

**THE STRESS RESPONSES OF PROBIOTIC
LACTOBACILLI AND A BIFIDOBACTERIUM
WITH SPECIAL EMPHASIS ON CLP FAMILY
PROTEINS**

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ACADEMIC DISSERTATION

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ABSTRACT

The use of food products containing probiotic microorganisms is of increasing economic importance. The health promoting effects of selected probiotic strains has been substantiated in controlled clinical studies. The probiotic nature of the health promoting bacteria is not well studied compared to the virulence of pathogenic bacteria. Microorganisms used in food technology and probiotics are exposed to technological and digestive stresses, such as temperature changes and low pH.

Virulence and stress responses are closely related in several Gram-positive bacteria while extremely little is known about the possible overlap of stress responses and the probiotic nature of the bacteria. The available data concerning stress responses of lactobacilli and bifidobacteria mainly cover physiological changes in these bacteria when subject to stress, such as high temperature and low pH, and their ability to survive in different challenges.

ClpATPases are a family of stress proteins that are known as virulence factors in a number of pathogenic bacteria, such as *Staphylococcus aureus* and *Streptococcus pneumoniae*, and regulators of several vital biological processes in Gram-positive bacteria with a low G+C content.

In the first part of this thesis, *clpL* ATPase encoding genes and their protein products were characterized in two potentially probiotic lactobacilli, *Lactobacillus rhamnosus* E-97800 and *L. gasseri* ATCC 33323. Southern blot analysis revealed that among four *L. rhamnosus* strains only *L. rhamnosus* E-97800 carried two *clpL* genes, assigned as *clpL1* and *clpL2*. Expression of both genes were induced after heat stress >20- and 3-fold, respectively. The *clpL2* region was found to be mobilized after prolonged cultivation of E-97800 at a high temperature. The sequence analyses revealed that *clpL2* shared 98 % identity to *L. plantarum clpL* gene and the *clpL2* is flanked by inverted repeat highly identical to the repeats of a functional insertion element in a *L. plantarum* strain. The data indicate that *L. rhamnosus* E-97800 has acquired *clpL2* region via horizontal gene transfer, probably the donor being a lactobacillar strain. Moreover, the low G+C content (40 %) of *clpL2* compared to *clpL1* (49 %) and to the average *L. rhamnosus* entries at GenBank (48 %) together with high identity of *clpL2* to the *L. plantarum clpL* gene indicate that the *clpL2* containing element has been transposed relatively recently. Homology searches using *clpL* genes as query sequences revealed that the number of paralogous *clpL* genes varies among lactic acid bacteria (LAB). However, the putative selective advantage of this extra *clpL* paralog to host bacteria remains to be studied, since the stress tolerance of the *clpL2*-deficient strain was not altered compared to its parental strain.

We demonstrated that a CIRCE element, which is known to mediate HrcA-dependent regulation, is located upstream of the *clpL1* in *L. rhamnosus* and *clpL* in *L. gasseri* which together with the strong induction fold of *clpL1* during heat stress suggest HrcA-mediated regulation of *clpL* genes. Moreover, we showed that purified HrcA protein is able to specifically bind to the promoter region of *clpL* in *L. gasseri*. Thus, the expression of

clpL is most likely regulated by the HrcA/CIRCE system in these lactobacilli representing a novel regulon.

In the second part of this work, two-dimensional electrophoresis-based (2-DE) tools were applied to investigate stress responses in selected probiotic bacteria. Since probiotic bacteria are adapted to grow in an environment rich in nutrients, the optimization of growth conditions for efficient metabolic labelling to examine protein synthesis kinetics in defined media is needed. A semi-defined medium for metabolic labelling with [³⁵S]methionine for *Bifidobacterium longum* was developed. This medium was shown to support efficient protein radiolabelling. In addition, chemically defined media and experimental conditions supporting efficient protein radiolabelling with [³⁵S]methionine were developed, and proved to be applicable for a number of lactobacillar strains to investigate their stress responses. Fluorescence 2-D difference gel electrophoresis (DIGE) was applied to study the heat shock response of *L. gasseri* ATCC 33323. In addition to classical chaperons DnaK and GroEL, four Clp AAA+ (ATPases associated with a variety of cellular activities) ATPases were detected and found to be increased in abundance after a heat shock. One of these, *clpL*, was deleted by using a thermosensitive vector. It was shown that the functional *clpL* gene is essential for the development of constitutive and induced thermotolerance in *L. gasseri*. The expression of several HSPs (heat shock proteins) was at the same level in both *clpL* deficient and its parental strain indicating that ClpL is not involved in modulation of the heat shock response in *L. gasseri*. Instead, ClpL probably prevents aggregation of non-native proteins generated by stress and thus the *clpL* gene might have industrial potential.

ABBREVIATIONS

2D PAGE	two-dimensional polyacrylamide gel electrophoresis
2-DE	two-dimensional electrophoresis
aa	amino acids
AAA+	ATPases associated with a variety of cellular activities
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
bp	base pair
CIRCE	controlling inverted repeat of chaperone expression
Clp	caseino-lytic protein
CtsR	class three stress regulator
Da	Dalton, a unit of protein mass
EBI	European Bioinformatics Institute
EDTA	ethylenediaminetetra-acetic acid
G+C	guanine-plus-cytosine
GI	gastrointestinal
GIT	gastrointestinal tract
GRAS	generally regarded as safe
HrcA	heat regulation at CIRCE
HRP	horseradish peroxidase
HSP	heat shock protein
HtrA	high temperature requirement A
IEF	isoelectric focusing
IPTG	isopropyl- β -D-thiogalactopyranoside
IR	inverted repeats
IS	insertion sequence
kb	kilo base
LAB	lactic acid bacteria
MALDI-TOF	matrix-assisted laser desorption / ionization – time of flight
MAM	methionine assay medium
mRNA	messenger RNA
NBD	nucleotide binding domain
<i>orf</i>	open reading frame
PCR	polymerase chain reaction
pI	isoelectric point
RBS	ribosome binding site
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sigB	alternative subunit of RNA polymerase
spp.	species

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, referred to in the text by Roman numerals **I-IV**.

I Suokko, A., K. Savijoki, E. Malinen, A. Palva, and P. Varmanen. 2005. Characterization of a mobile *clpL* gene from *Lactobacillus rhamnosus*. *Applied and Environmental Microbiology* 71:2061-1069.

II Savijoki, K., A. Suokko, A. Palva, L. Valmu, N. Kalkkinen, and P. Varmanen. 2005. Effect of heat-shock and bile salts on protein synthesis of *Bifidobacterium longum* revealed by [³⁵S]methionine labelling and two-dimensional gel electrophoresis. *FEMS Microbiology Letters* 248:207-215.

III Savijoki, K., A. Suokko, A. Palva, and P. Varmanen. 2006. New convenient defined media for [³⁵S]methionine labelling and proteomic analyses of probiotic lactobacilli. *Letters in Applied Microbiology* 42:202-209.

IV Suokko, A., M. Poutanen, K. Savijoki, N. Kalkkinen, and P. Varmanen. 2008. ClpL is essential for induction of thermotolerance and is potentially part of the HrcA regulon in *Lactobacillus gasseri*. *Proteomics*, in press.

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1 INTRODUCTION

Probiotics are live microbes which confer health benefit on the host. In humans, the most frequently used probiotics are bacteria belonging to genera *Lactobacillus* or *Bifidobacterium*. Both single species and mixed cultures (cocktails) are used. The probiotic features are strain-specific, but the factors contributing to the health promoting effects are largely unknown. Stress responses are a crucial part of the probiotic nature of a bacterium. This is exemplified in a debate about whether bacteria, such as *Lactobacillus delbrüeckii* subsp. *bulgaricus* and *Streptococcus thermophilus* used in yogurt fermentation, should be considered as probiotics at all, since while they confer well-documented health benefits like improving lactose digestion in lactase-deficient subjects and positively modulating the immune system, these bacteria are not very resistant to conditions in the stomach and small intestine, and generally do not reach the gastrointestinal tract (GIT) in very high numbers. Moreover, microorganisms used in food technology are exposed to technological stresses, such as temperature changes. Thus, there is need to know molecular basis of stress tolerance in bacteria marketed as probiotics with special health claims in much greater detail.

Both intestinal pathogens and probiotic bacteria must resist multiple stresses including the acidic pH of the stomach, bile acids and oxidative conditions provided by macrophages during passage or proliferation in its host. Although extensive studies have shown that stress responses and virulence overlap in Gram-positive pathogens (Frees *et al.*, 2007), little, if anything is known about the possible connection between stress responses and probiotic features of bacteria.

Interestingly, *clpC* ATPase in *L. plantarum* was one of the three stress-related genes induced in mouse GIT model (Bron *et al.*, 2004), and *clpL* is among the genes substantially down-regulated in a mutant of *L. plantarum* defective in the Agr-like two-component regulatory system that showed reduced adherence to a glass surface (Sturme *et al.*, 2005). These genes belong to the universal HSP/100 Clp AAA+ (ATPases associated with a variety of cellular activities) ATPases known to be essential in different stress responses in numerous Gram-positive bacteria. However, it is still an open question whether HSP/100 Clp family genes encode probiotic traits in lactobacilli and bifidobacteria. The research in this thesis derived from the hypothesis that stress responses are a crucial part of the probiotic nature of the bacterium, and that ClpATPases play an important role in probiotic characters.

In this thesis, I have examined the Clp family proteins of several bacterial species containing probiotic strains, such as *L. rhamnosus*, *Bifidobacterium longum* and *L. gasseri*. In order to investigate the distribution and expression of ClpATPases in bacteria, 2-DE based high-throughput methods were applied. The roles of *clpL* genes and their protein products in the stress responses of *L. rhamnosus* E-97800 and *L. gasseri* ATCC 33323 were studied in more detail.

