that the similarities between Lre0019 and PspC were most notable in the peptidoglycan-binding domain. The C-terminal of Lre0019 contained an amidase domain, which is found in several types of cell wall-degrading enzymes. Therefore, the function and the putative role of Lre0019 in colonisation and interactions with the immune system, require further studies. The C-terminal of the protein encoded by *lre0018* was similar to the Apf proteins from *L. johnsonii* and *L. gasseri* (Ventura *et al.*, 2002). This protein is also highly similar to Sep from *Lactobacillus fermentum* BR11 (Turner *et al.*, 2004). However, the Apf proteins lack the cell wall-anchoring LysM domains found in Lre0018 and Sep. The Apf proteins have certain similarities to S-layer proteins (Ventura *et al.*, 2002) and are probably involved in cell structure determination, although their mechanisms of action are currently unknown (Jankovic *et al.*, 2003). The function of Sep is also unknown, and no influence on cell shape has been detected (Turner*, et al.*, 2004).

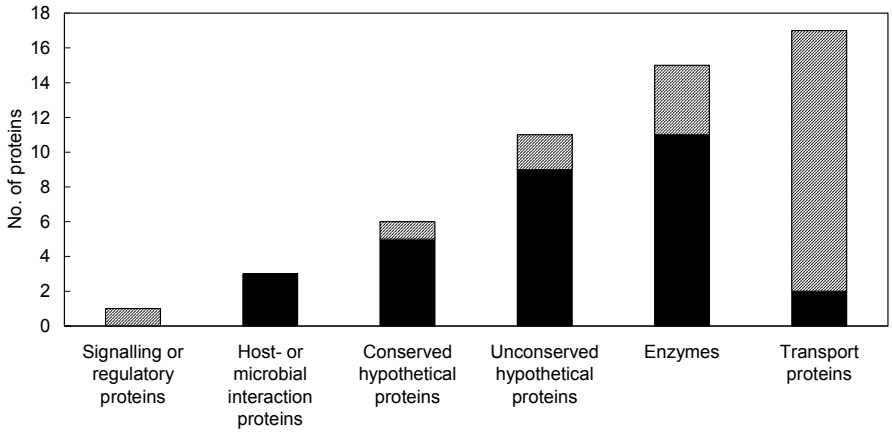

Fig. 4. The predicted functions of the extracellular (black) and transmembrane (hatched) proteins identified in *L. reuteri* strain DSM 20016. The original numbers of extracellular and transmembrane proteins presented in Paper I have been modified on the bases of the complete amino acid sequence deduced from the genome of *L. reuteri* ATCC 55730 presented in Paper II.

Analyses of bacterial genomes

The sequencing of complete genomes has introduced a new dimension to biology. In 1995 the genome of *Haemophilus influenzae* (Fleischmann *et al.*, 1995) was published as the first completely sequenced bacterial genome. Today, ten years later, the sequences of several hundred genomes are known. Several lactic acid bacterial genomes have been sequenced, completely or as draft genomes, for example the genomes of *L. lactis* (Bolotin *et al.*, 2001), *L. plantrum* (Kleerebezem *et al.*, 2003), *Lactobacillus gasseri* (http://genome.ornl.gov/microbial/lgas/), *L. johnsonii* (Pridmore *et al.*, 2004) and *L. acidophilus* (Altermann *et al.*, 2005). Knowledge of bacterial genomes provides new possibilities to characterise cellular processes and systems, intracellular networks and evolution. In other words,

genome research is an excellent base for creating hypotheses. However, experimental studies are required for biological understanding of the genomic data.

Extracellular proteins identified in L. reuteri *ATCC 55730 (II)*

The genome size of *L. reuteri* is 2.0±0.2 Mb and the draft genome of strain ATCC 55730, which covers between 90 and 95% of the genome, contains approximately 2100 putative protein-encoding genes. Analyses of this genome identified 126 genes encoding extracellular proteins. The total number of extracellular proteins in *L. reuteri* ATCC 55730 is therefore probably between 126 and 140 proteins and corresponding to between 6-7% of the total number of genes. In Fig. 5, the predicted functions of the 126 proteins are displayed.

