

**Factors Important for Persistence of
Lactobacillus reuteri in the
Gastrointestinal Tract**

**A Study of Extracellular Proteins, Stress Response and
Survival of Mutants in a Model System**

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Abstract

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Lactobacillus reuteri ATCC 55730 is a commercially available probiotic *i.e.* health promoting bacterium. The interest for lactic acid bacteria (LAB) as probiotics might be due to the tradition of use in food preservation, their non-pathogenic behavior and, a natural occurrence in the human gastrointestinal (GI) tract. Although examples of clinically proven probiotic effects are increasing, the understandings of bacterial-host interactions behind these effects are only beginning to be unravelled.

To put this thesis work into context, the field of probiotic research is described and health promoting mechanisms and perspectives of strain selection are discussed. The experimental work has focused on properties of *L. reuteri* ATCC 55730 that are tentatively important for persistence of this and other probiotic strains in the GI tract. To get an idea of the proteins on the surface of *L. reuteri* ATCC 55730, a draft genome sequence was scrutinized using bioinformatic tools. This resulted in the identification of a diverse set of 126 putative proteins with different possible means of surface attachment. The bacterium was also exposed to heat and acid stress and the response monitored by DNA microarrays. Acid stress is a well-documented way of investigating probiotic strains, since survival in the acidic GI tract is considered a prerequisite for a probiotic effect. Heat stress could be considered an artificially constructed environment for a probiotic bacterium, but is probably still relevant in elucidating mechanisms of persistence in other stressful conditions such as the passage of the GI tract. To further investigate genes revealed in the bioinformatical and DNA microarray analysis as well as a set of genes from the seven earlier identified two-component systems of *L. reuteri* ATCC 55730, 14 genes were mutated. These were then evaluated for ecological performance using a three-stage continuous culture system simulating a human colon using a real time PCR approach to monitor the relative abundance of the individual mutants. Two caseinolytic protease (Clp) genes, *clpE* and *clpL* that were induced in heat and acid stress respectively, both showed importance for persistence in the colon model.

Keywords: *Lactobacillus reuteri*, probiotics, heat stress, acid stress, surface proteins, gastrointestinal tract, survival, persistence, mutants, Clp

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Kanske tandkräm också....

man vill ju göra nåt
fint
eller åtminstone ville jag det
något som skulle stå kvar
en extra speciell tandborste
med tandkräm kvar som människorna
i nästa tid
skulle vilja gå o titta på
titta mamma! Där är bob hansson's tandborste
jag ville liksom duga
sätta ut något fint i gräset
som en sång mellan träden
för barn o ungar att nynna med i

men det vet ju alla
att så kan man inte hålla på

/Bob Hansson (Halleluja liksom)

Contents

Introduction	7
Objectives and outline of the thesis	8
Microbial ecology of the gastrointestinal tract	9
The gastrointestinal tract and the indigenous microbiota	9
Disorders related to the microbiota of the gastrointestinal tract	11
Lactic acid bacteria & probiotics	13
Effects of lactic acid bacteria	13
Probiotic strain selection	14
Probiotic research: Mechanisms known today and future perspectives	16
<i>Lactobacillus reuteri</i> ATCC 55730	17
Interaction of bacteria with the environment	18
Surface proteins (I)	19
Responses to changes in the environment	23
<i>Heat stress response (II)</i>	27
<i>Acid stress response (III)</i>	29
Biological importance of specific genes	32
<i>Construction of mutants</i>	32
<i>Evaluation of mutants in an artificial colon (IV)</i>	34
Concluding remarks	36
References	38
Acknowledgements	49

Appendix

Papers I-IV

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

- I. Båth, K., Roos, S., Wall, T. & Jonsson, H. 2005. The cell surface of *Lactobacillus reuteri* ATCC 55730 highlighted by identification of 126 extracellular proteins from the genome sequence. *FEMS Microbiology Letters* 253, 75-82.
- II. Båth, K., Wall, T. Jonsson, H. & Roos, S. Heat stress response in *Lactobacillus reuteri* ATCC 55730. *Submitted*
- III. Wall, T., Båth, K., Britton, R., Jonsson, H., Versalovic, J. & Roos, S. The early response to acid shock in *Lactobacillus reuteri* involves the ClpL chaperone and a putative cell wall altering esterase. *Submitted*
- IV. Båth, K., Costabile, A., Goulas, T., Jonsson, H., & Roos, S. Fourteen genes of *Lactobacillus reuteri* ATCC55730 putatively important for persistence in the intestine mutated and analysed in a artificial colon. *Manuscript*.

My contribution to the papers included in this thesis has been as follows:

- I. Took part in bioinformatical analysis and writing of the paper.
- II. Major part in planning, laboratory work and writing of the paper.
- III. Took part in planning and performance.
- IV. Major part in planning and laboratory work. Major contribution to writing together with S. Roos and H. Jonsson.

Introduction

Once upon a time, before food was easily accessible in grocery stores, people were more frequently exposed in their every day life to bacteria, both the pathogens and those that live in harmony with us. Since, e.g. of the oldest ways of preserving food is through naturally occurring fermentation (Ross, Morgan & Hill, 2002). However, in the industrialised part of the world we are now more prone to throw away food, since we can afford to buy new fresh groceries. Thus people in the past provided their gastrointestinal (GI) tract with vast amounts of microorganisms, both good and bad, on a daily basis. Today our surroundings are far more sterile and we encounter fewer microorganisms in our everyday life (Isolauri *et al.*, 2002). Many of us no longer consume as much naturally fermented foods such as sauerkraut, fermented milks, pickles, sourdough bread *etc.* Instead we consume a great deal of white bread and pasta, made from cereals, and a lot of sugar. Cereals did not become part of the staple diet until cultivation of crops begun (about 10 000 years ago). Prior to that, we consumed more meat, fish, eggs, fruit, vegetables and roots. Diet differs greatly between different areas of the world due to climate, culture and the economy. In Sweden cereals along with beans, dairy products, cooking fat and sugar currently constitute more than 70% of the daily calorie intake (Lindeberg, 2005). A question is whether this influences our health today and also if our digestive system is influenced. Could we possibly benefit from a dietary supplement to correct a disturbance of this system?

In the Western society today different diets are frequently introduced, which we are told we benefit from in different ways. There is e.g. the paleolithic diet (or the hunter/gatherer diet) that for instance is claimed to give a lower blood pressure and higher insulin sensitivity than the today normal cereal based diet (Jönsson *et al.*, 2006). There are also people with an anthroposophic lifestyle, with a diet comprising vegetables fermented naturally by lactobacilli (Alm *et al.*, 2002). A lot of studies show that by adding a daily dose of a bacterial culture to our diet we can promote health. An anthroposophic lifestyle has e.g. shown to reduce the risk for allergy in children (Alm *et al.*, 1999; Flöistrup *et al.*, 2006).

The interest in microorganisms as health-promoting additives to our diet began already 100 years ago (Metchnikoff, 1907), and live microorganisms that show positive health-promoting properties are today termed probiotics (Fuller, 1986). The possibility that certain microbes might be beneficial to human health is highlighted by numerous consumer products containing probiotic bacteria (Marco, Pavan & Kleerebezem, 2006). This thesis focuses on the lactic acid bacterium (LAB) *Lactobacillus reuteri* ATCC 55730 used in various probiotic products and shown to have beneficial effects on human health (Tubelius, Stan & Zachrisson, 2005; Weizman, Asli & Alsheikh, 2005). How can a single bacterial strain have an impact on our health and well-being even though the microbiota of our gut is very complex, consisting of approximately 800 species and more than 7000 strains (Bäckhed *et al.*, 2005)? An important factor could be that the gut mucosa is the major sites for induction and regulation of our immune system (Guarner, 2006). In humans about 60% of the total immunoglobulin produced is secreted into the GI tract (Brandtzaeg *et al.*, 1989), and about 70% of our immune system is estimated

to be located in the gut (Bengmark, 2002). Perhaps this provides an opportunity for a small change in the microbial composition to have a large impact. However, many of the details behind the beneficial effects of a single probiotic strain are still unknown, and basic knowledge of intestinal bacteria and their interactions with other microorganisms and with the host is a prerequisite for successful probiotic research and development (Parvez *et al.*, 2006).

Objectives and outline of the thesis

The work described in this thesis employs basic research to investigate properties of *L. reuteri* ATCC 55730. The main objective was to identify factors important for gastrointestinal persistence in this commercially available probiotic bacterium. A secondary objective was also to write a thesis that in a, read wise, pleasant way describe the field of probiotic research, discuss health-promoting mechanisms and provide a perspective of strain selection in order to place the research results in a context.

The starting point of this thesis is the draft genome sequence of *L. reuteri* ATCC 55730. In **Paper I** bioinformatical methods were used to give a putative picture of the extracellular proteins of this bacterium, in order to identify possible factors for host interaction. In **Paper II** and **III** the bacterium was exposed to heat and acid stress respectively, and DNA micro arrays were used to investigate the cellular stress response. Acid stress is a well-documented way of investigating probiotic strains, since survival in the acidic upper GI tract is considered a prerequisite for a probiotic effect. Heat stress, on the other hand, is an artificially constructed situation normally not encountered by *L. reuteri* but can still be used to identify persistence mechanisms used in other possibly stressful environments such as the GI tract. In **Paper IV** the importance of genes revealed in **Papers I-III** for ecological performance in an artificial colon was investigated. A schematic view of the work of this thesis is presented in Fig. 1.

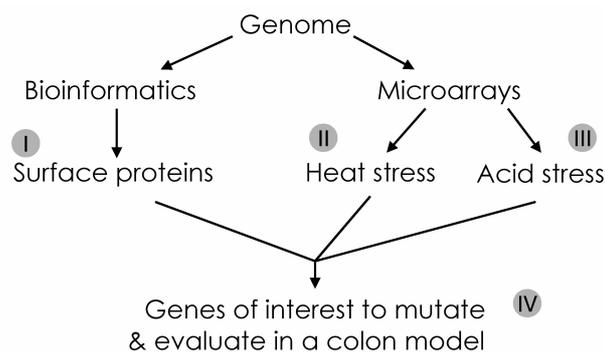


Fig. 1. Outline of thesis work. A schematic picture of how **Papers I-IV** in this thesis are interconnected.