2 REVIEW OF THE LITERATURE

2.1 Probiotics

About 10^{14} bacteria live in our body, the number being greater than the quantity of our cells (Reid *et al.*, 2003). The most bacteria rich body part of warm-blooded animals is the large intestine. Bacterial communities in the bowel can reach densities of 10^{11} per gram of content (Tannock, 2007). Overall, the gut microbiota makes a major contribution to human health and disease (Guarner and Malagelada 2003). Probiotic microbes are thought to act through a variety of mechanisms, such as the competition with potential pathogens for nutrients or enterocyte adhesion sites, degradation of toxins, production of antimicrobial substances, and immunomodulation (Silva *et al.*, 1987; Lewis and Freedman, 1998; Isolauri *et al.*, 2001).

Probiotics have been used for a long time to modify the intestinal microbiota. The premise for a microorganism being termed a “probiotic” include proper strain characterization, clearly documented efficacy in clinical studies, safety of use by the target population, instructions for route of administration, and dose applied (FAO/WHO, 2002). Probiotics are defined as “live microorganisms which, when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2001). In Japan, the Ministry of Health has acknowledged FOSHU (foods for specialized health use) status for several probiotic products that it has considered worthy of the health claims made about them.

The major organisms used as probiotics belong to the genera *Lactobacillus* and *Bifidobacterium*. The interest to use strains of these genera on potential probiotics is based on their association with healthy human intestinal tract (Limdi *et al.*, 2006; Boyle and Tang, 2006). Lactobacilli are also a natural part of the human diet since they are present in fermented foods, especially in fermented milk products. One of the best documented effects of probiotics is inhibition of diarrhoea. *Lactobacillus rhamnosus* and *L. reuteri* are effective against diarrhoea of infants (Rosenfeldt *et al.*, 2002; Szajewska and Mrukowicz, 2005; Szajewska *et al.*, 2001). A lot of data about the effectiveness of lactobacilli against diarrhoea related to antibiotic treatment have been reported. *Lactobacillus acidophilus* reduces the incidence of diarrhoea associated with clindamycin (Orrhage *et al.*, 1994) and ampicillin (Gotz *et al.*, 1979). *Lactobacillus rhamnosus* GG (Hilton *et al.*, 1996) and *L. acidophilus* (Black *et al.*, 1989) have reduced the incidence but not the duration of traveller’s diarrhoea. Bifidobacteria are the most abundant bacteria in gut of healthy breast-fed newborns (Harmsen *et al.*, 2000; Favier *et al.*, 2002). The bifidobacterium population then decrease to a lower but stable level in adults (Hopkins *et al.*, 2001; Satokari *et al.*, 2003). Bifidobacteria have been shown to prevent and ameliorate rotavirus infections in infants (Saavedra *et al.*, 1994; Bae *et al.*, 2002; Chouraqui *et al.*, 2004). While most probiotic bacteria belong to the genera *Lactobacillus* and *Bifidobacteria*, some strains belonging to the genera *Escherichia*, *Enterococcus*, *Bacillus*, and even *Saccharomyces* are also used as probiotics when supplemented with proved efficacy through clinical studies (Reid *et al.*, 2003).

The molecular basis of probiotic traits is largely unknown, but widely considered to be multifactorial and strain-dependent. When a probiotic bacterium reduces both the incidence and duration of gastroenteritis it is believed to happen via enhancement of the ability of the host to resist colonization by pathogen or by direct inhibition. Mucin gene expression was shown to lead to an inhibitory effect on enteropathogenic *E. coli in vitro* (Mack *et al.*, 1999) and probiotics have been shown to up-regulate mucin gene expression in a cell-culture model (Mattar *et al.*, 2002). Moreover, probiotics are proven positive modulators of the immune system, although the actual mechanism remains to be determined (Vaarala, 2003; Merk *et al.*, 2005). Substantial data have shown that probiotic strains affect many immunological parameters and the innate or non-specific immune system (reviewed by Senok *et al.*, 2005). In addition to probiotics, a concept for enhancing health not based on living organism, termed prebiotics, has been developed. An excellent definition of a prebiotic is “a nondigestible food ingredient beneficially affecting the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health” (Gibson and Roberfroid, 1995). Combinations of probiotics and prebiotics are known as synbiotics. The available data on synbiotics concern animals (Burns and Rowland, 2000; Pool-Zobel *et al.*, 1996; Femia *et al.*, 2002), but they might be of major importance in health food in the future if they also prove to be safe and effective to humans.

Molecular tools for studies of probiotic bacteria have been developed mainly during the last 10 to 15 years. Research on lactic acid bacteria (LAB) and probiotics has been boosted relatively recently by sequencing of the genomes of *Lactococcus lactis* subsp. *lactis* IL1403 (Bolotin *et al.*, 2001), *Bifidobacterium longum* (Schell *et al.*, 2002), *Lactobacillus plantarum* (Kleerebezem *et al.*, 2003), *Lactobacillus johnsonii* (Pridmore *et al.*, 2004), two *Streptococcus thermophilus* strains (Bolotin *et al.*, 2004), and *Lactobacillus acidophilus* (Altermann *et al.*, 2005). In addition to completed whole-genome sequencing projects, several adaptation-related LAB plasmids have been sequenced and annotated (Siezen *et al.*, 2005).

2.2 Protein quality control

A central dogma of molecular biology is the conversion of genetic information into active proteins. It has been estimated that about one fifth of all newly synthesized proteins are degraded by cellular proteases most likely due to errors in transcription and translation (Yen *et al.*, 1980). The function of chaperones and proteases in the quality control of proteins is based on their ability to fold/refold or degrade misfolded proteins. The quality control of proteins already occurs under favourable growth conditions but becomes particularly important under stress conditions. In *Escherichia coli*, the ribosome-associated trigger factor together with DnaKJ-GrpE system assist the *de novo* folding of at least 340 cytosolic proteins within a broad size range between 16 and 167 kDa (Deuerling *et al.*, 1999), whereas GroEL chaperone machinery helps to fold 250–300 newly synthesized proteins, highly preferring those with a size of 20 - 60 kDa (Houry *et al.*, 1999). The actual numbers could be far higher than these estimates, since substrates of these chaperones that are not prone to aggregation or are rapidly degraded by proteases can not be detected by the methods applied in the above-

mentioned studies. The GroEL chaperone system is the only one that proved to be essential in *E. coli* for growth at all growth temperatures (Fayet *et al.*, 1989). Interestingly, many of the proteins requiring the assistance of the GroEL system immediately after their synthesis are prone to aggregation and require GroEL for conformational maintenance several times in their lifetime (Houry *et al.*, 1999). Protein quality control is especially important under conditions of increased non-native proteins caused by environmental stressors such as bile salts or high temperatures.

Homeostasis in living organisms can be seen as a tendency to maintain a constant concentration of proteins and other compounds, leading to steady-state cellular processes. Although the physiological responses of bacteria to various stresses are highly specific the general objective is homeostasis of cellular processes. For example, after a heat shock the concentration of damaged proteins is increased. The cell responds to this by increasing the synthesis of heat-shock proteins (HSPs) over the basal level to enable folding, repair or degradation of damaged proteins until the concentration of damaged proteins returns to the level before stress.

2.3 The heat-shock response

Physiological stress responses include both global and rather specific responses; in the literature they are often divided into the general stress response and specific stress responses. Another common division is between Gram-negative and –positive bacteria, since the paradigms of regulation of the stress responses fundamentally differ between them.

The heat-shock response is one of the best known physiological responses in all kingdoms of life. The major heat shock proteins (HSPs) are conserved in evolution (Bardwell and Graig, 1984), and function in the folding of proteins in unnative conformations caused by stress (Gottesman *et al.*, 1997). One ubiquitous class of HSP consists of ATP-dependent proteases, which dispose of damaged proteins that cannot be folded correctly (Maurizi, 1998). HSPs are also important during optimal growth, since one third of the newly-synthesized cellular proteins do not spontaneously achieve their biologically active 3-dimensional structure. It has been estimated that one in seven nascent polypeptides is folded by chaperones (Teter *et al.*, 1999; Ewalt *et al.*, 1997) and one in five is degraded by proteases (Yen *et al.*, 1980) mostly due to errors in transcription or translation. Mutations and certain stresses also direct proteins to the quality-control system of protein synthesis in order to fold, or in the case of irreversible damages to degrade them. In bacteria, the heat-shock response is regulated by different mechanisms in Gram- and Gram+ model organisms (Wick and Egli, 2004; Schumann, 2003). The main difference is that in Gram- *E. coli* the response is mediated almost exclusively via promoter switching by competition between alternative sigma factors in binding to the RNA polymerase core enzyme (RNAP) whereas in Gram+ *B. subtilis* many promoters are up- or down-regulated (Narberhaus, 1999) in addition to promoter switching when bacteria faces hyperoptimal temperature.

Bacteria are able to sense the temperature in their environment by many ways. One of the best known mechanisms derives from the temperature-dependent secondary structure of *rpoH*-specific mRNA. At low temperatures, the untranslated region (UTR) of the 5'-end forms a secondary structure that prevents ribosome binding to it, leading to a low expression level. However, when the temperature rises above a threshold, the secondary structure 'melts down', allowing access to ribosomes and resulting in a higher level translation of σ^{32} (Tilly *et al.*, 1989).