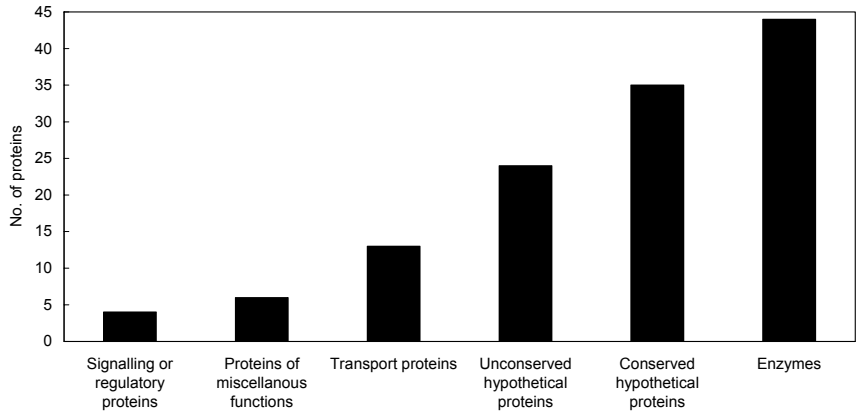

Fig. 5. The predicted functions of the extracellular proteins identified in *L. reuteri* strain ATCC 55730.

A number of the extracellular proteins of ATCC 55730 can be involved in host/environmental interactions. Four regulators and signal transduction components were found and in addition three ABC transporters were predicted to be involved in signal transduction. Furthermore, a periplasmic component of an ABC transporter, Lr1064, was classified as a member of the LraI family. Besides transport of metal ions, some proteins in this family are potential virulence factors and are involved in colonisation. In oral streptococci, a LraI protein adheres to salivary glycoproteins (Jenkinson, 1994) and in *Listeria monocytogenes* a member of this family has a role in entry into eukaryotic cells (Reglier-Poupet *et al.*, 2003). It is possible that this ABC transporter also has a role in adherence of *L. reuteri*. Another candidate for adherence to eukaryotic cells is an R28-like protein (LR1694). In *Streptococcus pyogenes*, R28 is involved in binding to human epithelial cells (Stålhammar-Carlemalm *et al.*, 1999). The R28-like protein in *L. reuteri* ATCC 55730 also displayed similarities to Lsp from *L. reuteri* strain 1023, which is important for colonising the gastrointestinal tract of mice (Walter, *et al.*, 2005). Furthermore, an orthologue of the Apf-like protein Lre0018 from strain DSM 20016 was identified in ATCC 55730. This protein was named

Lr0858 and in addition two paralogues, Lr0537, Lr1267, were present in strain ATCC 55730.

Summary of extracellular proteins identified in L. reuteri

Homologues to 24 of the extracellular and 15 of the transmembrane proteins identified in *L. reuteri* strain DSM 20016 were also present in the draft genome of strain ATCC 55730. The absence of the six remaining extracellular proteins was confirmed with PCR. For example, the PspC-like protein Lre0019 was lacking in ATCC 55730. Although only a minor group of protein were compared, this indicated marked differences between the two strains of *L. reuteri*. Preliminary results from a comparative study using microarray analyses revealed that approximately 20% of the genes in strain ATCC 55730 are lacking in DSM 20016 (Stefan Roos, unpublished data). These genes mainly consisted of plasmid-borne genes and phage-associated genes, but others were chromosomal genes not related to mobile elements. Obviously, the differences in the genomes result in different properties of the two strains. It is likely that different composition of cell surface proteins explains the different ability to adhere to mucus (Jonsson, Ström & Roos, 2001). The differences between ATCC 55730 and DSM 20016 are not surprising. Comparisons between the completely sequenced genomes of the two *L. lactis* strains MG1363 (ssp. *cremoris*) and IL1403 (ssp. *lactis*) also revealed large differences involving pro-phage and plasmids, but also plasmid integrations (Kok *et al.*, 2005). Furthermore, twenty strains of *L. plantarum* have been compared using microarrays (Molenaar *et al.*, 2005). This study exposed a chromosomal region of high plasticity utilised for adaptations and large differences in sugar metabolism between two groups of strains.

Taken together, few putative adhesion and colonisation factors were detected among the extracellular proteins in the two *L. reuteri* strains. No homologues to Mub (Roos & Jonsson, 2002) were present in the genome of ATCC 55730, even if this strain binds to mucus *in vitro* (Jonsson, Ström & Roos, 2001). However, the protein Lr1997 from ATCC 55730 was similar to both Lsp (Walter *et al.*, 2005) and Mub (Roos & Jonsson, 2002), although Lr1997 was only 97 amino acids and probably truncated. In addition, the genome contained surprisingly few proteins anchored with LPXTG domains. This class of proteins are abundant in pathogenic bacteria and several such proteins are classified as adhesion and/or virulence factors (Navarre & Schneewind, 1999). While *L. reuteri* only possess five such proteins, other lactic acid bacteria have several. *L. plantarum* (Kleerebezem *et al.*, 2003) and *L. johnsonii* (Pridmore *et al.*, 2004) encode 25 and 14 LPXTG respectively. This raises the question, which proteins in *L. reuteri* are mediating adherence to mucosal surfaces and interactions with the host. Expression and mutation analysis of extracellular proteins may exhibit such mediators.