Microbial ecology of the gastrointestinal tract

The gastrointestinal tract and the indigenous microbiota

The GI tract is specialised to carry out digestion of food (stomach and upper small intestine), absorption of nutrients (small intestine) and water salvage and waste storage (large intestine) (Tannock, 1995). In addition, the intestine is a complex ecological system. The microbiota is in constant communication with the mucosal surface of the intestine and thus with the immune system of its host. The normal interaction between the abundant, diverse and active microbiota of the GI tract and the host is a symbiotic relationship from which both partners benefit (Hooper, Midtvedt & Gordon, 2002). At birth, the GI tract is sterile, but colonization occurs directly after delivery and within a few months a relatively stable microbial population is established (Ewaschuk & Dieleman, 2006; Fanaro *et al.*, 2003). Each individual has a genetic predisposition to being colonized by different organisms. The delivery mode at birth, the environment in which the child is reared, the diet *etc.* are other factors that determines this individual microbiota. Thus, each person has a particular combination of predominant and subdominant species (Guarner, 2006). The intestine of an adult individual contain 500-800 species, of which 99% is comprised of 30-40 predominant species (Bäckhed, *et al.*, 2005; Hooper, Midtvedt & Gordon, 2002). Actually the number of microbial cells within the gut lumen exceeds the number of cells in the body by a factor of 10 (Bengmark, 2002; Guarner & Malagelada, 2003) and has been estimated to account for 35-50% of the volume of the human colon content (Isolauri, Salminen & Ouwehand, 2004).

Current knowledge about the human microbiota is far from complete. Earlier culture-based investigations and more recent 16S rRNA-based studies show temporal and individual variations in the microbial composition of our microbiota (Dethlefsen *et al.*, 2006). The human stomach harbours a relatively small number of microbes. The number is restricted because of the high acidity in the stomach (pH as low as 2). A maximum of 10^4 microbes per millilitre gastric juice can be detected. The only undisputed inhabitant is *Helicobacter pylori*, present in the gastric mucosa in some individuals. (Dethlefsen, *et al.*, 2006). However, a recent 16s rRNA study showed a diverse picture, with 128 bacterial species possibly representing both ingested and resident species of the stomach (Bik *et al.*, 2006). Interestingly, a number of lactobacilli, including for new species, were identified from a study of gastric biopsies (Roos, Engstrand & Jonsson, 2005). The transit time from the mouth to the ileum is short, about 4-6 h (Bourlioux *et al.*, 2003), which is also a limiting factor for colonization. However, the number of bacteria increases from about 10^4 up to about 10^7 per ml intestinal content in the jejunum. In the ileum the passage of the material for digestion is slower and microbial colonization is facilitated. The microbiota here is diverse and harbours e.g. lactobacilli and enterococci but also gram-negative facultative anaerobic bacteria and obligate anaerobes. A total of about 10^8 bacteria per millilitre of content is found in this niche (Metchnikoff, 1907). The large intestine is the main site of microbial colonization of the human GI tract. The transit time here is slow, normally about 55 hours (Bourlioux, *et al.*, 2003), and a large number of bacteria are present. The amount of bacteria increase from about 10^8 to 10^{11} - 10^{12} per ml

stool (Dethlefsen, *et al.*, 2006), representing about 800 species (Bäckhed, *et al.*, 2005) (Fig. 2). However, it is worth considering that there is a difference between the bacterial composition of the lumen and the mucosal membrane (Zoetendal *et al.*, 2002), which means that our picture of the GI colonization is even more incomplete.

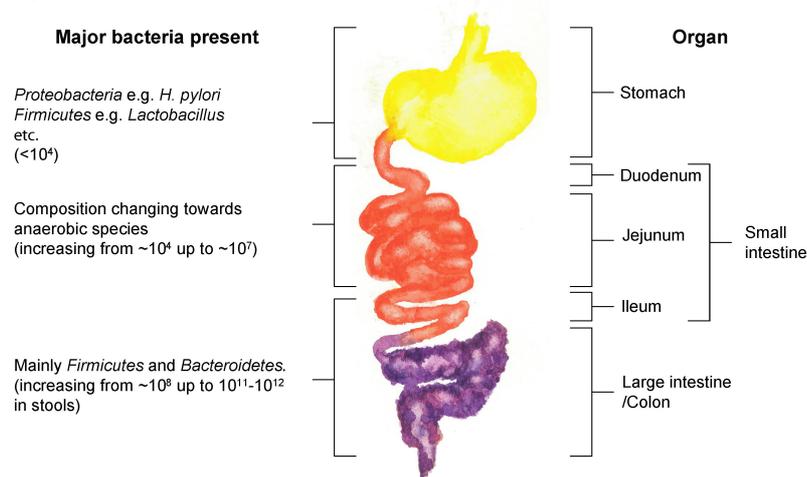


Fig 2. An illustration of the intestine and the major bacterial groups found in different parts. The microbiota of the GI tract is far from completely described. Data generated so far show temporal and individual variations in the microbial composition. (Illustration of data from Dethlefsen *et al.* (2006)).

By studying animals bred under germ-free conditions important information about the effect of the microbial community on its host can be obtained. The functions of the enteric microbiota can be divided into three groups: metabolic, protective and trophic (Fig. 3.) (Guarner, 2006). It has e.g. observed seen that germ-free animals require 30% more energy in their diet and that supplementation of vitamins K and B is necessary to maintain their body weight (Isolauri, Salminen & Ouwehand, 2004). The mucosal surface of our GI tract serves as a barrier between the microbiota and the host, and as mentioned above the major part of our immune system is situated there. The non-pathogenic bacteria, whether just passing or colonizing, could interact with the host through this mucosal surface and possibly prime or modulate our immune system in a positive manner. The balanced normal intestinal microbiota also strengthens the barrier effect of the mucosal surface, blocking the passage of bacteria and antigens into the blood stream (Parvez, *et al.*, 2006).

<p>Metabolic functions: Fermentation of nondigestible dietary residue and endogenous mucus Salvage of energy as short-chain fatty acids Production of vitamin K Adsorption of ions</p>
<p>Trophic functions: Control of epithelial cell proliferation and differentiation Development and homeostasis of the immune system</p>
<p>Protective functions: Protection against pathogens / The barrier effect</p>

Fig. 3. Effect of the microbiota on its host (Source: (Guarner, 2006)).

The microbes in our GI tract have the potential to act in a favourable, neutral or deleterious manner in relation to their host. Pathogenic bacteria are often considered to use a highly sophisticated mode of action during infection and the action of pathogenesis has already been thoroughly investigated, probably since when the pathogens strike they cause obvious medical problems. However, it would perhaps be a better survival strategy for a bacterial species to live in symbiosis with its host, *i.e.* giving something in return instead of merely exploiting. A group of indigenous inhabitants of the human GI tract, thought to be among the dominant inhabitants of the small intestine, are members of lactic acid bacteria (LAB). The most dominant common feature of the LAB is that a major proportion of their metabolic end-products is lactic acid. The organic acids produced tend to lower the pH of the gut (at least locally) and thus create a less desirable environment for harmful bacteria. The LAB also produces antimicrobial compounds (*e.g.* bacteriocins) with activity against closely related bacteria, but some strains also produce substances such as hydrogen peroxide, reuterin *etc.* with effects against gastric and intestinal pathogens and other microbes (Ljungh & Wadström, 2006).

Disorders related to the microbiota of the gastrointestinal tract

The human GI tract with its enteric microbiota is generally able to respond rapidly to invading pathogens and then return to balance after an infection. When this balanced interaction is disturbed, a chronic intestinal inflammation may occur. Diet, stress and side-effects of modern medical practices have been implicated as factors capable of exerting a negative influence on human health and nutrition (Buret, 2006; Collins, 2001; Kiliaan *et al.*, 1998; McKay, 2005). A possible outcome of these factors could be a deficiency or shift in the equilibrium of the gut microbiota, leading to an increase in chronic intestinal illness in the industrialised world of today. It is well accepted among the general public that emotional stress is a factor that can result in disturbances in intestinal health. Evidence in scientific studies also suggests that stress is a contributing factor in intestinal inflammatory disease, but the mechanisms involved remain to be identified. It is evident from animal work however, that stress is a potent modulator of inflammatory response (Collins, 2001), a cause of disturbance of the barrier effect (Kiliaan, *et al.*, 1998) and it has also been shown that stressful life events often precede disease relapses in patients with intestinal inflammatory disease (Collins, 2001; Kiliaan, *et al.*, 1998).

It has been known for about 50 years that people treated with antibiotics have an increased susceptibility to new infections (Bengmark, 2002). Antibiotic-associated diarrhoea is a frequent side-effect of antibiotics, and can affect as many as 25% of patients treated depending on the antibiotic used (Katz, 2006). In the past decade there has been a major increase in cases of antibiotic-associated diarrhoea, but in most cases the cause of the diarrhoea is unclear (Meier, 2005). One of the mechanisms behind these cases of diarrhoea is thought to relate to alteration of the microbiota, leading to a loss of colonization resistance. This predisposes the gut to colonization by potential pathogens such as *Clostridium difficile*, causing a

potentially severe form of toxin-induced colitis that accounts for 15-25% of cases of antibiotic-associated diarrhoea (Katz, 2006). A number of GI disorders connected to the microbiota and their symptoms are presented in Fig. 4.

IBD – Inflammatory bowel disease is a comprehensive term for Crohn's disease and ulcerative colitis. Originates in a combination of genetic, environmental and immune factors.

Crohn's disease - relapsing inflammation in the GI tract. Abdominal pain, diarrhoea, fever, and weight loss. Can affect any area of the GI tract, but most commonly the ileum. Causes diarrhoea, abdominal pain, fever and weight loss.

Ulcerative colitis – relapsing inflammation in the GI tract. Causes inflammation and ulcers in the lining of the rectum and colon. Causes diarrhoea which is often mixed with blood.

IBS – Irritable bowel syndrome is a functional disorder of which the underlying factors are uncertain, but it involves the immune system and possibly bacterial gastroenteritis. Problems of movement in colon causes abdominal pain, bloating, flatulence and diarrhoea.

Helicobacter pylori gastritis– causes chronic gastritis and induces the risk of peptic ulcer, gastric cancer, gastric mucosa-associated lymphoid tissue (MALT) lymphoma

Fig. 4. A summary of some gastrointestinal disorders connected to the microbiota of the GI tract, based on data from (Broekaert & Walker, 2006; Collins, 2001; Felley & Michetti, 2003).

As mentioned above, the enteric microbiota differs greatly between individuals and to date it has been difficult to get a complete picture of the microorganisms in the GI tract since only 60-80% of them have so far been cultivated (Suau *et al.*, 1999). In the current era of genetic techniques we may be able to expand our knowledge about the composition of our microbiota. Molecular biological techniques have shown that the microbial structure of the microbiota differs greatly between individuals (Eckburg *et al.*, 2005). Interestingly, connections between certain microbes or structures of microbial community e.g. and inflammatory disorders of the intestine have been observed (Seksik *et al.*, 2003). This connection makes it interesting to speculate whether it is possible to somehow manipulate the microbiota in a positive manner.