2.3.1 Regulation of the heat-shock response in (Gram-) *E. coli*

In *E. coli*, almost immediately after a mild heat stress (e.g. a shift from 30 to 42 °C) HSP synthesis increases to a maximum induction (~ 15-fold) within 5 min (Herendeen *et al.*, 1979; Yamamori and Yura, 1980). In this state, more than 20% of the total cellular proteins are HSPs (Herendeen *et al.*, 1979). Rapid HSP synthesis is followed by an adaptation phase in which the level of HSP gradually decreases. After 20 -30 min the steady-state level of approximately twice the level prior the heat stress is achieved (Lemaux *et al.*, 1978). In *E. coli*, HSP synthesis is activated during heat stress by two alternative σ factors, σ^{32} (Yura *et al.*, 1984) and σ^E (Erickson and Gross, 1989). σ^{32} and σ^E are associated with cytoplasmic and extracytoplasmic stress, respectively. More than 30 and 40 genes have been identified to belong to σ^{32} (Yura *et al.*, 2000) and σ^E (Dartigalongue *et al.*, 2001) regulons, respectively. The transcription of many of these genes is potentially activated by both regulators, and cross-talk occurs to at least some degree between the regulons, since the transcription of *rpoH* is activated by σ^E (Erickson and Gross, 1989). The level of σ^{32} is controlled both via its synthesis rate and its stability in a complex network of signals. Briefly, the major regulation of σ^{32} occurs at the level of translation; after a temperature increase the secondary structure of *rpoH* mRNA changes, making it more prone to translation (Morita *et al.*, 1999). The second major controlling point of the σ^{32} level (Fig. 1) is its rate of degradation by a set of proteases (Kanemori *et al.*, 1999; Morita *et al.*, 2000).

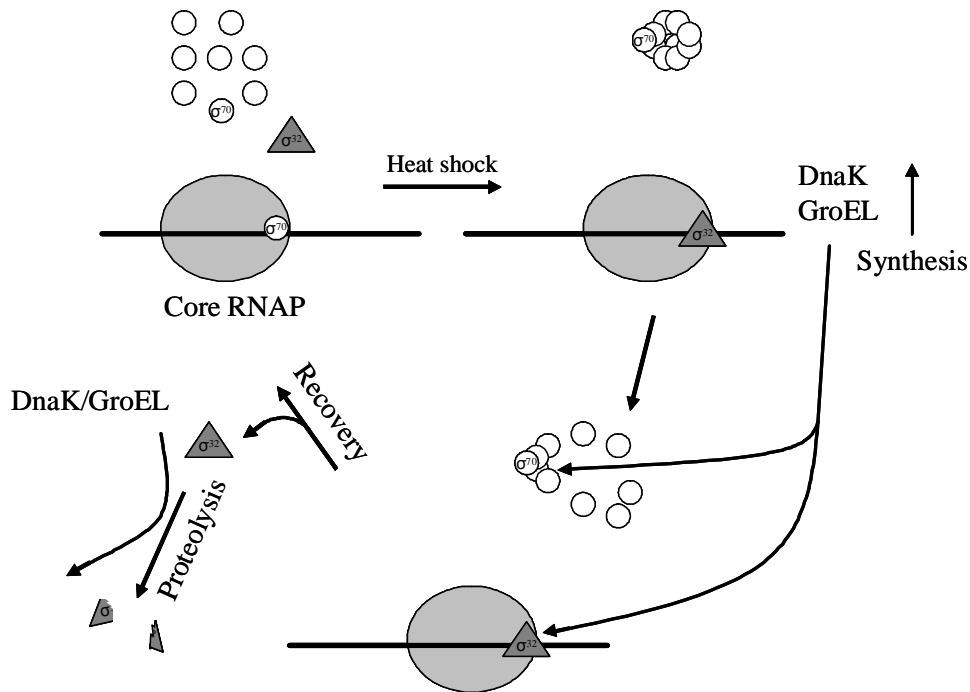


Figure 1. Heat shock leads to overcompetition of the heat-specific sigma factor and an increased HSP concentration. The present model of the function of DnaK/J-GroE and GroEL/S machines in the regulation of the heat-shock response in *E. coli* is shown. Heat shock leads to increased aggregation of heat-sensitive vegetative sigma factor (σ^{70}) (open circles) and subsequent overcompetition of the heat-specific alternative sigma factor (σ^{32}) (shaded triangle) in binding to the core RNA polymerase (RNAP), and to finally an increased concentration of molecular chaperones. During the shutoff period (recovery), the molecular chaperones, GroEL and DnaK, mediate proteolysis of σ^{32} leading to retake of σ^{70} (Blaszczak *et al.*, 1995; Tomoyasu *et al.*, 1998; Guisbert *et al.*, 2004).

2.3.2 Regulation of the heat-shock response in (Gram+) *Bacillus subtilis*

Bacillus subtilis is the model species for low G+C content Gram-positive bacteria. In *B. subtilis*, the expression ~200 genes are induced at least 3-fold in response to a heat shock (rapid temperature change from 37°C to 48-50°C) (Schumann, 2003). The heat-induced genes in *B. subtilis* can be categorized into six classes (Hecker *et al.*, 1996; Darmon *et al.*, 2002).

Class I comprises the classical chaperonin machines DnaK/J and GroEL/S, which are regulated by an HrcA repressor (Schultz and Schumann, 1996). HrcA binds to its palindromic operator sequence, CIRCE (controlling inverted repeats for chaperone expression) (Zuber and Schumann, 1994; Schultz and Schumann, 1996), which is usually located near or upstream from the structural gene. By binding to the CIRCE operator, according to the present model, HrcA forms a sterical hindrance to RNA polymerase repressing transcription of the following gene. The palindromic nature of the CIRCE operator suggests that HrcA is active as a homodimer. The fact that HrcA

from *Thermotoga maritima* was crystallized as a dimer (Liu *et al.*, 2005) further indicates that HrcA binds to its operator as a dimer.

Class II comprises a larger set of genes regulated by an alternative sigma factor SigB, whose expression is induced by several stress conditions and is thought to activate the general stress response (Petersohn *et al.*, 2001; Price *et al.*, 2001).

Into the class III are classified genes regulated by the CtsR (class three stress regulator) repressor, including several members of the Clp family. CtsR binds to the heptanucleotide repeat sequence located in the promoter area of a gene (Derré *et al.*, 1999).

The class IV contains only one gene (*htpG*; high temperature protein), presumably encoding a chaperone, which is induced about 10-fold after heat stress (Schulz *et al.*, 1997). Interestingly, indirect evidence suggest that *htpG* expression is controlled by an unidentified regulator that recognizes a heptameric regulatory site located on the *htpG* promoter (Schulz *et al.*, 1997). When the regulatory element was deleted, expression of *htpG* was reduced (Versteeg *et al.*, 2003); however, when the element was fused to a promoter of a gene with constitutive expression it showed heat inducibility (Versteeg *et al.*, 2003). Taken together, *htpG* seems to be regulated by a positive regulator.

The class V genes are under the regulation of a two-component signal transduction system, C_{ss}RS (control of secretion stress regulator and sensor) (Darmon *et al.*, 2002). This class currently comprises two genes (*htrA1* and *htrA2*) encoding putative membrane-associated proteases (Darmon *et al.*, 2002). The promoters of both genes contain a consensus octameric sequence. The observations that (1), the expression of *htrA1* and *htrA2* does not respond to puromycin (known to generate misfolded proteins in cytosol) in the medium (Darmon *et al.*, 2002) and (2) C_{ss}S function is essential under conditions of saturated secretion apparatus (Hyyryläinen *et al.*, 2001) suggest that C_{ss}S histidine kinase senses extracytoplasmic nonnative proteins (Darmon *et al.*, 2002).

Class VI comprises at least ten heat shock responsive genes that are controlled by regulators not described thus far.

The present model for the heat-shock sensing in *B. subtilis* (Fig. 2) is highly analogous to the titration model for σ^{32} -regulon in *E. coli*. In *B. subtilis*, the negative heat shock regulator (HrcA) is positively modulated by the GroEL/S chaperone system (Mogk *et al.*, 1998) while for σ^{32} in *E. coli*, the master (positive) regulator is sequestered or negatively modified by DnaK (Tilly *et al.*, 1983, 1989; Straus *et al.*, 1990; Blaszcak *et al.*, 1995; Tomoyasu *et al.*, 1998), and to at least some degree directed to the proteolysis via DnaK (Guisbert *et al.*, 2004). HrcA is a repressor protein that becomes aggregated and nonfunctional *in vivo* when GroEL is titrated away by nonnative proteins produced after a stress (Babst *et al.*, 1996; Mogk *et al.*, 1997) which leads to the derepression or increased transcription of heat-shock genes. HrcA binds to a heptanucleotide inverted repeat element (CIRCE) usually located near -10 and -35 boxes (Yuan and Wong, 1995). During the shutoff period nonnative proteins are diminished leading to a high concentration of free GroEL, which eventually leads to increased HrcA activation and repression of the HrcA-regulon.

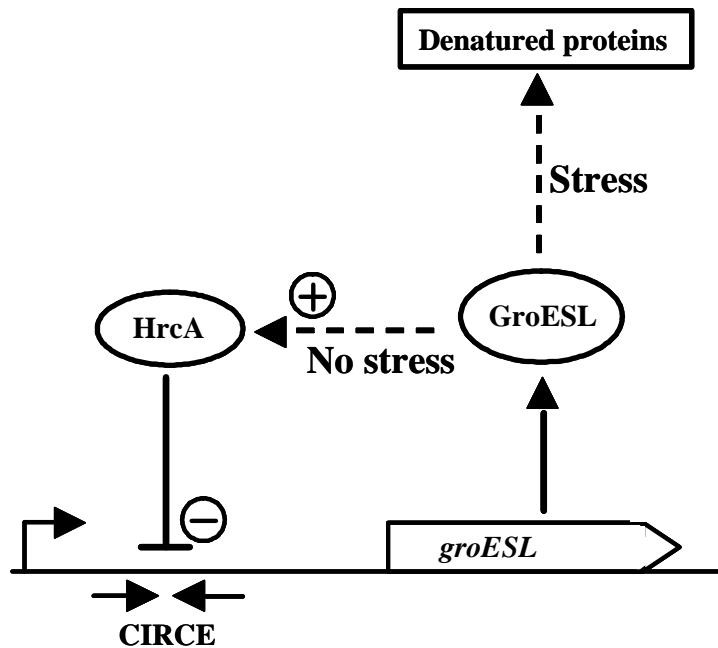


Figure 2. Heat shock leads to GroEL titration by non-native proteins and derepression of HSP expression. GroEL regulates its own expression in *B. subtilis*. An increased concentration of denatured proteins titrate GroEL which favours the aggregation of HrcA, and subsequently, the derepression of chaperone (DnaK and GroEL) machinery expression in order to maintain the proper folding status of cellular proteins (Yuan and Wong, 1995; Babst *et al.*, 1996; Mogk *et al.*, 1997).