Both methods utilised to search for extracellular proteins are intended for searching after a signal peptide. However, a number of proteins that are lacking signal peptides are still transported to the outside of the cell (Fekkes & Driessen, 1999). Experimental data have shown that the aggregation promoting protein, AggH, from *L. reuteri* is secreted, although this protein does not contain a signal

peptide (Roos, Lindgren & Jonsson, 1999). Such proteins will not be detected in studies like those in Paper I and II, but have to be identified with other methods.

Effects of an acidic environment (III)

Gene expression analysis with microarrays

Gene expression analysis with microarrays is a method extensively utilised in biological and medical research. In bacterial genomics, microarrays are extensively used to study stress responses and global gene regulation. In brief, this technique measures the relative differences in gene expression between two samples by comparing the amount of mRNA for each gene present on the array. Two samples can either be compared directly with each other, or indirectly by using a common reference. Several variables can be studied if a multifactorial design is utilised for the experiment (Yang & Speed, 2002). Therefore, the experimental design is of great importance for the results obtained. In addition, several technical and biological replicates and a suitable statistical method for the data analyses are required for reliable and reproducible results (van der Spek *et al.*, 2003). Microarray analysis is a suitable method for screening a genome or a large set of genes in order to identify interesting candidates for further characterisation, but additional methods such as quantitative PCR or Northern Blot analysis are still required to confirm the changed expression. Eventually, additionally methods are required to reveal the biological role and importance of the genes identified.

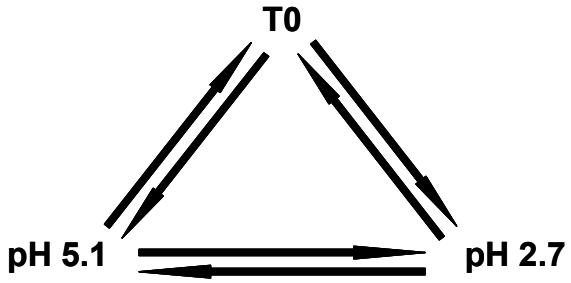

Fig. 6. The experimental design of the acid shock experiments. Comparisons were performed between untreated cells (pH 5.1), a treated control (pH 5.1) and acid-treated (pH 2.7) cells.

Acid shock experiments

Acid stress has been extensively studied in lactic acid bacteria. Growth and fermentation of these organisms creates an acidic environment, since various organic acids are produced (Axelsson, 2004). In addition, several species face an extremely low pH in the digestive tract. However, the physiological effects of long-term acid stress have been more investigated than the response of lactic acid bacteria to sudden acid shock.

In Paper III, the effect of low pH on the gene expression and survival of *L. reuteri* was studied in order to identify the genes involved in its response to acid shock. To mimic the entrance of the bacteria into the stomach after consumption, the pH was lowered by dilution into acidic medium (final pH 2.7) (Fig. 6). This method was also relatively non-traumatic to the cells in comparison to lowering the pH by adding HCl. Approximately 80% of the *L. reuteri* cells survived for 1 hour at pH 2.7 (data not shown). The changes in gene expression were measured after 5 and 15 minutes with microarrays. To estimate the effect of dilution, comparisons were made between acid treated cells, untreated cells and a treated control (i.e. dilution into the same pH). After statistical analyses with an empirical Bayes approach (Lönnstedt & Speed, 2002) and exclusion of genes only displaying minor changes in expression, 34 genes induced and 38 genes repressed specifically at pH 2.7 were identified.

The role of clpA *in acid adaptation*

The genes induced specifically at pH 2.7 included a stress response-associated gene; six genes encoding putative regulatory proteins; eight phage-associated genes; genes putatively involved in translation, metabolism or cell wall biogenesis; and several genes of unknown function. The stress response-associated gene was *clpA* (*lr1864*), which encodes an ATPase in the Clp family (Neuwald *et al.*, 1999). After 15 minutes this gene exhibited a 2-fold up-regulation at pH 2.7. Clp ATPases often have chaperone-like functions. Clp ATPases and other members of the Clp family are induced in response to various stresses in several bacteria, for example by acid stress in *Streptococcus mutans* (Len, Harty & Jacques, 2004) and by heat stress in *E coli* (Squires *et al.*, 1991), *L. lactis* (Ingmer *et al.*, 1999) and *Lactobacillus rhamnosus* (Suokko *et al.*, 2005). Interestingly, *clpC* (*lp_1019*) in *L. plantrum* is induced in the gastrointestinal tract of mice (Bron *et al.*, 2004), indicating that Clp proteins are also important *in vivo*. The *clpA* gene was therefore selected for further studies with mutation analyses. No difference in survival was found between the *L. reuteri* wild type and the *clpA* mutant in the late exponential phase when transferred to pH 2.7 (data not shown). However, when cells were diluted into synthetic gastric juice (Cotter, Gahan & Hill, 2001), but with enzymes excluded, the survival of the mutant was significantly lower ($P<0.001$) than that of the wild type (Fig. 7). In the wild type approximately 50% of the cells survived for 20 minutes, while in the mutant the corresponding figure was only 20%. This clearly demonstrated that *clpA*, although not essential, has a role in surviving acid shock.