Lactic acid bacteria & probiotics

Effects of lactic acid bacteria

The idea of yoghurt promoting gut health was raised already by the Ancient Roman historian Pliny the Elder, who wrote '...sour milk is being added to fresh milk which is wanted to curdle. This preparation is extremely wholesome to the stomach...' in *Naturalis historiae* (Plinius Secundus). In more modern times (100 years ago) Metchnikoff (Metchnikoff, 1907) also declared yoghurt to be a health-promoting additive to the human diet. The health-promoting organisms (responsible for the souring of milk) are today known to be lactic acid bacteria (LAB) and some of them are defined as probiotics. The definition of probiotics is a living microbial food ingredient that has a beneficial effect on human health (Fuller, 1986), and currently there are a large number of probiotic products on the market. The most extensively used and studied probiotic bacteria are the LAB, particularly *Lactobacillus* but also *Bifidobacterium* spp. Some species of other genera are also used e.g. *Bacillus* spp. (Guo *et al.*, 2006; Kim *et al.*, 2006), *E. coli* Nissle (Kokesova *et al.*, 2006) and the yeast *Saccharomyces boulardii* (Biloo *et al.*, 2006). The interest in LAB as probiotics might be due to their traditional use in preservation of food and the fact that they are normally not associated with pathogenesis and occur naturally all through the GI tract.

It has been shown that probiotics can impart a number of health benefits (modified from Parvez (2006) and Rastall (2005)), such as an ability to:

- synthesise and enhance the bioavailability of nutrients.
- modulate the immune system.
- reduce symptoms of lactose intolerance.
- decrease the prevalence of allergy.
- reduce the risk of certain cancers.
- reduce the risk of infectious diseases.

Originally, the probiotic effects were primarily thought to be a result of the improvement of the intestinal microbiota. However, many probiotic effects are in fact unlikely to be related to intestinal microbiota changes alone. Other factors include production of vitamins and a possible induction of host mucus production. Indeed, an important part of the beneficial effects of probiotics relates to their immunomodulatory effects; immune-enhancing as well as anti-inflammatory activity (Isolauri, Salminen & Ouwehand, 2004). An example of an interesting probiotic effect is the ability to reduce symptoms of atopic disease. Studies have shown that atopic children have a different microbial community structure than healthy children, in which the level of lactobacilli (Björkstén *et al.*, 1999) and bifidobacteria (Watanabe *et al.*, 2003) is significantly reduced. There is evidence that the incidence of atopic diseases is increasing in industrialised countries with a market economy. The reason is unknown, but it has been suggested that the increase in allergies is connected with a decreased microbial pressure in early childhood (Björkstén, *et al.*, 1999). There is evidence that some probiotic strains prevent allergies. *Lactobacillus rhamnosus* strain GG supplied to pregnant mothers and in a follow up treatment to the children themselves for 6 months gave

a 50% reduction in atopic disease among the children examined at 2 and 4 years of age (Kalliomäki *et al.*, 2001; Kalliomäki *et al.*, 2003). In another study, children with existing, moderate or severe, atopic symptoms were supplied with *L. rhamnosus* 19070-2 and *L. reuteri* DSM 12246. The severity of eczema and the frequency of GI symptoms were decreased after 6 weeks of consumption (Rosenfeldt *et al.*, 2004).

Lately, evidence that probiotics also play a role in oral health has increased (Meurman, 2005). It has e.g. been demonstrated that consumption of yoghurt containing *L. reuteri* reduces oral carriage of mutans streptococci (Nikawa *et al.*, 2004) and *L. reuteri* has also proven to be effective in reducing both gingivitis (inflammation of the gums) and plaque in patients with moderate to severe gingivitis (Krasse *et al.*, 2006). In another recent study oral administration of *L. reuteri* also improved the symptoms of infantile colic within just a week in 95 % of patients (Savino *et al.*, 2007). LAB has been administered to individuals with inflammatory disorders such as atopic and Crohn's disease as well as those with HIV and immunosuppression. Treatment resulted in a down regulation of over-expressed inflammatory responses and stabilisation of gut mucosal barrier (Rastall, *et al.*, 2005).

Probiotic strain selection

Previous criteria when selecting probiotic strains that are still used to some extent today include some or all of the following properties (Chermesh & Eliakim, 2006; Dunne *et al.*, 1999; Ewaschuk & Dieleman, 2006; Parvez, *et al.*, 2006). A probiotic has to be able to:

- withstand processing of the foodstuff and remain viable through the shelf life.
- withstand transit through the GI tract.
- adhere to intestinal epithelial cells.
- produce antimicrobial substances
- modulate the mucosal immune system.
- have a beneficial effect on the host.
- be of human origin.
- be safe (*i.e.* it must be non-pathogenic, free of transferable antibiotic resistance determinants, not degrade mucin *etc.*).

Most probiotic strains on the market today were originally selected on the basis of easily measurable parameters such as the ability to tolerate low pH or large amounts of bile salt or to survive passage through the GI tract. However, the selection of probiotics is perhaps not as straightforward as usually presented. In a human trial on *L. plantarum* 299v it was shown that irrespective of the subject's gastric acidity, the faecal count of supplemented bacteria reached the same level (Goossens *et al.*, 2005). A higher pH of the GI tract, might not, as one would assume, lead to better survival or colonization of a probiotic in the GI tract. Another question raised is whether it is really true that probiotics have to be alive to exercise their beneficial properties. In an animal model it was indicated that the effect of a probiotic could be, at least partly, mediated by its DNA (Katakura *et*

al., 2005). Thus it is important to bear in mind, that it might not only be live microorganisms that can exert a beneficial effect, but possibly also non-viable (dead) cells.

Adherence and colonization are considered to be important for the probiotic effect, but one has to consider that long-term intestinal colonization may only be possible during very early infancy and immediately after birth. No proof of a need for colonization has been reported and probiotics mostly reside only transiently in faecal samples from humans following dietary intake (Isolauri, Salminen & Ouwehand, 2004). A transient effect could still be of interest during *e.g.* acute diarrhoea or after an antibiotic treatment that disrupts the microbial community of the gut. Studies on lactose intolerance, diarrhoea and colon cancer shows that a daily dose of probiotics is needed for any measurable effect (Parvez, *et al.*, 2006; Rembacken *et al.*, 1999).

The effect of antimicrobial compounds in combating pathogens might also be discussed. Most compounds produced by the probiotic LAB are actually not directed specifically towards the pathogens but rather towards closely related species (Ljungh & Wadström, 2006; Ocana & Nader-Macias, 2004). The effects of LAB as a probiotic in various individuals might differ due to the original composition of the individuals' microbiota. In an extensive study with 11 commercial probiotic lactobacilli tested against 1079 freshly isolated indigenous *Lactobacillus* strains in joint pair cultivation, it was shown that the probiotic strains were incompatible with (*i.e.* suppressed the growth of) 60% of the indigenous isolates. Application of incompatible strains to mice (one dose) was shown to decrease the number of host-resident LAB in the short term (Shenderov & Glushanova, 2006). Whether the inhibition of host-resident LAB is good or bad might depend on the situation. It might just be a side-effect of no importance if the probiotic supplied is in balance with the resident microbiota and is still beneficial to the host. However, it could be a disadvantage to disturb the resident LAB in a GI tract in balance. Host-specific persistence behaviour was also shown in a human study, where a mix of five *Lactobacillus* strains was administered to 17 healthy volunteers. None of the volunteers harboured any of the strains before administration, but after a 22 days wash out period one of the volunteers still harboured one of the strains administered (*L. plantarum* DC13) (Klingberg & Budde, 2006). Thus it is tempting to speculate that in the future one could use a specific selection of a probiotic strain depending on the microbial composition of an individual.

Probiotic research: Mechanisms known today and future perspectives

At present, interest is being directed more towards specific effects of clearly defined strains and the focus is not on finding new probiotic strains but instead on determining what makes them beneficial (Fig. 5).

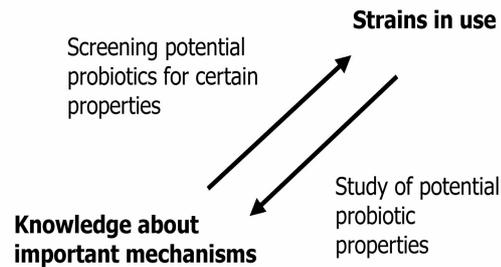


Fig. 5. A schematic view of the field of probiotic science. The focus today is not solely on screening for new strains according to existing criteria such as survival in acid, or production of antimicrobial compounds, but on understanding the mechanisms behind the probiotic effects so as to perform more targeted and specialised strain selection in the future.

Since it is still believed that a probiotic has to be viable at the time of consumption and persist passage through the GI tract to be effective, a well-documented way of investigating probiotics is by testing the survival in acid and bile (Bron *et al.*, 2006; Chou & Weimer, 1999; Lin *et al.*, 2006; Pieterse *et al.*, 2005; Taranto, Perez-Martinez & de Valdez, 2006). Also studies have been performed looking at the response of bacteria in e.g. a mouse intestine (Bron *et al.*, 2004; Walter *et al.*, 2003). This has to date resulted in a number of genes known to be involved in persistence of the bacterium during these conditions. The challenge today is to evaluate the importance of these genes for the probiotic effect. This would preferably be done by construction of knock-out mutants and comparison of their effect to the wild type in clinical studies. This is however not that easily accomplish due to e.g. practical and ethical issues regarding studies on humans and with legalisation regulating the use of genetically modified organisms (GMO). An alternative to the testing of GMO could be to compare effects of strains with different genetic composition and properties. This could give information about what is required for persistence and probiotic effects, but it would not target the importance of specific genes, since there is always more than one gene differing between two strains. As mentioned, part of the probiotic effect might be mediated by bacterial modulation of host cell function. The mechanisms underlying such modulations are currently being investigated *in vitro*. A number of studies on immune cell response and intestinal integrity have been performed (a recent summary can be found in Marco *et al.* (2006)) and observed responses and derived hypotheses now has to be confirmed in animal and human studies. Adherence has so far been studied *in vitro*, using different methods and cell lines (Riedel *et al.*, 2006; Roselli *et al.*, 2006). Different genomic approaches are also used to predict cell surface associated proteins (Broekaert & Walker, 2006; van Pijkeren *et al.*, 2006) and homologues to proteins already known to adhere to

intestinal cells and mucus (Boekhorst *et al.*, 2006a). However, it is important to reflect on genes of unknown function and possibly unknown motifs for binding that will not be detected in these studies.