2.3.3 Regulation of the heat-shock response in (Gram+) LAB and bifidobacteria

Lactococcus lactis has become a model organism for several reasons. In addition to being widely used as a starter in the dairy industry, *L. lactis* has a relatively straightforward type of metabolism, obtaining most of its energy from lactic acid fermentation (Benthin *et al.*, 1994), and the function and energetics of sugar and amino acid transport is well characterized in *L. lactis* simplifying energy calculation and modelling (Poolman, 1993). Moreover, the *L. lactis* strains isolated from the dairy environment provide a bacterial model system of multiple auxotrophic phenotypes since it has adapted to the excess of nutrients over several hundreds or even thousands of years (Godon *et al.*, 1993).

The CtsR regulon has been characterized in *L. lactis* (Varmanen *et al.*, 2000). Indirect evidence suggests that HrcA-dependent regulation has been conserved in *L. lactis*. Firstly, a complete CIRCE was needed for efficient thermoinduction of the *dnaK* operon (van Asseldonk *et al.*, 1993). Secondly, it has been shown that an anti-HrcA serum can detect a protein from *Streptococcus thermophilus*, a close relative of *L. lactis*, which is able to bind to a CIRCE sequence (Martirani *et al.*, 2001). Thirdly, in another closely-related bacterium, *S. mutans*, deletion of the *hrcA* gene led to the derepression of the *GroEL* operon (Lemos *et al.*, 2001). However, the inability of *L. lactis* HrcA to complement the *B. subtilis* *hrcA* mutant (Wiegert *et al.*, 2004) remains to

be explained. Notably, the salt stress response of *L. lactis* is highly similar to the heat-shock response (Kilstrup *et al.*, 1997), which is not the case in *B. subtilis*, possibly reflecting the adaptation of *L. lactis* to the dairy environment where these two stressors co-exists.

The most striking difference in heat shock response regulatory strategies between the LAB model organism *L. lactis* and *B. subtilis* is that the genome of *L. lactis* does not carry a heat-specific alternative sigma factor (Wegmann *et al.*, 2007). No information is available about the regulation of the heat-shock response in lactobacilli. This is at least partly due the LAB model organism status of *L. lactis* with feasible genetic transformation and gene inactivation techniques that are not yet available for most lactobacilli.

Bifidobacteria are Gram-positive anaerobic, non-motile, non-sporulating, non-gas-producing, usually catalase-negative micro-organisms belonging to the *Actinobacteriae* group. The most extensively studied organisms of this group are *Streptomyces coelicolor* and *Corynebacterium glutamicum*. Little is known about the heat-shock responses of bifidobacteria. Apparently, the *Actinobacteriae* group is diverse and inhabits various ecological niches and this is most likely why the stress responses are regulated and organized very differently among bacteria in this group. For example, the *S. coelicolor* A3 genome codes for 65 alternative sigma factors (Bentley *et al.*, 2002) while only one can be localized into the genome of *Bifidobacterium longum* NCC2705 (Schell *et al.*, 2002). However, studies on bifidobacterial stress responses and their regulation have been boosted by the genome sequencing of *Bifidobacterium longum* NCC2705 (Schell *et al.*, 2002) and *B. breve* UCC2003 (cited in Ventura *et al.*, 2006). An HrcA encoding gene was recently characterized in *B. breve* and, interestingly, the operon encoding *hrcA* was shown to be highly expressed after osmotic stress but not during heat shock (Ventura *et al.*, 2005a). This possibly reflects its ecologic niche, the GIT, where the temperature is rather constant while osmotic conditions fluctuate due to the variations in the diet. Recently, two *clpP* peptidase encoding genes were found to be highly expressed as a bicistronic unit in *B. breve* after heat shock (Ventura *et al.*, 2005b). Moreover, a transcriptional regulator of *clp* gene expression in *Streptomyces* (Bellier and Mazodier, 2004), ClgR (for the *clp* gene regulator), was shown to bind specifically to the promoter area of *clpPIP2* from *Bifidobacterium breve*, strongly suggesting that it is also a *clp*-specific regulator in bifidobacteria (Ventura *et al.*, 2005b). Notably, purified ClgR was able bind to the promoter region of *clpPIP2* only in the presence of crude lysate from heat-stressed *B. breve* cells. In addition, the binding activity was lost upon proteolysis of the heat-stressed crude lysate. Moreover, in pull-down assays using purified recombinant ClgR and heat-stressed crude lysate, a protein of 56 kDa was co-purified. Taken together, these results indicate a novel positive proteinaceous modulator of ClgR in bifidobacteria (Ventura *et al.*, 2005b).

Some organisms use more than one transcriptional regulator to control the production of certain HSPs indicating that the HSP concentration needs to be fine-tuned both in the absence of stress and under challenging conditions. For instance, in *Agrobacterium tumefaciens* and *Caulobacter crescentus* the control of heat-shock gene expression is mediated via both CIRCE and σ^{32} (Mantis and Winans, 1992; Reisenauer *et al.* 1996; Roberts *et al.*, 1996; Segal and Ron, 1993; Segal and Ron, 1995). In *Streptococcus*

salivarius, the ClpP (Chastanet and Msadek, 2003), and *Staphylococcus aureus* (Chastanet *et al.*, 2003), the classical chaperones, GroEL and DnaK, are under the dualistic regulation of HrcA and CtsR regulators. In *S. aureus*, only two classes of heat-shock genes can actually be identified, since CtsR and HrcA respond to the same signals and the HrcA regulon is embedded in the CtsR regulon (Chastanet *et al.*, 2003). In *Streptococcus* group these two heat shock regulons partially overlap since DnaK is regulated by HrcA but GroEL is regulated by both HrcA and CtsR (Chastanet and Msadek, 2003; Chastanet *et al.*, 2003).

2.4 Physiological adaptation and the general stress response

A brief pre-treatment of bacteria with stress can lead to physiological adaptation to forthcoming more severe stress caused by the same stressor. A well-studied example of physiological adaptation is the acid adaptation of enteric bacteria (Foster, 1999; Foster and Hall 1990; Tiwari *et al.*, 2004; Koutsoumanis and Sofos, 2004). The phenomenon has also been studied and well documented in lactobacilli (Lemay *et al.*, 2000; Lorca *et al.*, 2002; Lorca and Valdez, 2001). For example, *L. acidophilus* CRL 639 cells subjected to sublethal acid stress (pH 5.0 for 60 min) were found to confer resistance against subsequent exposure to a lethal pH (pH 3.0) (Lorca *et al.*, 2002).

Recently, heat adaptation was shown to improve the technological characteristics of *L. helveticus*, including proteinase and peptidase activities during its propagation in cheese whey (Di Cagno *et al.*, 2006). Desmond and co-workers (2004) found only moderately increased stress tolerance after overproduction of the GroEL chaperone system (up to 20% of the total cellular protein) during heat stress of *L. paracasei* and *L. lactis* (Desmond *et al.*, 2004). This fact might reflect the involvement of other factors than GroEL system during heat stress. Overexpression of GroEL was achieved using plasmid vectors which might cause cellular stress via their metabolic burden (Ricci and Hernandez, 2000).

It was later observed that when cells were initially pre-treated (adapted) with a mild stress prior to severe challenge, resistance was also induced for some other stresses (for example, see Völker *et al.*, 1992). In nature, bacteria are only rarely in the exponential growth phase due to suboptimal growth conditions. However, early in the development of molecular biology it became widely accepted practice to almost exclusively study exponentially growing *Escherichia coli* cells. The general stress response is strongly connected to the stationary phase of growth and, mostly because of this discrepancy, the concept of the general stress response in bacteria is relatively recent. While stress-specific proteins defend the cell against particular environmental insults such as osmotic or oxidative stress, all the proteins whose synthesis is increased in response to multiple stress conditions providing resistance to subsequent stresses are considered to belong to the general stress response (Foster and Hall, 1990; Tao *et al.*, 1989). In this mode of stress response a bacterium survives an otherwise lethal stress due to the increased concentration of the proteins synthesised after the sub-lethal stress.

Cells of *L. collinoides* pretreated with heat stress are 1860-fold and 190-fold more tolerant against subsequent acid and ethanol challenges, respectively, than non-adapted control cells (Laplace *et al.*, 1999). *L. rhamnosus* GG cells pre-treated with a high hydrostatic pressure were able to resist an otherwise lethal temperature (Ananta and Knorr, 2004). However, pre-adaptation to a stress condition does not always induce cross-protection. This was exemplified by acid-pretreated *L. collinoides* showing a 30-fold lower survival rate after heat stress compared to non-adapted cells, indicating that acid stress could not induce thermotolerance in this bacterium (Laplace *et al.*, 1999). The storage stability of *L. rhamnosus* HN001 was substantially increased after a sublethal stress such as heat or osmotic stress (Prasad *et al.*, 2003). The largest increase in the storage stability of *L. rhamnosus* HN001 was observed after sublethal heat stress during stationary phase (Prasad *et al.*, 2003). It was demonstrated for *L. paracasei* (Desmond *et al.*, 2004) that heat adapted cells showed increased tolerance against spray-drying, which otherwise cause a substantial loss of viability. Survival of lyophilization of *Lactobacillus delbrückii* subsp. *lactis* is considerably increased after osmotic or heat stress (Koch *et al.*, 2007).