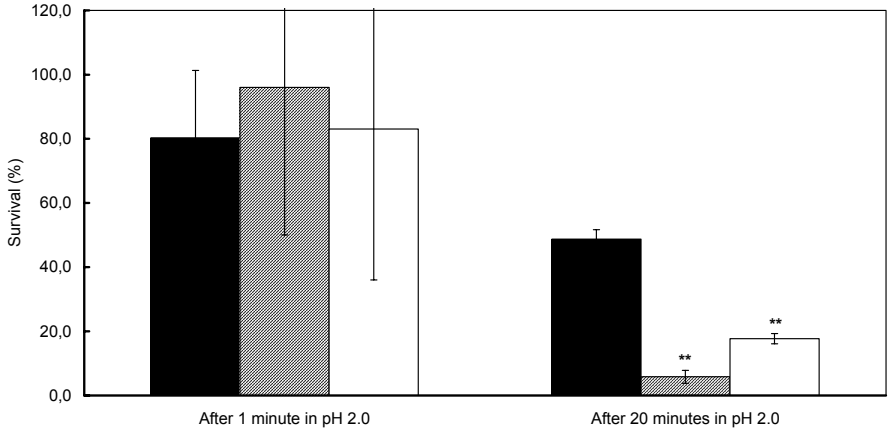

Fig. 7. Survival of *L. reuteri* wild type (black), *hpk3* mutant (hatched) and *clpA* mutant (white) in synthetic gastric juice pH 2.0. The data are presented in percent of the number of cells at the start of the experiment (N=3). The asterisks indicate statistically significant ($P < 0.001$) differences to the wild type in Student's t-test**.** Error bars represent standard deviation.

Altered cell wall and cell membrane composition

In addition to *clpA,* the expression of genes putatively involved in cell envelope biogenesis increased at pH 2.7. Changing the cell wall and the cell membrane is a known adaptation mechanism to an acidic environment. Alterations of the cell envelope could increase the cells resistance to acidic compounds. The permeability of protons in *S. mutans* is possibly dependent on esterification of lipoteiochoic acids. Inactivation of genes in the *dlt* operon, which encodes the genes required for this reaction, provided an acid-sensitive phenotype (Boyd *el al*., 2000). In the acid shock experiment in *L. reuteri*, a gene (*lr1516*) encoding a putative esterase and a neighbouring gene (*lr1515*), an extracellular protein of unknown function, were strongly induced (*lr1516* >6-fold and *lr1515* >10-fold after 15 minutes). In addition, the putative esterase contained a penicillin-binding domain. Proteins with such domains are often involved in peptidoglycan synthesis (Arbeloa *et al.*, 2004; Hesek *et al.*, 2004). Interestingly, in *L. lactis* such proteins have been linked to acid resistance (van de Guchte *et al.*, 2002). Altering the composition of fatty acids in the cell membrane is another mechanism for adapting to external stress factors (Quivey *et al.*, 2000). This response could also decrease the amount of protons entering the cell. Two genes *(lr0811, lr1797)*, predicted to be involved in lipid metabolism, were also induced at pH 2.7. Further studies are required to investigate the possible role of these genes in acid adaptation.

Gene regulation

Six genes encoding regulatory proteins were up-regulated by the acid treatment. This indicates further responses and adaptations to the new environment. Five of these were similar to characterised transcriptional regulators in other species.

However, no indications of the cellular processes, in which they are involved, were found. Interestingly, the final regulatory protein was RR7 from a two-component system resembling the essential YycFG in *B. subtilis* (Fabret & Hoch, 1998). Since YycFG is involved in cell homeostasis and cell division (Howell *et al.*, 2003), the altered expression of RR7 is possibly a step to inhibit growth in an unsuitable environment, rather than a mechanism to increase acid tolerance.

Inhibited growth and translation

The genes repressed at pH 2.7 were linked to different types of cellular processes than the genes that were induced. The majority of the encoded proteins are putatively involved in transport and metabolism; energy conversion; cell division; replication; protein translocation; or translation. This implies that the processes important for growth and protein synthesis decreased under these conditions.