As mentioned above the probiotic strains on the market today are selected on the basis of easily measurable parameters such as tolerance to acid and bile. Hopefully, the future perspective will be selection for strains possessing well-defined health benefits.

***Lactobacillus reuteri* ATCC 55730**

L. reuteri ATCC 55730, isolated from the breast milk of a woman living in the Peruvian Andes, is a bacterium used today in various probiotic products. *L. reuteri* are Gram-positive, rod-shaped, hetero-fermentative bacteria that commonly inhabit the gut of mice, poultry, and pigs as a member of the normal microbiota (Hammes & Hertel, 2005), and has also been found in the human GI tract (Ahrné, Molin & Axelsson, 1992). It was recently shown in samples from 226 women in Sweden, Denmark, Israel, South Africa, South Korea, Japan and Peru that 12% of mothers had *L. reuteri* in their breast milk (Sinkiewicz & Nordström, 2005). *L. reuteri* has been shown in several clinical studies to promote health and improve defence against illness, such as GI tract problems (Rosenfeldt *et al.*, 2002; Shornikova *et al.*, 1997), atopic disease (Rosenfeldt *et al.*, 2003), colic in babies (Savino, *et al.*, 2007) and oral gingivitis (Krasse, *et al.*, 2006). There are products manufactured specifically for ingestion but also for oral health (*e.g.* chewing gum). The bacteria also produce a broad-spectrum antimicrobial compound called reuterin when using glycerol as an electron-acceptor (Talarico *et al.*, 1988). For production of reuterin this bacterium needs vitamin B12. The production of B12 uses a large set of genes and *L. reuteri* is the only commercially available probiotic strain known to possess this property (Taranto *et al.*, 2003). B12 has been shown to affect inflammation (Bottiglieri, 1996; Scalabrino *et al.*, 2003) and also the nervous system (Ahluwalia, 2004) and thus, could partially be responsible for the effects of *L. reuteri* as a probiotic.

In a large double blind study involving 14 child care centres in Israel infants were supplied *L. reuteri* ATCC 55730. The *L. reuteri* group had significant decrease of days with fever, clinic visits, child care absence, and antibiotic prescription compared to the control group (Weizman, Asli & Alsheikh, 2005). In another double blind study of 262 employees at TetraPack in Sweden it was shown that after 80 days consumption of *L. reuteri* ATCC 55730 there was a remarkable decrease in reported sick-leave of the *L. reuteri* group compared to the control (Tubelius, Stan & Zachrisson, 2005). Hence, *L. reuteri* has shown to be a diversely effective probiotic strain and it is thus interesting to further investigate properties of this bacterium.

Interaction of probiotic bacteria with the environment

All living organisms have to interact with the environment and cope with the changes that can occur there. As indicated above, a reasonable prerequisite for a bacterium to benefit our health lies in its interaction with the host. For the bacterium to achieve this interaction it has to persist in the GI tract for some time, even though permanent colonization is not needed. A vast number of properties could be of importance for this persistence, and the nature and outcome of the bacterial-host interactions are certainly worthy of study. In this work, the probiotic bacterium itself is the focus of attention and the perspective is its ability to live and perform in the host intestine.

There are different approaches to investigate the interactions taking place in the intestinal ecosystem. For example, they can be investigated from the host perspective, looking at the eukaryotic (human or animal) cells and how they respond to the bacteria by *e.g.* immunological responses. Another way of elucidating mechanisms important for the probiotic affect is by looking at the response when the bacterium is exposed to an environment where it is assumed to be active. This can take the form of comparison of cultures exposed to different environments, using *e.g.* two-dimensional gel electrophoresis to study the response on the protein level (De Angelis & Gobbetti, 2004) or DNA microarrays investigating the global gene expression (Pieterse, *et al.*, 2005). These methods can also be used to look at the response in eukaryotic cells exposed to a probiotic bacterium (Di Caro *et al.*, 2005). Another approach that could be employed here is the *in vivo* expression technology (IVET), a genetic system designed to be of general use in a wide variety of bacterial-host systems (Mahan, Slauch & Mekalanos, 1993). This system has been used to find genes of lactobacilli specifically induced in the mouse GI tract (Bron, *et al.*, 2004; Walter, *et al.*, 2003). These are all methods that can be used for screening for interesting genes and proteins for further investigation through *e.g.* analysis of knock-out mutants, over expression of the genes or possibly recombinant production of the proteins.

A growing opportunity at present is the sequencing of a vast number of bacterial genomes (Gordon *et al.*, 2005). This gives rise to new possibilities for learning more about bacteria and their potential probiotic properties. The genomes are currently being screened for genes involved in housekeeping functions, metabolism, transport, regulatory systems, stress responses and production of surface proteins, bacteriocins and antimicrobial compounds. However, it is significant that 30-40% of the genes determined are of unknown function (Rastall, *et al.*, 2005). Let us not forget that these might also be important and their biological functions have to be investigated.

Surface proteins (I)

A conceivable way of interaction with the host is via the bacterium's outer surface, more specifically via its extracellular proteins. Hence it was deemed interesting to predict what the surface of a probiotic bacterium might look like and to identify some features common among probiotics or unique for a certain strain. To get a conceivable picture of possible host-interaction of *L. reuteri* ATCC 55730, the draft genome sequence was used to bioinformatically predict an image of the proteinaceous compounds on its surface. Predicting the function of surface proteins is quite a challenge, since they can be large and have a complex set of domains and since the sequence conservation of functionally overlapping proteins from different species of bacteria can be relatively low. Still, the identification and characterisation of domains and repeats can play an important role in elucidating the function of the extracellular proteins (Boekhorst *et al.*, 2006b).

Surface proteins are known to be involved in transport (both import and export), signal recognition and transduction, virulence and binding and many extracellular proteins are enzymes involved in *e.g.* cell wall biogenesis and metabolic processes. A crucial function of the bacteria is of course transport, which is needed for import of nutrients and export of metabolites and other compounds with *e.g.* antimicrobial effect. A large class is the ABC transporters, known to import *e.g.* ions and amino acids and export *e.g.* bacteriocins (Nes *et al.*, 1996). An example of signal recognition and transduction is the two-component systems discussed below, where the signal recognition component is localised in the cell membrane. Many surface proteins involved in virulence have been well characterised in pathogens (Palumbo & Wang, 2006; Schmidt, Riley & Benz, 2003; Schwarz-Linek, Höök & Potts, 2006; Tuomanen, 1999) and they have also been shown to be involved in adhesion of LAB to Caco-2 cells (Buck *et al.*, 2005) and mucus (Roos & Jonsson, 2002). Many proteins on the surface of bacteria have demonstrated moonlighting properties. Again, one example is the ABC transporters, of which several have also been shown to be involved in adherence of streptococci (Kolenbrander *et al.*, 1998) and campylobacter (del Rocio Leon-Kempis *et al.*, 2006) and in *L. reuteri* there is the collagen binding protein CnbP (Roos & Jonsson, 2002).

Using a variety of tools available on the Internet, the genome sequence of *L. reuteri* ATCC 55730 was screened for genes encoding a signal peptide that would enable transport of the protein through the cell membrane (Nielsen *et al.*, 1997) or transmembrane (TM) helices directing the proteins to the membrane of the cell. Several predicted membrane proteins possessed only one TM helix, most often located close to the amino (N) terminal of the protein. Since these did not contain a cleavable signal peptide, they could be considered to be cell membrane-anchored proteins attached via an N-terminal TM helix. The bioinformatical screening revealed 126 putative extracellular proteins, 94 with an N-terminal signal sequence and 32 with a putatively uncleaved N-terminal TM anchor. A functional categorisation of these putative proteins showed that 47% were of unknown function and 35% coded for enzymes. Other functional categories identified were transport and regulator or signal components (Fig. 6). Even though closely related species have been sequenced and their extracellular proteins have

been studied, 24 genes were found only in *L. reuteri* ATCC 55730, indicating that this bacterium has some unique properties. In line with several papers dealing with a prediction of surface proteins we excluded proteins predicted to have more than one TM helix from **Paper I**. However, in a biological perspective there is no good reason to exclude these, since among these there are also membrane-bound proteins with extracellular domains and proteins involved in transport. The contribution of membrane proteins to colonization and other interactions of lactobacilli is unknown. However, some membrane proteins in other bacteria have been assigned such functions. For example, OmpA from *E. coli* has been ascribed important functions in host-bacterial interactions and its surface exposed loops interact with host components (Shin *et al.*, 2005). Thus, further studies of this group of proteins is relevant.

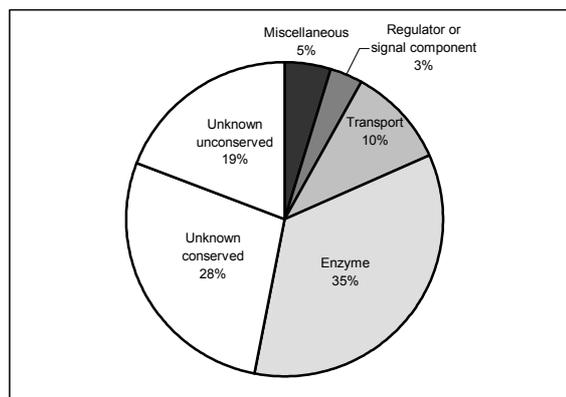


Fig. 6. Function categorisation of the predicted extracellular proteins of *L. reuteri* ATCC 55730.

As mentioned, in order to be secreted across the membrane, proteins have to have a signal peptide, but there are then different means of attaching the protein to the cell surface (Fig. 7). Proteins with a signal sequence but with no putative domains indicating attachment to the cell wall are predicted to be secreted out of the cell. A well-documented group of surface proteins attached to the cell wall have a carboxyl (C) terminal LPXTG motif and were first described in Gram-positive cocci (Fischetti, Pancholi & Schneewind, 1990). The protein is cleaved between the threonine and glycine residues and the carboxyl group of the threonine residue is linked to the peptidoglycan of the cell wall. Another group of cell wall-associated proteins is the lipoproteins, attached at their N-terminus via the cysteine residue of the so-called lipobox to the long chain fatty acids of the plasma membrane. As mentioned earlier, there are also hydrophobic tail proteins anchored in the membrane by a C- or N- terminal TM helix followed by positively charged amino acids (Krogh *et al.*, 2001). Lys-M is a domain that attaches the protein to the cell surface by interacting with the peptidoglycan in the cell wall (Steen *et al.*, 2003). This domain is often found in a variety of enzymes involved in cell wall degradation (Bateman & Bycroft, 2000; Carroll *et al.*, 2003; Fukushima *et al.*, 2006).