LAB growing in the stationary phase and/or subjected to starvation can also develop multiple stress resistance (general stress response) (Kim *et al.* 1999; Hartke *et al.*, 1994), but further studies are needed to get a comprehensive view of the general stress response and particularly its regulation in LAB. While the alternative sigma factors regulate the general stress responses in the model bacteria *E. coli* and *B. subtilis*, their counterparts in LAB are not yet characterized. The SigB-dependent general stress response does not occur in strictly or facultatively anaerobic bacteria including LAB (Hecker *et al.*, 2007). However, a small HSP encoding gene is preceded by a putative sigB-dependent promoter in *L. plantarum* (Spano and Massa, 2006). *L. plantarum* WFCS1 encodes for three alternative sigma factors yet to be characterized (Kleerebezem *et al.*, 2003). The genome of *B. longum* NCC 2705 and *B. breve* UCC2003 contain one and two genes showing homology to alternative sigma factors (Ventura *et al.*, 2006) but their putative contribution to stress responses awaits investigation.

2.5 Clp family

Clp proteins are important to the various cellular processes under both the normal physiological condition and during the stress. Clp family proteins have been studied for almost two decades, since Katayama and co-workers (1988) purified a novel protease to homogeneity from *E. coli* cell extracts. They found this protease to possess caseinolytic activity *in vitro*, and hence named it as Clp (Caseinolytic protease). Katayama and co-workers (1988) showed that Clp is a two-component protease and proposed a model in which an ATP-binding regulatory subunit (named ClpA) interacts with and activates the proteolytic activity of the protease component (named ClpP). Clp family proteins constitute a conserved protein family that can be divided into structurally distinct but functionally closely-related subfamilies: ClpP peptidases (Maurizi, 1998) and Clp/HSP100 AAA⁺ ATPases which act as chaperones when alone (Wawrzynow *et al.*, 1996) but confer substrate specificity to the protease complex when associated with ClpP peptidase.

2.5.1 ClpP peptidase

ClpP is a serine peptidase subunit of the ATP-dependent protease complex. Alone, ClpP has only peptidase activity (Katayama *et al.*, 1988) and requires association with an ATPase subunit in order to be an active protease. In the functional protease, two adjacent barrel-shaped ClpP heptamers (Maurizi *et al.*, 1990) containing 14 active sites inside the proteolytic core are associated at one or both ends of the barrels with a ring-like hexameric (Maurizi, 1991) Clp/HSP100 AAA⁺ ATPase. ClpATPases select the substrate(s) to be degraded. According to the present model of Clp-dependent proteolysis, which is based primarily on studies with *E. coli*, ClpATPases bind to the substrates labelled with ATP which is followed by unfolding and translocation of the substrates through a narrow proteolytic chamber, resulting in degradation in an ATP-dependent manner (Wickner *et al.*, 1999; Sauer *et al.*, 2004). The number of paralogous genes encoding ClpP peptidase within in a genome varies among bacteria. Most eubacterial genomes code for only one ClpP, while some bacteria belonging to the actinobacteria group were found to possess several orthologs. Two *clpP* genes are present in *Mycobacterium tuberculosis*, four were found in the cyanobacterium *Synechocystis*, and at least five are present in *Streptomyces coelicolor* (Viala *et al.*, 2000). All genomes of the genus *Lactobacillus* so far sequenced have been found to code for only one *clpP* gene. However, in *Bifidobacterium breve*, two *clpP* genes are present and expressed as a bicistronic operon (Ventura *et al.*, 2005b). Moreover, in *L. monocytogenes*, a Gram-positive pathogen and model organism for intracellular growth, two *clpP* genes were found (Chastanet *et al.*, 2004), and at least one of them being essential for intracellular parasitism under stress conditions (Gaillot *et al.*, 2001).

2.5.1.1 ClpP in *B. subtilis*

ClpP is an essential protein in *B. subtilis* during conditions leading to increased misfolding of proteins, such as high temperature (Gerth *et al.*, 1998). Moreover, ClpP is essential for competence development, degradative enzyme synthesis, motility, and sporulation (Msadek *et al.*, 1998). In 2-DE gel electrophoresis studies of *clpP* deficient *B. subtilis*, a number of substrate candidates for proteolysis via ClpP have been identified (Kock *et al.*, 2004a). Interestingly, among them are proteins catalysing the first step of certain biosynthetic pathways (Kock *et al.*, 2004a), indicating a regulative role for ClpP. This type of regulation of biosynthetic pathway by ClpP was recently demonstrated, as MurAA, the first enzyme in peptidoglycan biosynthesis, is targeted for ClpP-dependent degradation in *B. subtilis* (Kock *et al.*, 2004b). Another example highlighting the regulatory role of ClpP comes from a recent study demonstrating that an anti-sigmafactor of the *B. subtilis* extra cytoplasmic sigma factor (ECF sigma factor) σ^W is degraded mostly by ClpXP and ClpEP protease complexes after alkaline stress (Zellmeier *et al.*, 2006), which leads to activation of σ^W . It has been estimated that 1650 (Gerth *et al.*, 2004) and 3000 (Østerås *et al.*, 1999) tetradecameric ClpP complexes are respectively present in exponentially growing and starving *B. subtilis* cells. Interestingly, the high number of ClpP complexes relative to the ATPase-binding subunits suggests that there is no competition between ClpATPases for ClpP (Gerth *et al.*, 2004). Protein turnover rate is substantially reduced in the *B. subtilis clpP* mutant

during the exponential growth phase, stationary phase and heat-stress (Kock *et al.*, 2004a), indicating that ClpP is a major protease in the organism. Most strikingly, in the absence of ClpP even under normal growth conditions bulk protein formed dead-end protein aggregates at high level (Kock *et al.*, 2004a), suggesting that the chaperone activity of classical chaperons DnaK and GroEL and/or ClpATPases is somehow linked to the presence of ClpP peptidase in *B. subtilis*. However, these genetic evidences for this connection between proteolysis and chaperone activity awaits support from *in vitro* studies.

2.5.2 Clp/HSP100 AAA+ ATPases

The Clp/HSP100 family belongs to the ring-forming AAA+ (ATPases associated with diverse cellular activities) superfamily of ATPases (Neuwald *et al.*, 1999). ClpA or ClpX are the regulatory subunits (Kessel *et al.*, 1995; Flynn *et al.*, 2003) in proteolytic Clp complex in *E. coli*. Association of ClpX with ClpP has been shown by mutational studies to be mediated by a conserved I/(VM)-G-F/(L) tripeptide motif located in the surface of the AAA or nucleotide-binding domain (NBD) facing ClpP (Kim *et al.*, 2001). Moreover, in completely sequenced bacterial genomes the tripeptide motif is found in most ClpA, ClpC, ClpE and ClpX subfamily members (Kim *et al.*, 2001). In addition, at least one Clp/Hsp100 protein with the motif is found in each completely sequenced organism possessing a ClpP ortholog (Kim *et al.*, 2001). In contrast, the tripeptide is not present in any of the ClpATPases in the genomes of *Methanobacterium thermoautotrophicum* and *Mycoplasma genitalium* lacking a gene encoding ClpP (Kim *et al.*, 2001). Most Clp/HSP100 AAA⁺ ATPases have two distinct 230-residue AAA domains while some AAA⁺ ATPases, exemplified by ClpX, contain only one NBD (Maurizi and Xia, 2004). ClpA (Guo *et al.*, 2002) and ClpX (Singh *et al.*, 2001) have been crystallized as hexamers.

2.5.2.1 ClpATPases in low G+C content Gram-positive bacteria

Many ClpATPases, such as ClpX, ClpC, and ClpE act as a substrate selector part of the bipartite cellular protease (Frees *et al.*, 2007) while the function of others, such as ClpL, remains largely unknown. Some ClpATPases, have been exclusively found in Gram-positive bacteria to date, such as ClpE (Derré *et al.*, 1999) and ClpL (Huang *et al.*, 1993). In Gram-positive bacteria, ClpA has not yet been found, but ClpC as an evolutionary equivalent of ClpA (Shanklin *et al.*, 1995), ClpX (Krüger *et al.*, 2000; Wiegert and Schumann, 2001), and ClpE (Gerth *et al.*, 2004) are most likely the regulatory subunits associated with the ClpP protease.

Many secreted proteins, like trypsin, degrade practically all proteins they meet. Any protein containing accessible lysine or arginine is cleaved by trypsin. In contrast, intra-cellular proteases have to be highly specific to prevent unfavourable proteolysis of cellular proteins. Thus, there is a great challenge for the right protease to degrade the correct protein at the correct time. The substrate specificity of different ClpATPases is further increased by specific adaptors. Developments have recently been made in

demonstrating how substrate specificity of ClpC ATPase is mediated in *B. subtilis* (Kirstein *et al.*, 2006; 2007).

ClpC

A developmental cycle termed competence leading to increased DNA intake in *B. subtilis* is controlled by ClpCP-mediated proteolysis (Turgay *et al.*, 1998). According to the current model, ComK, the transcriptional activator of competence genes in *B. subtilis*, is targeted at ClpCP during exponential growth in a MecA-dependent manner (Persuh *et al.*, 1999; Turgay *et al.*, 1998). In the stationary phase, a quorum-sensing induced protein, ComS, overcompetes MecA from binding to ComK, which leads to proteolysis of MecA, increased stability of ComK, and finally increased transcription of ComK-activated genes (Persuh *et al.*, 1999; Turgay *et al.*, 1998). Recently, Kirstein *et al.* (2006) showed that MecA not only enhances the substrate specificity of ClpCP but also catalyses the oligomerization step of ClpC. MecA is the first adaptor protein shown to be involved in oligomerization of Clp/HSP100 ATPase. It remains to be studied whether this trait is specific to MecA from *B. subtilis* or is ubiquitous for adaptor proteins.