A view of complex gene regulation

Gene regulation is complex. The experimental model described above, provides a detailed but complex view of the changes that takes place when a bacterium is diluted into an acidic pH. The most striking observation was that many genes strongly induced or repressed in the treated control (diluted in pH 5.1) were unchanged or only expressed minor changes when the cells were diluted into a lower pH. The conclusion was that the lowered pH counteracted the effects of dilution. Was this a secondary effect, or was it a controlled response to the acidic environment? The majority of these genes were down-regulated in the control after treatment. Subsequently, these genes were expressed to a higher level at pH 2.7 than in the control. Several of these genes were likely to be down-regulated by dilution, for example the quorum sensing gene *luxS* (Xavier & Bassler, 2003). Other genes have previously been reported to be involved in response to acid stress, e.g. the arginine deiminase pathway (*lr1518*, *lr1517* and *lr1020*). This pathway produces ammonia and hence contributes to an alkalisation of the environment. Interestingly, the arginine deiminase pathway is probably regulated by the combined effects of arginine availability, energy depletion, catabolite repression and oxygenation rather than by low pH (reviewed by van de Guchte *et al.*, 2002). Furthermore *hpk3*, which encodes a two-component sensor protein, belonged to the set of genes. The *hpk3* was inactivated and the survival of the disruption mutant in synthetic gastric juice was tested in the same way as the *clpA* mutant. The *hpk3* mutant was significantly more sensitive than both the wild type and the *clpA* mutant ($P<0.001$ and $P<0.01$ respectively) (Fig. 7.). The corresponding genes of *hpk3* in *L. lactis* (O'Connell-Motherway *et al.*, 2000) and *L. acidophilus* (Azcarate-Peril *et al.*, 2005) are additionally involved in acid tolerance. Taken together, this implies that the counteraction of the effects of dilution by the low pH was of importance for the *L. reuteri* cells. However, further studies are required to examine these effects and to comprehend the gene regulation of *L. reuteri*.

The expression of several genes changed in different ways in the treated control and at pH 2.7, i.e. they were induced at pH 2.7 and repressed in the control or vice versa. The gene encoding one of the extracellular Apf-like proteins (Lr0537)

exhibited the most outstanding profile. This gene was highly induced in the treated control, but strongly repressed at pH 2.7. Of the remaining genes encoding Apf-like proteins, one (*lr0858*) displayed a similar profile, while the expression of the other (*lr1267*) did not change in this experiment (data not shown). This supports the speculations that the Apf-like proteins are involved in maintenance of cell shape. Furthermore, this indicates that the role of *lr1267* partly differs from that of *lr0537* and *lr0858*.

Signal transduction and gene regulation (IV)

There are numerous examples of systems controlling and modulating gene expression in response to environmental signals in bacteria. Some systems are only found in one or in a limited number of species and are likely to only be involved in a specific regulation mechanism. Other systems are widely spread among different species and different variants are specialised for regulation of a number of behaviours. Little is known about mechanisms for sensing the environment and gene regulation in *L. reuteri*. However, the genome of *L. reuteri* contains genes encoding seven complete two-component signal transduction systems. Detailed characterisations of these systems would provide a first insight into the signal transduction pathways of this organism.

Characterisation of the L. reuteri *two-component systems*

Analyses of the amino acid sequence of the seven histidine kinases (Hpks), revealed that they had the same core structure as the class I, EnzV-like, two-component sensors, with two transmembrane helixes surrounding the sensor domain, a dimerization domain with the active site histidine and an C-terminal kinase ATP-binding domain (West & Stock, 2001). In addition, Hpk3, 4, 6 and 7 contained HAMP domains. The HAMP domains are conserved linker domains, which are found in approximately 25% of all histidine kinases and may be involved in the interactions of the protein. The structure of Hpk7 differed from the other 6 Hpks, since it contained one additional region, a PAS domain. PAS domains bind to various kinds of small regulatory molecules, for example to heme, NAD and ATP (Koretke et al, 2003). The seven response regulators (RRs) in the complete two-component systems (TCSs) all displayed the classical structure of the OmpR family proteins (West & Stock, 2001. These contained an N-terminal receiver domain containing the phosphoryl-accepting asparatate residue and a C-terminal DNA-binding regulatory domain. The structural characterisation revealed that the proteins have the residues and domains required for functioning in signal transduction.