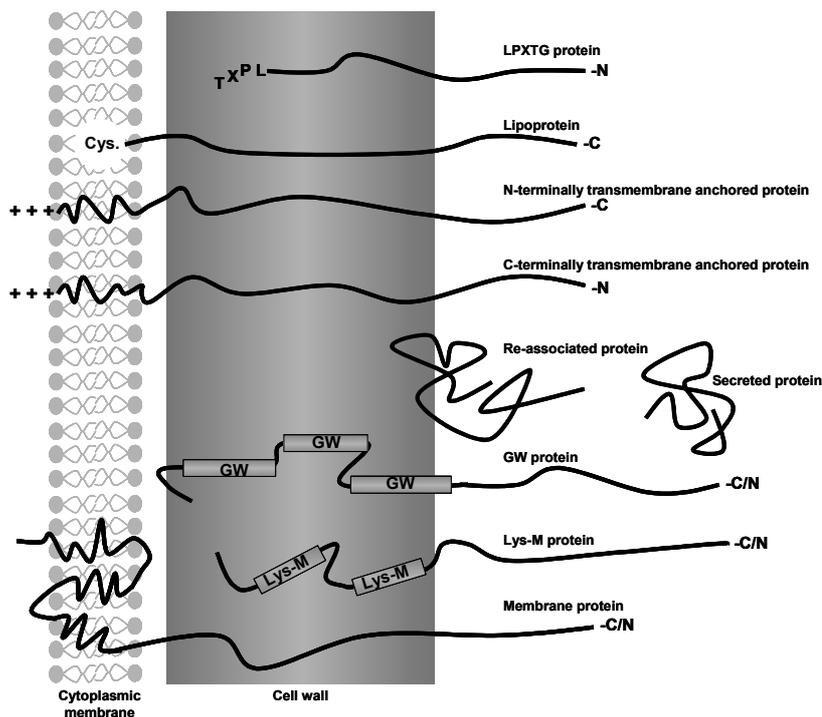


Fig. 7. Mechanisms of anchoring proteins to the cell surface identified in *L. reuteri*. The 126 predicted extracellular proteins include, LPXTG proteins, lipoproteins, N-terminal TM anchored proteins, C-terminal TM anchored proteins, secreted proteins, secreted and re-associated proteins, GW proteins and LysM proteins. In addition to extracellular proteins, membrane proteins can be important for interactions with the host, other microbes and the environment.

When predicting the surface-associated proteins of *L. reuteri*, a much more diverse picture than first envisaged emerged. Among the 126 predicted extracellular proteins we found 32 possessing an N-terminal anchor, this is a group of proteins not always considered when discussing surface associated proteins but it has also been noticed in the genome project of *Lactobacillus plantarum* (Boekhorst, *et al.*, 2006b; Kleerebezem *et al.*, 2003). In addition, during the process six genes encoding proteins highly similar to known lipoproteins but lacking the cysteine residue of the lipobox were detected. These proteins were all positively charged, having a high isoelectric point ($pI > 9$), and we predicted these to be secreted by means of their signal sequence and then re-associated with the cell surface via electrostatic forces. The collagen binding protein CnBP earlier characterized in *L. reuteri* (Roos *et al.*, 1996) was found to be among these. The hypothesis of re-associated proteins is strengthened by the fact that CnBP can be released in large amounts from the cell surface of *L. reuteri* by extraction with LiCl solution.

Only five genes encoding true LPXTG motifs were identified, however *L. reuteri* ATCC 55730 seems to possess some defect genes encoding proteins otherwise similar to known LPXTG proteins. This apparent genome decay has earlier been described in *Streptococcus thermophilus* (Hols *et al.*, 2005) and more recently in *L. salivarius* (van Pijkeren, *et al.*, 2006) and it could be speculated that the specific

loss of LPXTG proteins reflects an adaptation to a new environment where these proteins are not selected for. We also found genes encoding proteins similar to known lipoproteins with a cystine residue close to the signal sequence and since many of these contained an atypical lipobox, we suggested a modified, broader consensus of the lipobox in *L. reuteri* ATCC 55730 than previously described ([LIVFAGTM] - [AISVTLF] - [AGSIVTFLYC] - C - [GLSIAFETW]) (Sutcliffe & Harrington, 2002). Another group contained a set of eight genes, all being similar to each other, encoding a signal peptide but no means of anchoring. Several of them contained enzymatic domains suggesting a surface location. These proteins were also found to contain similar repeats which all harboured the dipeptide glycine-tryptophane (GW). Repeats containing this peptide have been found previously in *Listeria*, where they are shown to be attached to the cell wall. In this organism proteins with GW repeats are large surface proteins known to be important for interaction and virulence (Cabanés *et al.*, 2002). The GW motif is also found in repeats in a number of *Streptococcus pneumoniae* proteins (Yother & White, 1994). No proteins with GW repeats had been identified previously in *Lactobacillus*. However, recently genes encoding such repeats were also found in *Lactobacillus salivarius* (van Pijkeren, *et al.*, 2006).

Several of the extracellular proteins described in **Paper I** could be speculated to be involved in interactions with the host, but from among these, two genes containing GW proteins were chosen to be mutated for further characterization (Table. 1A). The procedure for construction of mutants and investigation of their ecological performance will be discussed later on (in **Paper IV**).

Table 1A. *L. reuteri* ATCC 55730 genes mutated in order to investigate their biological importance for ecological performance. (This table will expand with more genes during this thesis, the complete table is Table 1D.)

Gene	Predicted product
Predicted surface protein	
<i>Lr1610</i>	Gw1
<i>Lr1611</i>	Gw2

Responses to changes in the environment

The response in a bacterium when exposed to challenging environmental change is often termed a "stress response". It can be interesting to consider bacterial stress in the light of emotional stress in humans. In behavioural science the "biopsychosocial model of stress" is defined (Selye, 1982). This model includes three parts (i) an external component, (ii) an internal component and (iii) an interaction between the external and the internal component. In short, the external component is the environmental events that precede the stress response. The internal component is the neurological and physiological reaction, which includes three stages: (a) the alarm stage, (b) the resistance or adaptive stage and (c) the exhaustion stage. The interaction is the ongoing relationship between the individual and its environment, and here the emphasis is on the meaning an event has for the individual, *i.e.* not on the physiological response (Lazarus, 1984).

In an attempt to compare research concerning the stress response in bacteria to Selye's biopsychosocial model (Selye, 1982), a schematic diagram was drawn up (Fig. 8). It can be seen that most studies concentrate on the effect of one external component when investigating the internal response, *e.g.* by using a pure culture, exposing bacteria to a stress and evaluating the response at a couple of time points. Selye (1982) states that with so many factors contributing to emotional stress it can be difficult to define the concept of "stress" and few people actually define stress in the same way or even attempt to make a clear-cut definition. The same is true for bacteria; the level of "stress" differs for different bacteria. This can partly be explained by the environment from where it is isolated, as what is considered a stress condition for a bacterium isolated from *e.g.* soil is most often different from that for a bacterium isolated from *e.g.* the GI tract. The level of the external component can also differ, *i.e.* the intensity of the stress can be changed by using *e.g.* a higher temperature or a lower pH. Of course, the time during which the bacterium is exposed to stress is also important. Thus giving a homogeneous definition of stress is clearly difficult, since the natural environment for a bacterium differs greatly, just as different individuals respond to different extents to the same emotional stress depending on their background, *i.e.* their upbringing.

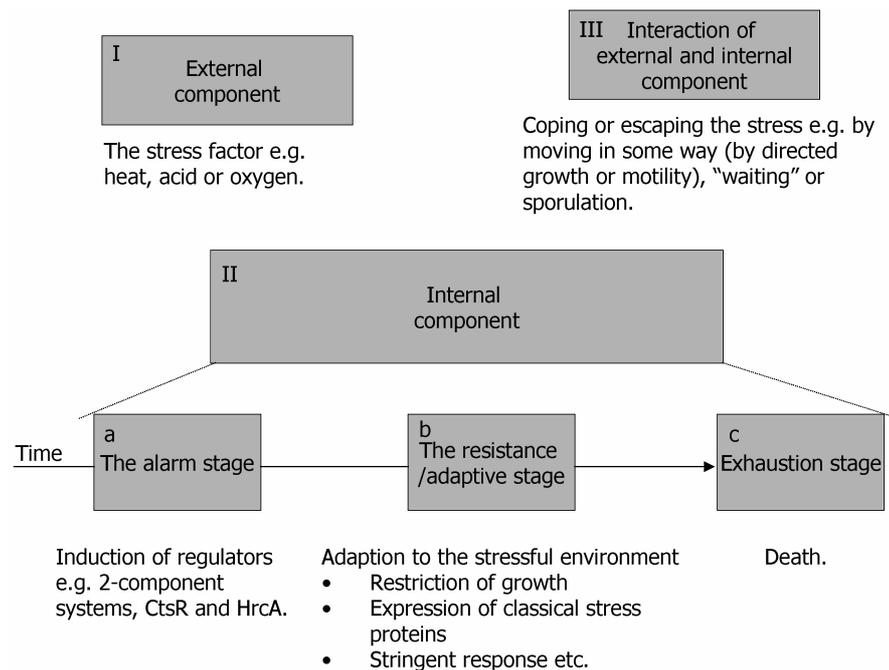


Fig. 8. A definition of stress illustrated using a model for human emotional stress. The grey boxes represent the different stages in Seyle's (1982) biopsychosocial model of stress, while suggestions of different stages of bacterial stress are written underneath.

It is generally considered that live bacteria are a prerequisite for a probiotic effect. Consequently, a bacterium used as a probiotic has to be able to survive the process of product formulation and then persist in the challenging environment of the gut and intestine. Functional properties and also robustness, reflecting the stability of a probiotic strain, is thus an important field of study. More specifically, it is crucial to understand how the bacteria respond to changes in the environment. The exposure to different types of stress can be a tool in revealing genes and proteins involved in biological performance of the bacterium.

A type of system that is important for sensing the environment and possibly responding to stress is the two-component signal transduction system. Such a system is classically characterised by a sensor histidine protein kinase (HPK) that senses conditions in the environment and thereafter transfers a signal to a response regulator (RR) that elicits the response by effecting a number of genes (West & Stock, 2001) (Fig. 9). This type of two-component system is known to control e.g. phosphate and nitrogen availability, osmotic response, chemotaxis, respiration, transport, autolysis, haemolysis, haemagglutination, toxicity, encapsulation and surface proteins (Hoch, 2002). Such systems are considered to be the predominant means by which bacteria sense and respond to their environment (Skerker *et al.*, 2005), by detecting external cues and responding by changing the gene expression. In agreement with this, bacteria with a complex life style (e.g. *Myxococcus xanthus*), bacteria found in varied environments (e.g. *Pseudomonas*) or bacteria with numerous alternative metabolic strategies (e.g. δ - and ϵ -proteobacteria) have a large number of two component signal transduction

systems while few have been identified in the reduced genomes of parasitic bacteria likely to have a relatively constant external environment (Alm, Huang & Arkin, 2006).