ClpX

A small amount of controversial data has been published concerning the function of ClpX in stress responses in Gram-positive bacteria. Deletion of *clpX* lowered the heat tolerance of the *B. subtilis* (Gerth *et al.*, 1998) while *clpX* inactivation increased the heat tolerance of *S. aureus* (Frees *et al.*, 2003; 2004). The expression of *clpX* is not heat-inducible in *S. lividans* (Viala and Mazodier, 2003) or *Caulobacter crescentus* (Østeras *et al.*, 1999) while it can be induced by heat shock in *L. lactis* (Skinner and Trempey, 2001). Recently, *clpX* was shown to be among the essential genes in *Streptococcus pneumoniae* R6, while *clpP* is not (Robertson *et al.*, 2003), suggesting that ClpX also functions in ClpP-independent way(s) in this organism. No ClpX encoding gene has been characterized to date from either lactobacilli or bifidobacteria.

ClpE

ClpE is a ClpATPase with an N-terminal zinc-finger domain (Derré *et al.*, 1999). Apparently, ClpE has the tripeptide motif known to mediate association with the functional protease (Kim *et al.*, 2001). Recently, ClpE was shown to be able to associate with ClpP (Gerth *et al.*, 2004). ClpE is the first Clp protein to be induced after (<10 min) heat stress, its copy number is lowest in exponentially growing cells, and its half-life is shortest among the Clp proteins in *B. subtilis* (Gerth *et al.*, 2004). Interestingly, whereas *clpE* from *B. subtilis* is heat-stress-inducible its mutant has no obvious phenotype (Derré *et al.*, 1999). However, the *clpE* mutant of *L. monocytogenes* showed attenuated virulence (Nair *et al.*, 1999). In *B. subtilis* (Gerth *et al.*, 2004) and *L. lactis* (Varmanen *et al.*, 2003), respectively, expression of ClpE is strongly and moderately repressed by CtsR under physiological conditions. Moreover, *clpP* expression shows prolonged derepression both in *B. subtilis* (Miethke *et al.*, 2006) and in *L. lactis* (Varmanen *et al.*, 2003), suggesting ClpE-dependent modulation of the CtsR regulon. Whereas CtsR is partially stabilized after heat stress in *clpC* or *clpE*

mutant strains, it is fully stabilized in the *clpCE* double mutant of *B. subtilis* (Miethke *et al.*, 2006), indicating that ClpEP has a crucial role of in the proteolysis of CtsR. The ATPase activity of ClpE derivative from *B. subtilis* lacking the N-terminal zinc finger domain was reduced to about one tenth of the native ClpE ATPase activity *in vitro* (Miethke *et al.*, 2006). Mutational analysis of the zinc-finger domain of ClpE from *L. lactis* revealed that it participates in restoring the basal level of ClpP after stress (Varmanen *et al.*, 2003). These findings indicate that the zinc finger domain is essential to the function of ClpE.

ClpB

ClpB function differs fundamentally from other molecular chaperones. Like many ClpATPases ClpB is able to form homohexamers (Lee *et al.*, 2003). Instead of associating with cellular protease (Parsell *et al.*, 1994; Woo *et al.*, 1992; Kim *et al.*, 2001), it cooperates with DnaK/Hsp70 chaperone machinery forming a bi-chaperone system in order to disaggregate stress-damaged proteins (Doyle *et al.*, 2007). In *E. coli* they act synergistically in the presence of an aggregated substrate, since the ATPase activity of these machineries together is 2-fold higher than the sum of their individual ATP hydrolysis rates (Doyle *et al.*, 2007). Unusually, *clpB* is not present in the *B. subtilis* genome, but was found to be carried in the genomes of several other *Bacillus* species (Namy *et al.*, 1999). ClpB is needed for induced thermotolerance and virulence in Gram-positive pathogenic *L. monocytogenes* (Chastanet *et al.*, 2004), and induced thermotolerance in *S. aureus* (Frees *et al.*, 2004). In some Gram-positive bacteria, the induction of *clpB* by heat was demonstrated while no obvious phenotype was associated with respective mutants (Ingmer *et al.*, 1999; Grandvalet *et al.*, 1999). Recently, it was shown that in Gram-positive halophilic lactic acid bacteria *Tetragenococcus halophilus*, the structure of ClpB varies depending on stress and ATP (Sugimoto *et al.*, 2006). In the presence of ATP, ADP, or its slowly-hydrolysable analog, ATP γ S, it forms hexamers (Sugimoto *et al.*, 2006). *E. coli* ClpB (Zolkiewski *et al.*, 1999) and *Thermus thermophilus* (Watanabe *et al.*, 2002) ClpB hexameric forms are also induced by ATP and ATP γ S, while in the presence of ADP only 2- 5-mers were detected. Moreover, when *T. halophilus* recombinant ClpB_{Tha} was incubated at high temperature only mono- and dimers were detected (Sugimoto *et al.*, 2006). Future studies are needed to assess whether the structure and function of ClpB varies between species.

ClpL

A ClpL encoding gene is missing from the genome of *B. subtilis*, but present in the genomes of Gram-positive bacteria with a low GC content, such as *Streptococcus pneumoniae* (Kwon *et al.*, 2003) and *Staphylococcus aureus* (Frees *et al.*, 2004) and in a lactococcal plasmid (Huang *et al.*, 1993). The conserved I/(VM)-G-F/(L) tripeptide motif needed for interaction between a ClpATPase and ClpP (Kim. *et al.*, 2001) can not be found in ClpL. ClpL protein has been shown to function as a chaperone *in vitro* (Kwon *et al.*, 2003), and to be important under heat stress conditions in several bacteria (Kwon *et al.*, 2003; Frees *et al.*, 2004). Moreover, ClpL is involved in virulence gene expression in *Streptococcus pneumoniae* (Kwon *et al.*, 2003), and is important in the

pathogenesis of *Streptococcus pneumoniae* (Polissi *et al.*, 1998; Hava and Camilli, 2002).

The expression of *clpL* is substantially increased in several stress conditions and in the stationary phase in *Oenococcus oeni* (Beltramo *et al.*, 2004), suggesting that it is a crucial part of stress responses and has a role in stationary-phase-related protein turnover in this organism. ClpL ATPase has been shown to be essential at high temperature, since ClpL deficient cells of *Streptococcus pneumoniae* has a greater generation time (Kwon *et al.*, 2003). Moreover, the ability of heat-adapted *clpL* deficient *S. aureus* and exponentially-growing *clpL* deficient *Streptococcus thermophilus* cells to form colonies is reduced after exposure to a lethal temperature (Frees *et al.*, 2004). Frees *et al.* (2004) showed that the survival rate of ClpL-deficient *S. aureus* at lethal temperature was reduced 10- and 100-fold, respectively, in the genetic background of a lower and higher expression level of ClpL. Moreover, *clpB* inactivation had only a minor effect on the ability of *S. aureus* to form colonies after exposure to a lethal temperature with the genetic background of a higher ClpL expression level, while the *clpB* deficient strain with the genetic background of a lower ClpL level lead to a very low survival rate under the same conditions (Frees *et al.*, 2004).

Recently, a ClpL encoding gene in *L. reuteri* was shown to be up-regulated at low pH (Wall *et al.*, 2007). Moreover, a *clpL*-disruption mutant of probiotic *L. reuteri* showed a decreased survival rate at low pH when compared to its parent culture (Wall *et al.*, 2007). These data might indicate a role of ClpL in *L. reuteri* during adaptation to an acidic environment such as in the gastric juice. Taken together, these results point towards ClpL having a role in the removal of aggregated or otherwise heat-damaged proteins by a still unknown mechanism.

2.6 Proteomic approaches for studying potentially probiotic bacteria

Over 500 bacterial genome sequences are currently (year 2007) available in public databases, representing hundreds of different species as well as multiple strains of the same species. Many studies have focused on changes in the transcription of genes of interest. However, these studies are not able to measure whether the genetic information is translated into proteins, and do not take into account the stability and activity of the corresponding protein products. Therefore, an overview of the protein composition of a bacterium under particular conditions is of great importance to researchers. Proteomics can be defined as the qualitative and quantitative comparison of proteomes, the protein complements of a genome, under different conditions to further unravel biological processes.

Most proteome studies use two-dimensional gel electrophoresis (2-DE) for protein separation. It was simultaneously invented by Klose and O'Farrell already in the 1970s'. In 2-DE, proteins are first separated according to their isoelectric point and then molecular weight. It enables efficient separation and relative quantification of complex protein mixtures, which is very useful in kinetic studies. The relative amount of a protein at different environmental conditions can be measured e.g. by using fluorescent

dyes. Kinetics of protein synthesis under different conditions can be measured e.g. using radioactive amino acids in pulse-chase experiments. Bacteria are perfectly suited to proteomic studies due their single-cell organization and the relatively small number of proteins encoded in their genomes. The proteome is highly dynamic and considered to largely reflect the physiological status of the cell. For example, protein profiles in optimally growing and stressed cells can be extremely diverse. In *B. subtilis* cells, the synthesis of approximately 400 and 150 proteins is substantially decreased and increased, respectively, when the growth is stopped by glucose starvation (Bernhardt *et al.*, 1999). When the general stress response of *B. subtilis* was analyzed by 2-DE-based proteomics more than 60 uncharacterized genes belonging to the σ^B -regulon were found (Petersohn *et al.*, 2001).