Indications of putative functions of the two-component systems in *L. reuteri* were obtained by comparison to characterised systems in other species. In addition, genes likely to be part of the same operon as the genes encoding the Hpks and the RRs were examined. Four of the complete systems were remarkably similar to previously described systems (e-value $<e^{-58}$, amino acid identity >50% for at least one of the components). TCS2 were highly similar to TCS31 in

Lactobacillus sakei and TCS4 to TCS48 in the same species (Morel-Deville, Fauvel & Morel, 1998). Mutation analysis of the RR in TCS31 displayed a phenotype that was more sensitive to heat, acid, oxygen and hydrogen peroxide and that grew more slowly than the wild type. However, this mutant was 15-fold more resistant to the antibiotic vancomycin than the wild type. The phenotype of the *rrp48* mutant survived heat shock well and was tolerant to oxidative stress (Morel-Deville, Fauvel & Morel, 1998). The *L. reuteri* Hpk3 and RR3 showed high similarity to *lrA* and *llkinA* respectively from *Lactococcus lactis*. Strains, with mutated *lrA* or *llkinA*, are sensitive to acid stress (O'Connell-Motherway *et al.*, 2000). This system is also found in *L. acidophilus* (Azcarate-Peril *et al.*, 2005). Finally, TCS7 was part of an operon homologous to the *yycFGHIJK* operon in *Bacillus subtilis* (Fabret & Hoch, 1998; Howell *et al.*, 2003). This operon contains the two-component system YycFG, which is widely spread among gram-positive bacteria. Furthermore, YycFG is essential in several species, for example in *B. subtilis* (Fabret & Hoch, 1998) and *Staphylococcus aureus* (Clausen *et al.*, 2003), although not in *L. lactis* (O'Connell-Motherway*, et al.*, 2000) and *Streptococcus mutants* (Lee, Delaney & Elkhateeb, 2004). In *B. subtilis*, the YccFG system is likely to be involved in regulation of cell division and cell wall homeostatis (Howell *et al.*, 2003). The specific functions of two-component system, i.e. which signals trigger the response and which genes they regulate, are hard to predict from amino acid and homology studies. Therefore, the predicted functions have to be confirmed with experimental analyses.

Phenotype of the rr2 *mutant*

Paper IV describes the construction and analysis of a mutant of response regulator 2. The phenotype of the *rr2* mutant was studied in order to identify differences compared to the wild type. Properties tested were for example growth, antibiotic sensitivity and stress tolerance, which are often regulated by two-component systems in other bacteria and also by TCS31 in *L. sakei* (Morel-Deville, Fauvel & Morel, 1998). In addition, properties believed to be important for the ecological performance of *L. reuteri*, such as adhesion and production of reuterin, were examined. The properties tested and the phenotype of the wild type and the *rr2* disruption mutant are highlighted in Table 1. There were no differences between the mutant and the wild type in growth, fermentation patterns, enzymatic activity, reuterin production, or tolerance to acidic pH (pH 4), alkaline pH (pH 8), bile, oxygen and tetracycline. However, the mutant was more sensitive than the wild type to sodium chloride (Fig. 8) and to the antibiotic ampicillin. In addition, the *rr2* mutant exhibited significantly increased binding to mucus compared to the wild type (data not shown) and the cells of the *rr2* mutant showed slight aggregation when studied under the microscope (Fig. 9). The MIC (Minimal Inhibitory Concentration) value for ampicillin was 3 μg ml^{-1} for the mutant and 6 μg ml^{-1} for the wild type, when grown on solid media. In liquid media *L. reuteri* was less sensitive to ampicillin. Both the wild type and the *rr2* disruption mutant grew in MRS with an addition of 8 μg ml^{-1} ampicillin, although the growth rate of the mutant was notably lower. When grown in MRS containing NaCl, similar results were obtained. Although the *rr2* mutant grew in MRS with 4% NaCl added (w/v), the growth rate was clearly affected.

Table 1. The phenotype of *L. reuteri* ATCC 55730 *rr2* mutant compared to the wild type

Phenotype tested	**Comparison between *L. reuteri* wild type and *rr2* mutant**
Growth in MRS 37ºC	No difference
Growth in MRS 30ºC	No difference
Growth in microaerophilic conditions	No difference
Growth in MRS pH 4	No difference
Growth in MRS 4% NaCl	Reduced growth in *rr2* (Fig. 8)
Growth on MRS 0.3% bile	No difference
Adhesion to mucus	Increased adherance in *rr2*
Production of reuterin	Produced by both
Fermentation pattern (API 50CH)	Identical profile
Fermentation pattern (Rapid ID32 Strep)	Identical profile
Enzymatic activity (API Zym)	Identical profile
Tetracycline resistance	Equal resistance
Ampicillin resistance	Wt MIC 6 μg ml^{-1}, RR2 MIC 3 μg ml^{-1}

Genes putatively regulated by response regulator 2

Microarray analyses were performed in order to identify genes putatively regulated by this two-component system. The gene expression of the *rr2* mutant was compared with that of the wild type cells in late exponential phase before and after 40 minutes of incubation with 8 μg ml^{-1} ampicillin. Preliminary results, based on three biological replicates before addition of ampicillin and two biological replicates after 40 minutes incubation, revealed 40 genes that were differently expressed in the mutant in comparison with the wild type. Of these, 18 genes were expressed lower in the mutant and 22 genes higher. The genes expressed higher in the *rr2* mutant than in the wild type included two putative glycosyl transferases probably involved in cell wall synthesis. Several genes expressed lower in the mutant also encode proteins with a connection to stress response or cell surface biogenesis.