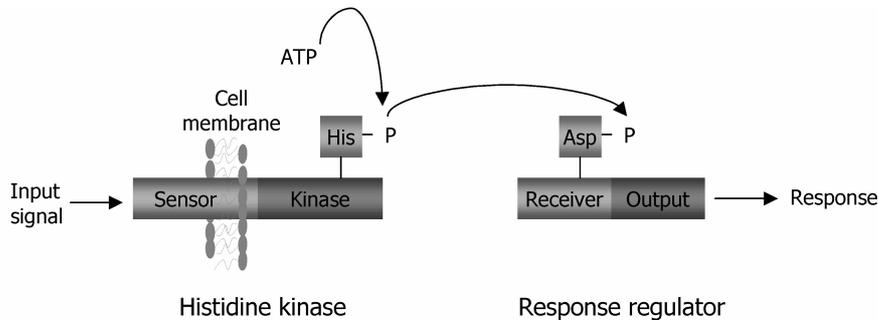


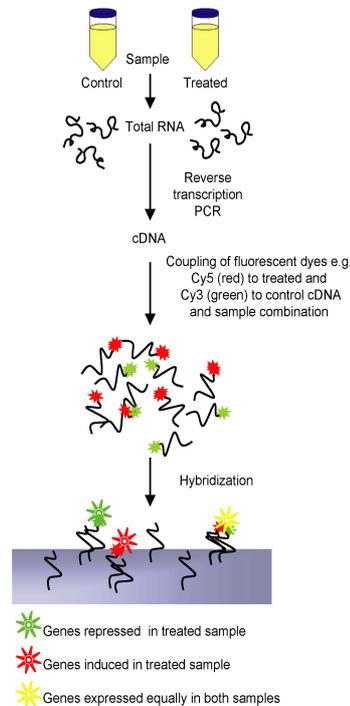
Fig. 9. Two-component systems. When exposed to an appropriate stimulus, the HPK is activated to autophosphorylate a specific histidine residue. The phosphorylated histidine serves as a high-energy intermediate and the phosphoryl group is subsequently transferred to an aspartic residue on the RR. Phosphorylation of the RR usually creates an active form of the protein (probably by causing conformational changes), which mediates the output response. (Picture modified after (Morel-Deville, Fauvel & Morel, 1998).)

Seven complete two-component systems have been identified in *L. reuteri* ATCC 55730 and one RR has also been mutated (rr2) (Wall, 2005). In addition to this, one more RR and five HPK's were successfully mutated in this thesis work (Table. 1B). As mentioned above, the procedure for construction of mutants and investigation of their ecological performance will be discussed later on (in **Paper IV**).

Table 1B. *L. reuteri* ATCC 55730 genes mutated in order to investigate their biological importance for ecological performance.

Gene	Predicted product
Predicted surface protein genes	
<i>lr1610</i>	Gw1
<i>lr1611</i>	Gw2
RR of two-component system	
<i>Lr677</i>	Rr2
<i>Lr1057</i>	Rr5
HPK of two-component system	
<i>lr0392</i>	Hpk1
<i>lr0679</i>	Hpk2
<i>lr0819</i>	Hpk3
<i>lr0856</i>	Hpk4
<i>lr1749</i>	Hpk6

A way of studying the regulation of genes in response to different stress conditions is by the use of DNA microarrays. With this technique it is possible to compare mRNA levels of each individual gene in a genome or a large set of genes between two samples.



RNA is extracted from the samples, reversely transcribed into complementary DNA (cDNA) and labelled with fluorescent dyes (either Cy3 or Cy5). The cDNA is then hybridized to the microarray. The microarray used in the present case was a glass-slide spotted with 60-mer oligonucleotides representing each of more than 1800 genes from a draft genome sequence of *L. reuteri* ATCC 55730. The hybridized microarray slides are then scanned for fluorescence and data are extracted from the image (Fig. 10). To get reliable results, it is important to use both biological and technical replicate including dye swaps, where the different dyes are used for both the treated and the control sample (Zhou & Yang, 2006).

Fig. 10. Outline of a DNA microarray experiment.

DNA microarray is a good technique for investigation of changes in gene expression of all or a large set of genes in a cell at a certain point. This information could be used for formulation of hypotheses and *e.g.* for further characterisation of the biological function of certain genes. Looking at the mRNA levels provides the major picture of regulation, but it must be borne in mind that RNA is short-lived and that its production does not necessarily result in production of a protein because regulation can be controlled at other levels such as the translational level. DNA microarrays are currently used to study gene expression in a large range of organisms but the use in studies of *Lactobacillus* is so far relatively limited to, *e.g.* studies on response to bile in *L. plantarum* (Bron, *et al.*, 2006) and the carbohydrate utilization in *L. acidophilus* (Barrangou *et al.*, 2006).

Heat stress response (II)

A bacterium exposed to heat primarily suffers from denaturation of proteins. However, the cell wall and membrane of the bacterium are also influenced by a melting of lipids and a change in the state of the peptidoglycan. At higher temperatures, the ribosomes and DNA start to denature (Teixeira *et al.*, 1997). At the cellular level, the response to stress is the production of specific gene products. These products are variously termed heat shock proteins (HSPs) or cell stress proteins and were originally identified as molecules produced in response to the presence of unfolded proteins within the cell. They are called molecular chaperones and function by interacting with denatured or only partially folded proteins, facilitating correct folding. In both prokaryotes and eukaryotes molecular chaperones are classified as essential proteins, and there is significant conservation of sequences between prokaryotic and eukaryotic chaperones (Henderson, Allan & Coates, 2006).

It has been shown that the classical HSPs are not produced solely as a response to stress in order to protect the bacteria. These chaperones have also been shown to be involved in other mechanisms. One example is the GroEL heat shock protein of *Lactobacillus johnsonii* La1, which has been shown to be cell surface-associated, bind to mucin and epithelial cells and mediate aggregation of *Helicobacter pylori* (Bergonzelli *et al.*, 2006). Another more extreme example is found in *Enterobacter aerogenes* that lives in the mouth of the antlion (*Myrmeleon bore*). The GroEL of this bacterium is actually a toxin that paralyzes the prey of these insect-consuming larvae. What is even more fascinating about this is that GroEL of *Enterobacter aerogenes* differs from the GroEL of *E. coli* by only 11 amino acid residues, and it has been shown that a substitution of a single residue can make the difference between being toxic or not (Yoshida *et al.*, 2001).

In experimental disease models for *e.g.* arthritis, type 1 diabetes and allergies, microbial HSPs or peptides derived from HSPs have been shown to have protective effects (van Eden, van der Zee & Prakken, 2005). The immunoregulatory qualities of HSPs might stem from their evolutionary conservation, which enables the cross-reactivity between microbial HSPs and self-HSPs (van Eden, van der Zee & Prakken, 2005). Highly speculative, could the HSPs of the probiotic bacteria in fact be a means to modulate and prepare our immune system for encountering pathogens with HSPs highly similar to those of the probiotic? The relevance of heat stress studies for improved understanding of the action of lactobacilli in the GI tract could be argued. Heat stress is a condition that bacteria living in the GI tract normally never encounter. The genes induced during a heat stress response *e.g.* in a probiotic LAB are most probably not evolved for this specific situation but might instead be used in other stressful conditions such as the acidity, bile or enzymes in the GI tract or in encountering the immune system. In fact, there are numerous reports on classical HSPs with important roles in virulence, colonization and interaction with the host (Lemos, Luzardo & Burne, 2007; Michel *et al.*, 2006).

The heat stress experiment described in this thesis (**Paper II**) was performed by exposing a culture of *L. reuteri* ATCC 55730 at late stationary phase to 50 °C for 5, 15 and 30 minutes. The expression profile was investigated using DNA microarrays (described above) comparing heat-stressed cells with cells grown at 37 °C. The genes induced during heat stress were mainly involved in replication, recombination and repair of DNA, but several genes encoding classical HSPs (*i.e.* GroEL, GroES, DnaK and ClpE) were also induced as a response to elevated temperature. In addition, *relA* was induced and the expression of many metabolic systems was turned down, indicating activation of the stringent response (Braeken *et al.*, 2006). The regulators of the classical HSPs, *ctsR* and *hrcA*, were induced and a possible dual regulation by both HrcA and CtsR of the *groESL* operon was identified. HrcA is known to recognise the so-called CIRCE motif and regulate the *dnaK* and the *groESL* operones. CtsR has been shown to regulate the caseinolytic proteases (Clp's) by binding to the so-called CtsR box. A dual regulation of the *groESL* and the *dnaK* operon has previously been identified in *Staphylococcus aureus* (Chastanet, Fert & Msadek, 2003). In total there are six Clps in the *L. reuteri* genome, only one of which (*clpE*) was induced as a response to an elevated temperature. This is also the only one of the Clps harbouring a CtsR box upstream of the gene.

As discussed above, two-component signal transduction systems are important in sensing and responding to changes in the environment and one of the response regulators described earlier (*rr5*) was induced in this study. We also encountered genes with a specific function usually not related to stress (*e.g.* a L-ldh). The function of the proteins encoded by these genes during heat stress remains to be determined. This is also true for the many genes of unknown function induced during heat stress. The genes *rr5*, *clpE*, *ldhL1* and the gene encoding a RelA/SpoT domain protein (*rdpR*) were all mutated for further analysis (Table 1C). The procedure for construction of mutants and investigation of their ecological performance is discussed below (in **Paper IV**).

Table 1C. *L. reuteri* ATCC 55730 genes mutated in order to investigate their importance for ecological performance.

Gene	Predicted product
Predicted surface protein genes	
<i>lr1610</i>	Gw1
<i>lr1611</i>	Gw2
RR of a two-component system	
<i>lr1057*</i>	Rr5
<i>lr0677</i>	Rr2
HPK of a two-component system	
<i>lr0679</i>	Hpk2
<i>lr0392</i>	Hpk1
<i>lr0819</i>	Hpk3
<i>lr0856</i>	Hpk4
<i>lr1749</i>	Hpk6
Genes induced in response to heat	
<i>lr1057*</i>	Rr5
<i>lr0004</i>	ClpE
<i>lr0167</i>	L-ldh
<i>lr1056</i>	RdpR

*Same mutant

Acid stress response (III)

The acidity or alkalinity of an environment has profound effects on the activity and stability of macromolecules such as enzymes, so it is not surprising that the growth and metabolism of bacteria are influenced by pH. Drastic variations in pH can harm bacteria by disrupting the plasma membrane or inhibiting the activity of enzymes and membrane transport proteins. Changes in external acidity might also alter the ionisation of nutrient molecules and thus reduce their availability to the bacterium. The ability of a probiotic culture to persist in an acidic environment is crucial when passing through the gut and also for stability of a fermented food product. Generally microorganisms have their maximum growth between pH 6-8 while LAB have a maximum between 6-6.5 (Konings, 2002).