While the potential of gel-based proteomic methods to stress response studies is considerable, there are certain drawbacks. Even though thousands of proteins can be separated and analyzed with 2-DE in a single experiment, not all the proteins from an organism are present in a gel. Usually only the soluble part of the total proteome is available for study since the proteins of low solubility (e. g, membrane-derived proteins) are not present in the gel. Also very large and small as well as acidic (low pI) and basic (high pI) proteins are poorly represented in 2-DE analysis. These drawbacks have prompted the development of gel-free alternatives to complement 2-DE-based proteomics. In this approach the proteins are digested into peptides followed by peptide separation by multidimensional liquid chromatography, and analysis online by electrospray ionization mass spectrometry (Washburn *et al.*, 2001; Link, 2002) or offline by MALDI-TOF MS (Zhang *et al.*, 2004).

Many research groups have begun to study the pathophysiology of several low GC% Gram-positive pathogens, such as *Staphylococcus aureus* as well as the intimate interactions between probiotic bacteria and their host. Several proteomic studies have been published concerning technologically relevant physiological responses in LAB: The cell wall/surface-associated proteome of *L. salivarius* has been assessed (Kelly *et al.*, 2005) and a non-gel based proteomic study applied to *Bifidobacterium infantis* (Vitali *et al.*, 2005). A proteomic study revealed 34 proteins to be differentially expressed in *Bifidobacterium longum* after bile salt stress (Sánchez *et al.*, 2005) The cytosolic proteome of *L. plantarum* as a function of the growth cycle was recently published (Cohen *et al.*, 2006). The physiology of the stress responses of several lactobacilli has also been investigated by means of proteomics. At least 20 proteins were up-regulated after heat shock in *Lactobacillus delbrückii* subsp. *bulgaricus* (Gouesbet *et al.*, 2002) and *L. acidophilus* (Broadbent *et al.*, 1997) although all of them were not identified. In *L. sanfranciscensis*, thirteen proteins showed differential expression after exposure to high pressure demonstrating that they might be part of the machinery regulating or alleviating the effects of stalled protein synthesis (Drews *et al.*, 2002). More than 20 proteins were increasingly synthesized in *L. collinoides* (Laplace *et al.*, 1999) and *L. sanfranciscensis* (De Angelis *et al.*, 2001) after subjection to acid stress. The identities of most of these proteins were strain-specific. A few proteins were common to both. They were known heat shock proteins suggesting that there is a general stress response in these bacteria.

3 AIMS OF THE STUDY

Representative strains of bacterial species used as probiotics were chosen for this study. The aim was to investigate the responses of these bacteria to various stresses simulating the natural conditions bacteria have to face, such as a temperature increment in the production, acidity during the storage period, and the presence of bile salts in the gastrointestinal tract. Moreover, the role of ClpL ATPases in the stress tolerance of these bacteria was assessed.

The specific goals of the present study were:

- 1) To characterize two orthologous *clpL* genes in *L. rhamnosus* E-97800 (Study I);
- 2) To gain insights into the stress-specific proteome of *Bifidobacterium longum* by 2-DE and immunoblotting (Study II);
- 3) To study the heat-shock responses of *L. brevis*, *L. reuteri* and *L. rhamnosus* using 2-DE-based proteomics (Study III);
- 4) To investigate the specific role of ClpL ATPase in the stress responses of *L. gasseri* ATCC 33323 by mutational studies, DIGE, and mass spectrometric identifications (Study IV).

4 MATERIALS AND METHODS

The bacterial strains and plasmids used in this work are listed in Table 1. The methods are described in detail in the original publications and are summarized in Table 2.

Table 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant property or genotype(s)	Article	Source or reference
Bacterial strains			
<i>Lactobacillus rhamnosus</i>			
ATCC 7469 ^T	type strain	I	ATCC ^a
E-97800	human fecal isolate	I, III	VTT ^b ; Kontula <i>et al.</i> , 1999
GG (ATCC 53103)	human isolate	I	ATCC ^a
1/6	isolated from cheese	I	Varmanen <i>et al.</i> , 1998
GRL1056	<i>clpL2</i> -deficient derivative of E-97800		This work
<i>Lactobacillus plantarum</i>	type strain	I	ATCC ^a
ATCC 14917 ^T			
<i>Lactobacillus plantarum</i>	<i>upp::ISLp11</i> ;	I	Nicoloff and Bringel, 2003
HN38	spontaneous mutant of <i>L. plantarum</i> FB335		
<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> ATCC 25302 ^T	type strain	I	ATCC ^a
<i>Lactobacillus gasseri</i>			
ATCC 33323	neotype, DSM 20243	IV	ATCC ^a
AS1	ATCC 33323 derivative with 0.9-kb deletion in <i>clpL</i>	IV	This work
AS2	ATCC 33323 harboring plasmid pKTH2095	IV	This work
AS3	AS1 harboring plasmid pKTH2095	IV	This work
AS4	AS1 harboring plasmid pAS2	IV	This work
<i>Lactobacillus reuteri</i> SD 2112		III	Ruiz-Palacios <i>et al.</i> 1996
<i>Lactobacillus brevis</i> ATCC 8287	probiotic	III	Rönkä <i>et al.</i> , 2003
<i>Bifidobacterium longum</i> 3A		II	Bioferme Ltd., Kaarina, Finland
<i>Lactococcus lactis</i> ssp. <i>lactis</i> MG1363	laboratory strain	III	Gasson, 1983
<i>Escherichia coli</i> TOP10F'	cloning host	I	Invitrogen
<i>Escherichia coli</i> M15(pREP4)	cloning host with inducible expression	IV	Qiagen

Plasmids	Relevant property or genotype(s)	Article	Source or reference
pCR2.1-TOPO	cloning vector	I	Invitrogen
pTN1	conditionally replicating vector for an allelic replacement	IV	Neu and Henrich, 2003
pAS1	pTN1 with 1.1-kb fragment containing 0.9 kb in-frame deletion in the <i>clpL</i>	IV	This work
pQE30	IPTG-controlled overexpression	IV	Qiagen
pKTH5202	pQE30 with 1.1-kb fragment encoding HrcA	IV	This work
pKTH2095	lactobacillar expression vector	IV	Savijoki <i>et al.</i> , 1997
pAS2	pKTH2095 with 2.4-kb fragment containing the <i>clpL</i> region	IV	This work

^a ATCC, American type culture collection

^b VTT, Technical Research Centre of Finland, Espoo, Finland

Table 2. Methods used in this study

Method	Described and used in
Genetic methods	
Isolation of total DNA	I
Isolation of plasmid DNA	I, IV
Cloning to pCR2.1-TOPO (for sequencing)	I
Cloning to pTN1 (for an allelic replacement)	IV
Cloning to pQE30 (for overproduction)	IV
Cloning to pKTH2095 (for complementing)	IV
Vectorette genomic library kit (for sequencing)	I
DNA sequencing	I
Southern and dot blot hybridization	I
Northern hybridization	I, IV
5'-end mapping of a transcript	I
Protein assays	
SDS-PAGE	IV
Western blotting	II, III, IV
Expression of His-tagged recombinant protein	IV
2D-PAGE	II, III, IV
Mass spectrometry	II, IV
Protein and peptide identifying	II, IV
Physiological characterization	
Bioscreen C growth monitoring system	I, IV

5 RESULTS AND DISCUSSION

To be able to function as a probiotic, bacteria have to tolerate several stress factors, such as an acidic pH and bile salts, throughout the GIT. Several bacterial species are unable to survive the challenging conditions in the stomach, while others can survive the passage by utilizing various defence mechanisms. These mechanisms often involve changes in gene expression and phenotype. A common model stress condition used to examine the stress responses of a microbial system is heat shock.

This work consisted of two parts in connection with the following objectives: 1) to characterize the *clpL* genes and their products in two potentially probiotic *Lactobacillus* strains and 2) to utilize and evaluate the efficiency of 2-DE-based proteomic approaches for the investigation of stress responses in potentially probiotic bacteria.

5.1 Characterization of *clpL* genes and their products from selected LAB

Clp family proteins are well-known virulence factors in a number of pathogenic bacteria, including *Streptococcus pneumoniae* and *Staphylococcus aureus* (Frees *et al.*, 2007), but the role of Clp family proteins in the probiotic nature of bacteria has not been widely studied. Genes encoding ClpL ATPase and their products were characterized in *L. rhamnosus* E-97800 (I) and *L. gasseri* ATCC 33323 (IV), respectively, representing *L. casei* and *L. acidophilus-L. delbrückii* groups of lactobacilli, which are rich in strains with probiotic attributes.

5.1.1 Distribution and expression of *clpL* genes and their products in selected LAB

The composition and expression of the ClpATPase protein family in selected probiotic strains *L. rhamnosus* E-97800, *Lactobacillus brevis* ATCC 8287 and *Lactobacillus reuteri* SD 2112 was investigated by Western blotting using antibodies raised against the ClpE protein of *Lactococcus lactis* (Varmanen *et al.*, 2003). *L. lactis* possesses five ClpATPases, including ClpX, ClpE, ClpC and two ClpBs, of which ClpE and the two ClpBs have been recognized using ClpE-specific antibodies (Ingmer *et al.*, 1999; Varmanen *et al.*, 2003). The low expression level of the *clpC* gene observed in *L. lactis* can at least partially explain the lack of detection of this Clp protein in *L. lactis* (Ingmer *et al.*, 1999; Varmanen *et al.*, 2000), whereas ClpE antibodies presumably do not recognize ClpATPase proteins having only one nucleotide binding domain, like ClpX. The Western blotting results obtained from protein samples extracted from probiotic bacteria growing under standard conditions and after applying heat stress revealed the appearance of 3 to 5 proteins of between 83 and 115 kDa cross-reacting with the ClpE antibody (Figure 3). Interestingly, the number of cross-reacting proteins was found to be higher in *L. rhamnosus* E-97800 than in other two probiotic strains, implying that *L. rhamnosus* may encode for more members belonging to the ClpATPase family than other strains under study.