Lr1121 was similar to asparagine synthetases in *Bacillus subtilis* (38% identity) (Yoshida, Fujita & Ehrlich, 1999). These belong to the AsnB family, the members of which are able to use glutamine and ammonia for synthesis of asparagine. *B. subtilis* has at least three asparagine synthetases named AsnB, AsnH and AsnO. Mutation analyses of the corresponding genes expose a physiological role of AsnB in vegetative cells and of AsnO in sporulating cells. Interestingly, AsnH has been suggested to be involved in cell surface organization. This gene is less efficient in asparagine synthesis than asnB (Yoshida, Fujita & Ehrlich, 1999), but instead the *asnH* mutant is sensitive to penicillin and forms abnormal colonies on high-salt plates (Yoshida, Fujita & Ehrlich, 1999). This indicates that also Lr1121 affects the organisation of the cell surface.

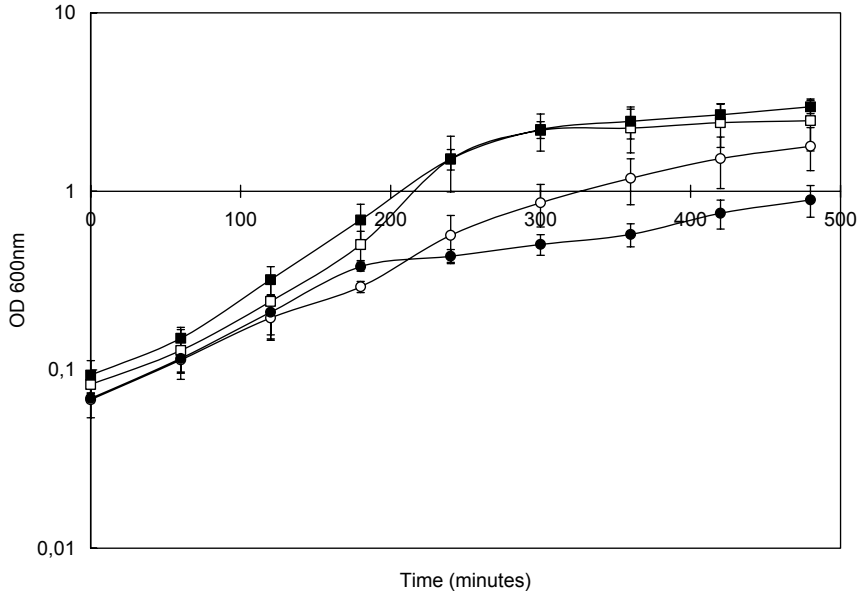

Fig 8. The growth of *L. reuteri* ATCC 55730 wild type and *rr2* mutant at 37ºC in MRS or in MRS + 4% NaCl. □ represent the wild type in MRS, ■ the *rr2* mutant in MRS, ○ the wild type in MRS-NaCl and ● the *rr2* mutant in MRS-NaCl. Error bars represent standard deviation (n=3).

The gene *lr2108* was similar to *asp1* in *Lactobacillus sakei* (Marceau *et al.*, 2004) and to the stress-response gene *gls24* in *Enterococcus faecalis* (Giard *et al.*, 2000). *L. sakei* also encodes a second copy of this gene *asp2*, while the draft genome sequence of *L. reuteri* only contained one. The Asp proteins in *L. sakei* were identified with two-dimension gel electrophoreses, and were up-regulated when the bacterium was grown in medium containing 4% NaCl. However, although clearly more sensitive to low temperatures than the wild type, the *asp1* mutant was not affected by NaCl.

The CydABCD system is widely spread among gram-negative and gram-positive bacteria. The *cydA* and *cydB* genes encode the two subunits of the cytochrome *bd* oxidase complex, while *cydC* and *cydD* encode a heterodimeric ABC transporter. The CydCD transporter is required for assembly of cytochrome *bd* in *B. subtilis* (Winstedt *et al.*, 1998). In *E. coli* CydCD also functions as an exporter of the reductants cysteine (Pittman *et al.*, 2002) and glutathione (Pittman, Robinson & Poole, 2005). The *L. reuteri* gene *lr1233*, which was expressed lower in the *rr2* mutant than in the wild type, encodes the CydD protein. In *E. coli*, the phenotype of the *cydD* mutant differs from that of the *cydC* mutant. The *cydD* mutant is sensitive to e.g. benzylpencillin and dithiothereitol, although this is reversed by addition of exogenous cysteine (Pittman *et al.*, 2002) or glutathione (Pittman, Robinson & Poole, 2005).