Acid stress can often be defined as a combined biological effect of low pH and weak organic acids present in the environment. Weak acids in their uncharged protonated forms can diffuse across the cell membrane and dissociate inside the cell and lower the internal pH. The lower the external pH, the more undissociated weak acids will be available to cross the membrane and affect the internal pH (Bearson, Bearson & Foster, 1997). Bacteria have developed different mechanisms to tolerate a sudden drop in external pH. Gram positive bacteria control an acidic environment by utilising proton pumps (e.g. the F₁F₀ ATPase proton pump), proteins involved in repair or degradation of damaged cell components, alkalinisation of the external environment and alterations in the cell envelope (Cotter & Hill, 2003; De Angelis *et al.*, 2001; van de Guchte *et al.*, 2002). Neutrophilic bacteria (such *E. coli*) maintain their internal pH close to neutral, producing a large proton gradient. Acid stress response may be particularly important in LAB,

whose growth is accompanied by the production of organic acid, which results in acidification of the media, arrest of cell multiplication, and possible cell death. Fermentative bacteria have developed an alternative mechanism. In these organisms the internal pH decreases as the surrounding pH drops during growth, thus maintaining a constant pH gradient instead of a constant internal pH (Siegumfeldt, Rechinger & Jakobsen, 2000), supposedly making these bacteria more acid-tolerant.

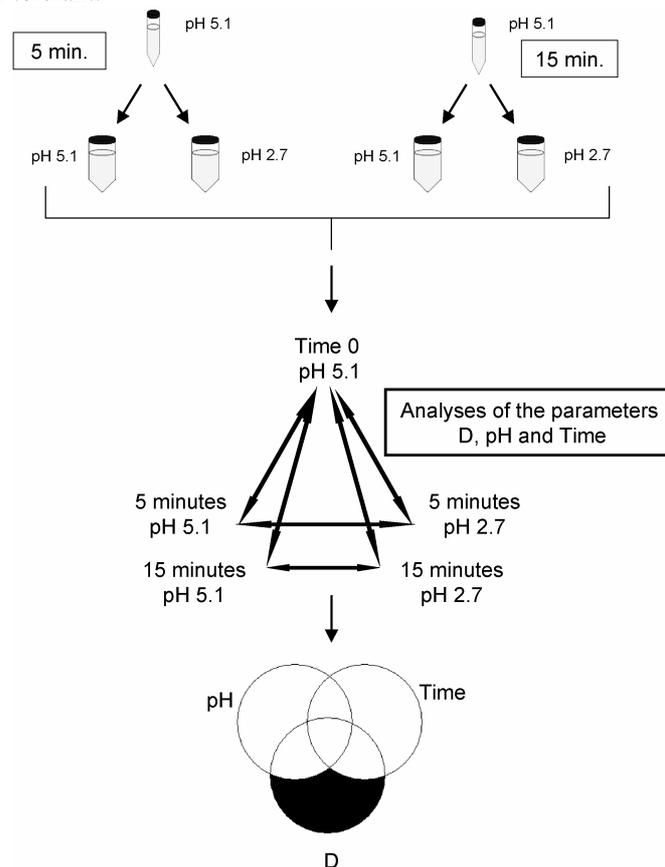


Fig. 11. Outline of the acid stress DNA microarray experiment. The experiment was designed considering three different factors; pH, time and dilution (D). Using a linear model, genes only affected by D (illustrated in black in the schematic Venn-diagram) could be distinguished from genes affected by pH and time specifically. This illustration was modified from a picture kindly supplied by Dr. Wall.

DNA microarray experiments were also performed to investigate acid stress in *L. reuteri* ATCC 55730 (**Paper III**) (for set-up see Fig. 11). Similarly to heat stress a drop in pH normally results in denaturation of proteins and an increased expression of chaperones and classical stress proteins such as the HSPs. However, in contrast to the heat shock experiment, in this study only few of the classical HSPs or other well-characterized stress proteins were induced as a response to a drop in external pH. In fact only one previously known classical stress protein gene was induced, namely *clpL*. This Clp is related to *clpE* induced in the heat stress experiment (**Paper II**) but since no CtsR box was found upstream of *clpL*, it

could be suggested that the Clps are regulated differently and are specialised in their function in *L. reuteri* ATCC 55730. The objective of this study (**Paper III**) was to mimic the acidity of the stomach and, thus a very low pH (2.7) was chosen. The response to this level of acidity was different from the response to a milder and more long-term acid stress used in most experiments dealing with acid stress (Lim, Ehrlich & Maguin, 2000; Lorca, Font de Valdez & Ljungh, 2002). See Fig. 8 for the reasoning about different conditions of stress above. The same is true for the heat stress experiment, where the stress was also relatively severe. It could be speculated that milder conditions would have resulted in a greater overlap in response between heat (**Paper II**) and acid (**Paper III**) stress. Genes putatively involved in cell envelope biogenesis were also induced in response to the acidic environment. These genes could be important for bacterial persistence in the acidity. One of these genes, a cell wall-altering esterase (*lr1516*) contains a putative penicillin-binding domain although it is not a classical penicillin-binding protein (PBP). PBPs are associated with peptidoglycan synthesis (Spratt & Cromie, 1988), and thus a change in the cell wall of the acid stressed bacteria can be postulated.

The genes *clpL* and *lr1516* were mutated for further characterisation (Table 1D). The procedure for construction of mutants and investigation of their ecological performance will be discussed below (in **Paper IV**).

Table 1D. *L. reuteri* ATCC 55730 genes mutated in order to investigate their biological importance for ecological performance.

Gene	Predicted product
Predicted surface protein genes	
<i>lr1610</i>	Gw1
<i>lr1611</i>	Gw2
RR of two-component system	
<i>lr1057*</i>	Rr5
<i>lr0677</i>	Rr2
HPK of two-component system	
<i>lr0679</i>	Hpk2
<i>lr0392</i>	Hpk1
<i>lr0819</i>	Hpk3
<i>lr0856</i>	Hpk4
<i>lr1749</i>	Hpk6
Genes induced in response to heat	
<i>lr1057*</i>	Rr5
<i>lr0004</i>	ClpE
<i>lr0167</i>	L-ldh
<i>lr1056</i>	RdpR
Genes induced in response to acid	
<i>lr1864</i>	ClpL
<i>lr1516</i>	Cell wall altering esterase

*Same mutant

Biological importance of specific genes

The biological importance of specific genes for survival, persistence, competition *etc.* can be investigated using different approaches and different models ranging from a simple laboratory culture experiment to a complex human/animal study. There are advantages and disadvantages with both extremes. Using the more limited laboratory culture is cheaper and the interpretation of the results might be more straightforward. On the other hand more unexpected effects and the importance of the complex system might be overlooked. Animal models are obviously good when it comes to natural similarities although human trials are of course the optimal way of studying probiotic effects. However, both animal and human trials are expensive, sampling can be difficult and ethics are also an issue. Considering these facts, the use of the three stage continuous culture system mimicking the human large intestine (here termed an artificial colon), introduced by Gibson *et al.* (1988) and validated by Macfarlane *et al.* (1998), was considered to be a good alternative for an evaluation of the *L. reuteri* mutants. The three-stage continuous culture system allowed us to study the persistence and competitive ability of the mutants in a complex human-like ecosystem.

Construction of mutants

In order to investigate the biological importance of different previously described genes of interest, a number of *L. reuteri* ATCC 55730 genes were mutated. Two of the putative surface proteins with GW repeats (*gw1* and *gw2*), two genes induced as a response to acid stress (*clpL* and *lr1516*, a putative cell wall-altering esterase) and four genes induced as a response to an elevated temperature (*clpE*, *ldhL1*, *rdpR* and *rr5*) were all mutated. In addition to *rr5* one more RR had previously been mutated (*rr2*) (Wall, 2005) In addition the HPK of the same two-component system (*hpk2*) and four other HPK's (*hpk1*, *hpk3*, *hpk4* and *hpk6*) were also mutated (Table X.). Mutants were constructed by interruption of the target gene through site-specific integration of a plasmid harbouring an antibiotic resistance gene (for details see Fig. 12 and Fig. 13).

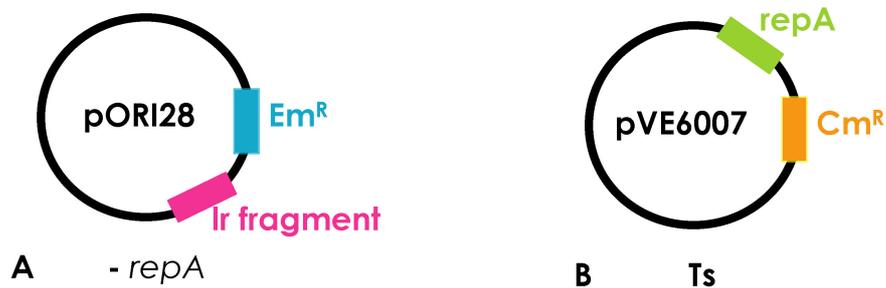


Fig. 12. Plasmids involved in construction of mutants. (A) pORI28 with a fragment of the target gene (Ir fragment in pink) inserted, pORI28 harbours a erythromycin resistance gene but lacks *repA* and thus need this gene from another plasmid to be able to replicate. (B) The helper plasmid pVE6007 which carries *repA* and a chloramphenicol resistance gene, the replication of this plasmid is temperature sensitive.