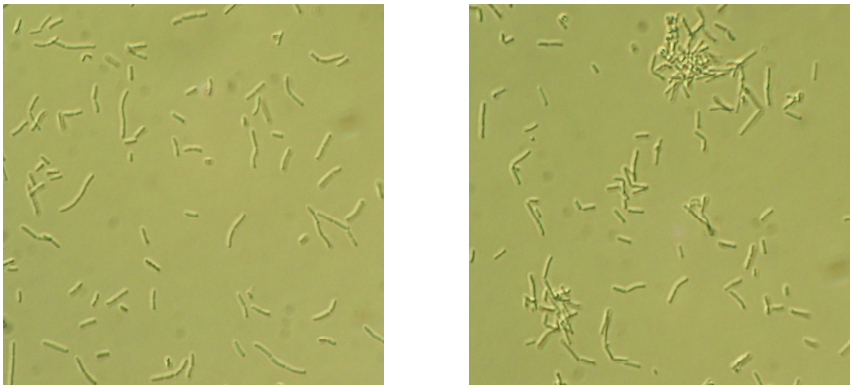

Fig. 9. Morphology of the *L. reuteri* ATCC 55730 wild type (A) and *rr2* mutant (B).

The putative role of two-component system 2

In summary, the *rr2* mutant was more sensitive to NaCl and ampicillin than the wild type although neither growth itself nor fermentative capability were affected. Several of the genes, with a changed expression in the mutant, were tentatively associated with stress response, cell surface organisation or transport. This indicates that the biological role of RR2 and TCS2 in *L. reuteri* is to modify the cell envelope in a general response to unfavourable external conditions rather than to mediate adaptation to osmotic stress or antibiotic resistance specifically. Alterations of the cell wall in response to osmotic stress have been studied in e.g. *Lactobacillus casei* (Piuri, Sanchez-Rivas & Ruzal, 2005). Changes of the cell surface could also explain the increased ability of the bacteria to aggregation and to adhere to mucus. TCS2 is therefore designated Lea (*L. reuteri* Envelope Altering).

Men strunt är strunt och snus är snus, om ock i gyllne dosor,
och rosor i ett sprucket krus är ändå alltid rosor.
Gustaf Fröding

Conclusions and future perspectives

Understanding of the commensalistic bacteria inhabiting the gastrointestinal is crucial for comprehending this complex ecosystem both in health and disease. In this thesis the lactic acid bacterium *Lactobacillus reuteri* was examined using three different approaches, in order to reveal and analyse properties important for its ecological performance. *L. reuteri* encodes at least 126 extracellular proteins, although the functions of the majority of these proteins are unknown. The early response to acid shock of *L. reuteri* involves the chaperone ClpA and possibly also mechanisms to alter the cell envelope. Furthermore, the genome of *L. reuteri* contains seven complete two-component signal transduction systems. TCS2 (Lea) probably modifies the composition of the cell wall in response to unfavourable environmental conditions. The surface of the cell is clearly important not only for interactions with the environment, but also as a shield protecting bacteria from various stresses. These findings provide a better insight in the adaptation mechanisms and life strategies of this bacterium.

However, this thesis only touches the surface of the genetic properties of *L. reuteri* important for its ecological performance. Underneath there is an enormous depth of undetected signals, mechanisms and processes. The cell surface-associated and secreted proteins must be characterised in order to reveal their functions, anchoring mechanisms and biological roles.

An ability to adapt to acid conditions is essential for surviving the passage through the stomach. Several candidate genes important for responding to acidic shock and interesting for further studies were identified in this project. However, several other factors, such as bile, enzymes and other microorganisms, also affect the bacteria in the gastrointestinal tract. To understand the gene expression *in vivo*, these parameters must be studied in detail, singly and in combination.

Of the seven two-component systems in *L. reuteri*, the functions of six systems are unknown. Detailed characterisations of these systems and their effects on the gene expression are required to understand their biological role. Such knowledge would provide a comprehension of the signal transduction pathways and gene regulation mechanisms in this bacterium. Learning about gene regulation would hopefully increase our understanding of how this organism adapts and interacts in its ecological niche. In a longer perspective this could result in a better insight into how the intestinal microflora affects their host in health and disease.

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