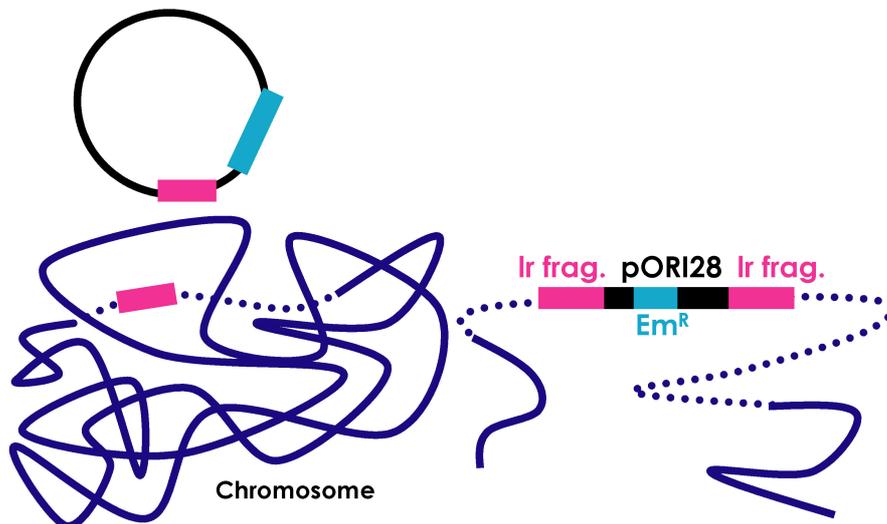
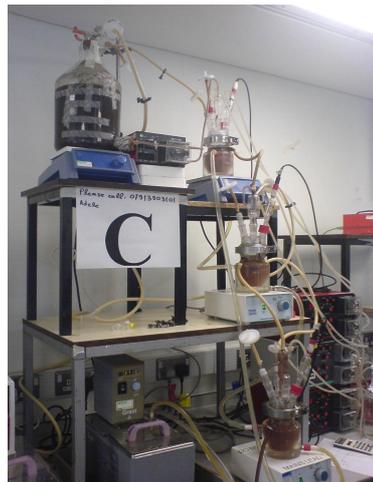


Fig. 13. Schematic view of the homologous recombination utilized for construction of mutants. The target gene is marked with a dotted line in the chromosome and the pink boxes (Ir frag.) illustrates the fragment of the gene that was amplified and inserted into pORI28. The pORI28 construct was electroporated into *L. reuteri* containing pVE6007. When these bacteria then were grown at high temperature (45°C) pVE6007 could not replicate. Thus with erythromycin in the growth media and without the helper plasmid only bacteria with an incorporated pORI28 harbouring the erythromycin resistance gene will grow.

When testing the mutants from the heat and acid stress experiments, the first obvious approach was to test their survival at an elevated temperature or in an acidic environment. When the survival of the mutants from the heat stress experiment was compared with that of the wild type, the *clpE* and *rdpR* mutants both showed decreased survival at 50 °C after 2.5 and 3 h incubation. The *clpE* mutant had a decreased survival already after 2 h. The *ldhL1* mutant was also affected after 3 h (**Paper II**). When the wild type and some of the mutants were grown at 45 °C, the *clpE* mutant exhibited unexpected behaviour. After 12 h the wild type and the other mutants had established a stationary phase, but in contrast, the cell density of the *clpE* mutant had instead dropped. Since no aggregation of the bacteria was observed under the microscope, autolysis is a possible explanation for this phenotype. However, this mutant did show a disturbed cell morphology with a mycelial like growth under the microscope (**Paper II**). A change in morphology was also observed already in 37 °C in a double mutant of *clpE* and the associated proteolytic domain *clpP* of *Listeria monocytogenes*. This mutant was also sensitive to higher temperatures and did not survive at 42 °C (Nair *et al.*, 1999). There were no clear differences between the wild type and the *clpL* and esterase mutants from the acid stress experiment when diluted in MRS with pH 2.7. However, the survival was also examined in synthetic gastric juice (lacking enzyme and bile) with pH 2.0. After 50 minutes, the survival of the *clpL* mutant and the esterase mutant was significantly lower than that of the wild type (**paper III**).

Evaluation of mutants in a colon model (IV)

Paper I, II and III of this thesis all resulted in the identification of a set of genes possibly of importance for ecological performance. Some of these genes were mutated and evaluated in the respective paper. The selection of mutants from the previous works was increased by mutants of genes from two-component signal transduction systems originally identified by Wall (2005) (all genes mutated can be found in Table 1D). In **paper IV** the whole collection of mutants were evaluated in a colon model.



This three-stage continuous culture fermentation system was designed to mimic the spatial, temporal, nutritional and physiochemical characteristics of the microbiota in the proximal (vessel 1) and distal (vessel 2 and 3) colon (Macfarlane, Macfarlane & Gibson, 1998). The temperature was set to 37°C and the pH of vessels 1, 2 and 3 were set to 5.5, 6.2 and 6.8 respectively. The gut model was set up and inoculated with a fresh human faecal sample (Fig. 14).

Fig. 14. The three-stage continuous culture system.

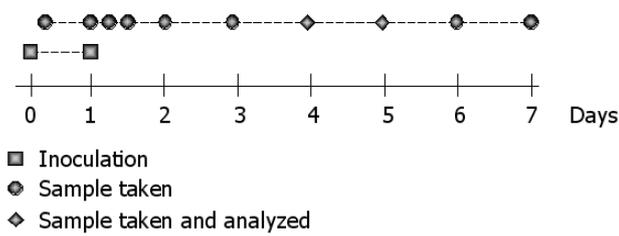


Fig. 15. Outline of the experiment evaluating performance of mutants in an artificial colon.

After equilibration of the system a mix of fifteen strains, the fourteen mutants described above and a strain with a mutated inactive transposase (*lr0849*) (termed the control-mutant), were inoculated in the top vessel. This inoculation was repeated after 24 h. Samples were then taken from each vessel from 8 h up to 7 days after the first inoculation to collectively follow the persistence of all mutants in the system (Fig. 15). The bacteria were grown on plates with erythromycin and vancomycin to select for *L. reuteri* mutants. Samples taken before inoculation of the mutants showed no growth of *L. reuteri* (Fig. 16). The total *L. reuteri* mutant count dropped from the initial value of 10^7 to around 10^3 after five days. Thereafter, a tendency to stabilisation could be seen. The fact that the total number of mutants was rapidly reduced could be explained by the fact that they multiply at a rate that is too slow to compensate for the dilution effect.

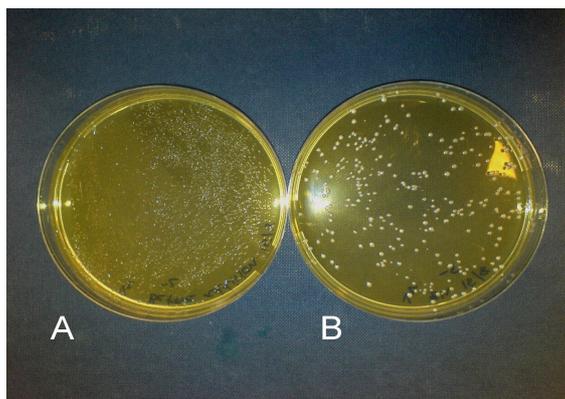


Fig. 16. Growth of samples from the artificial colon. (A) 10^{-2} dilution of sample before inoculation of the *L. reuteri* mutants and (B) 10^{-5} dilution of a sample taken 8 h after first inoculation. The appearance of *L. reuteri* compared to bacteria from the indigenous microflora of the artificial colon was clearly visible. There was no growth on the plate from the 10^{-5} dilution of the sample taken before inoculation of *L. reuteri* mutants.



Fig. 17. Schematic diagram of the design of specific real time PCR primers. The primers were used to individually detect each mutant in samples taken from the artificial colon experiment.

A real time PCR based approach was used to determine the relative abundance of individual mutants. Since specific detection of the wild type was not possible, a control-mutant was constructed to facilitate comparison of persistence between the mutants. Using primers specific for each mutant (Fig. 17) the ratio of mutants on days four and five after the first inoculation was investigated.

The data from this experiment are so far only based on samples from one artificial colon experiment and so it is difficult to draw conclusions on the definite importance for survival of the individual genes. It would therefore be interesting to perform the same experiment again. Furthermore, a large amount of samples were taken and not all have been investigated so far. Even though the conditions and composition in the different vessels were a somewhat different, the result from each can to some extent be considered as replicates. Comparisons show that three mutants were clearly less persistent than the control-mutant in all three vessels, indicating that *clpE*, *rr2* and *hpk1* are important for persistence in the human colon. There were also tendencies for low persistence of other mutants, e.g. *ldhL1*, *clpL*, *gw2* and *hpk4* mutants. It is interesting to see that it is the same mutants that are sensitive to acid and heat that are influenced by the environment and competition in the artificial colon. However, it is clear that not all genes induced in separate response to heat or acid are important for persistence of the bacterium in the colon. The bacterium has a complex matrix of signals and protective strategies all working together to perform in the GI tract.

To our knowledge, this way of using a real time based PCR approach to detect the relative abundance of a mix of mutants and evaluate their persistence in a complex model has not been published previously, and hopefully opens up a new perspective on how to evaluate mutant survival in a biological system. This way of using real time PCR could also possibly provide new opportunities for evaluation of mutants in important *in vivo* studies.

Concluding remarks

From the work described in this thesis it can be concluded that some of the classical HSPs such as the Clps are important for survival during stress and for ecological performance. Genes encoding Clps were induced in response both to heat and acid stress, though the response seems to be specialised since different Clps were induced and also differences in regulatory motifs were observed. However, both these chaperones were important for the persistence of *L. reuteri* ATCC 55730 in a colon model and thus probably for the ecological performance in the GI tract. The importance of these genes for the health promoting effects still has to be unravelled. The bioinformatics study of extracellular proteins described in this thesis revealed a diverse set of proteins with different means of attachment. Both stress response and identification of surface proteins exposed a complex picture where most of the genes actually have an unknown function. To be able to understand the intricate mechanism enabling a bacterium to persist in the GI tract

and interact with the host much effort has to be put in studies of these unknown genes. Another issue that complicates the determination of the function behind genes is the possible moonlighting properties that sometimes give a false realisation of the gene function.

When looking at previously used criteria for strain selection of probiotics, the only things being undoubtedly important are beneficial effects and safety. However, it is of interest to understand the mechanisms underlying the promotion of health to be able to find new ways of selecting strains with more defined effects. This could eventually lead to new probiotics with a more justified and accepted role in treatment of gastrointestinal derived disorders. The field of probiotic science is now moving from selecting strains by these previously described criteria based on readily measurable parameters such as survival in acidic conditions, towards understanding the underlying mechanisms behind GI tract persistence and in the long run the health-promoting effects. By screening genomes in different ways, *e.g.* by predicting surface proteins or gene homologues to already characterised proteins, or by studying genes and proteins involved in stress response, using *e.g.* 2D gel electrophoresis and DNA microarrays, we can get a glimpse of the bacterium's strategy for ecological performance. However, it is important that we do not end up only identifying more and more genes that are potentially important for persistence. To move further, we also need to evaluate these genes by studies of the probiotic effects of mutants compared to their wild type.

"Foxes have holes and birds of the air have nests,
but the Son of Man has no place to lay his head."
(Luke 9:58)

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/Kaizers orchestra (De involverte)

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