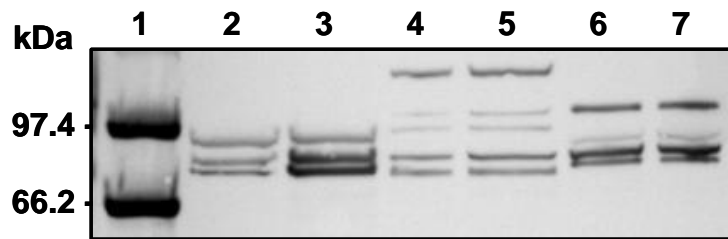


Figure 3. The number of ClpE cross-reacting proteins varies among selected LAB. Western blot analysis of *L. reuteri*, *L. rhamnosus* and *L. brevis* strains. Lane 1, Molecular weight marker; lanes 2 and 3, *L. reuteri* SD 2112; lanes 4 and 5, *L. rhamnosus* E-97800, lanes 6 and 7, *L. brevis* ATCC 8287. An equal amount of protein (5 μ g) extracted from cells before (lanes 2, 4, 6) and 15 min after (lanes 3, 5, 7) shifting the cells to 50 °C was subjected to NuPAGE™ 4-12% Bis-Tris ZOOM™ gels with MOPS running buffer. ClpE cross-reacting proteins were detected by Western blotting with anti-ClpE antibodies (1:3000) and HRP-conjugated goat anti-rabbit IgG (1:3000) combined with a colorimetric reaction.

Degenerate primers previously utilized to obtain partial coding regions of the *clpC*, *clpB* and *clpE* genes in *Lactococcus lactis* (Ingmer *et al.*, 1999) were applied to the amplification of a PCR product from *L. rhamnosus* E-97800. The 550-bp PCR product obtained was cloned into an *E. coli* vector, transformed into an *E. coli* strain and sequenced. Fifteen clones with inserts were found to contain an insert with homology to the ClpATPase-encoding genes. One of these clones was shown to carry an insert sharing 96% identity with a corresponding fragment of the ClpL-encoding gene, named as *clpL1*, in *L. rhamnosus* strain RW-9595M (Provencher *et al.*, 2003), while others carried an insert with 98% identity to a *clpL* gene previously identified in *L. plantarum* (Kleerebezem *et al.*, 2003). The complete nucleotide sequence of this *clpL* gene, named as *clpL2*, and its flanking regions was obtained using the Vectorsite library kit. The *clpL2* gene was found to be flanked by repeats with high sequence homology to inverted repeats of IS*Lpl1*, which has been a functional IS-element in *L. plantarum* (Nicoloff and Bringel, 2003).

As demonstrated by southern hybridization experiments with *clpL1*- and *clpL2*-specific probes, only the *L. rhamnosus* E-97800 strain among the four *L. rhamnosus* strains studied was harbouring both *clpL*-encoding genes (Figure 2 of article I). The *clpL2*-specific probe was able to hybridize with DNA samples of E-97800 and *L. plantarum* ATCC 14917. The *clpL1*-specific probe hybridized with DNA samples of all four *L. rhamnosus* strains and *L. paracasei* ATCC 25302. These results clearly suggest that *L. rhamnosus* E-97800 carries two copies of *clpL* encoding genes, while the other strains in the experiment carry only one *clpL* gene. Based on genome mining of *L. gasseri* ATCC 33323 (Figure 2 of IV) and *L. plantarum* (data not shown) four and five potential genes encoding ClpATPases were found, respectively. A FASTA search with *clpL2* as a query sequence revealed that at least *L. reuteri* and *Oenococcus oeni* possess two copies of ClpL-encoding genes (data not shown) indicating that whether the functions of ClpL paralogs are redundant or not, they both can withstand selective pressure among LAB.

The expression of *clpL1* and *clpL2* in *L. rhamnosus* E-97800 was found to be induced, >20- and 3-fold, respectively, during heat shock (Figure 3 of I). Moreover, the expression of both *clpL* genes increased drastically on entering the stationary phase (Figure 3 of I). The *clpL1*-specific transcripts could not be detected in the early exponential phase (Figure 3 of I) indicating very strict control of expression. The expression of *clpL* in *S. pneumoniae* (Robertson *et al.*, 2002) and *Oenococcus oeni* (Beltramo *et al.*, 2004) as a function of the growth phase is highly similar to that observed for *clpL1* and *clpL2*. ClpL protein expression increased 4.4-fold in *L. gasseri* ATCC 33323 after heat shock (Figure 3 of IV). This heat-shock induction pattern of ClpL was verified with northern analysis of *clpL* (Figure 4 of IV). The relative amount of ClpL was shown to be increased by high pressure in *L. sanfranciscensis* (Pavlovic *et al.*, 2005), and the expression of *clpL*-specific mRNA were induced by low pH in pathogenic *Streptococcus mutans* (Len *et al.*, 2004), suggesting that ClpL is essential during stress conditions in these organisms. In *S. thermophilus*, the expression of *clpL* was induced by both heat and cold shocks (Varcamonti *et al.*, 2006).

5.1.2 Genetic stability of *clpL* genes in *L. rhamnosus* E-97800

Since only the strain E-97800 among the four *L. rhamnosus* studied was found to carry two copies of the *clpL* gene, we aimed to characterize the *clpL2* region. The region containing the *clpL2* gene was sequenced and found to be flanked by inverted repeats with high sequence homology (>90%) to terminal inverted repeats of the IS30-related insertional element IS*Lpl1* (Figure 1 of I) which was recently shown to be functional in *L. plantarum* HN38 (Nicoloff and Bringel, 2003). A ClpL encoding gene has been part of the transposon-like structure in a lactococcal plasmid (Huang *et al.*, 1993). The *clpL2* gene was almost identical to a gene present in the genome of *L. plantarum* WCFS1 (Kleerebezem *et al.*, 2003) and shares over 90% identity with the *clpL* gene from a lactococcal plasmid (Huang *et al.*, 1993). These observations support the idea that the ClpL encoding gene has spread horizontally among bacteria and could possibly help hosts to adapt to new environments and prompted us to study the genetic stability of *clpL* genes in *L. rhamnosus* E-97800.

The genetic stability of *clpL2* was investigated by plating appropriate dilutions of the parental culture (grown overnight at 37°C) and the cultures grown for seven (7-day culture) and 14 (14-day culture) serial passages at 30, 37, and 45 °C on MRS agar plates. The individual clones were examined for the presence of *clpL1*, *clpL2* and *pepX* (a control) by colony-PCR (data not shown). Indeed, *clpL2* was found to mobilize from *L. rhamnosus* E-97800 during prolonged cultivation at high temperature. The frequency of mobilization was temperature-dependent, since according to PCR-analyses of 20 individual clones, *clpL2* was present after 7 or 14 days of cultivation at 30 °C (data not shown) or 37 °C (six clones from 20 are shown in Figure 4) but not present in all clones when E-97800 was cultivated at 45 °C for 7 and 14 days (Figure 4). One of the colonies from 7-day culture at 45 °C devoid of *clpL2* was designated GRL1056 and subjected to Southern and dot blot hybridization to confirm the loss of *clpL2* (Figure 6 of I).

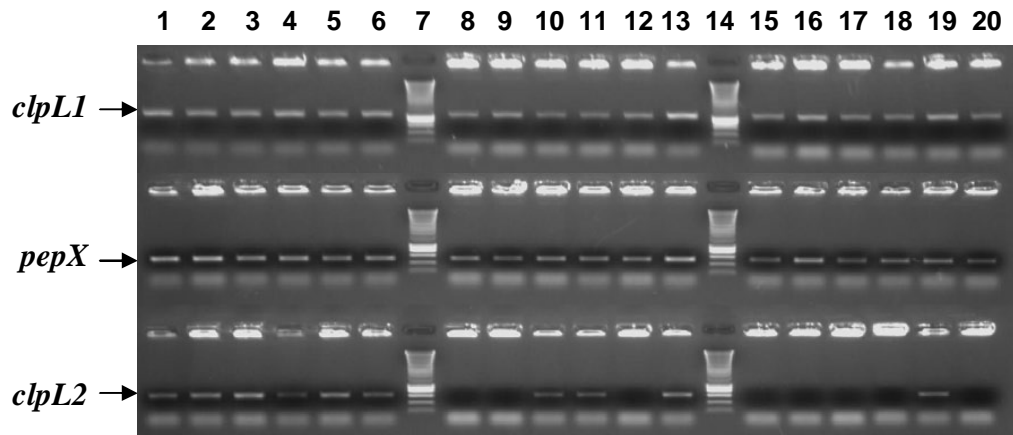


Figure 4. The *clpL2* gene is lost from *L. rhamnosus* E-97800 during prolonged heat stress. Electrophoresis of colony-PCR of E-97800 with *clpL*- and *pepX*-specific oligonucleotides. Lanes 1 to 6, clones from overnight cultivation at 37 °C; lanes 8 to 13, clones from 7-day culture at 45 °C; lanes 15 to 20, clones from 14-day culture at 45 °C. The lanes 7 and 14 contained a molecular weight marker. The gel was stained with ethidium bromide.

The next questions arising from the results were whether: (1) *clpL2* is encoded by a plasmid in E-97800, (2) *clpL2* provides any selective advantage to the host, (3) the transposing event of *clpL2* into E-97800 is recent, and (4) the *clpL2* mobilization event was random or controlled.

We were able to extract one 14-kb plasmid from E-97800, but according to southern hybridization, *clpL2* was not carried by this plasmid (data not shown). However, we cannot exclude the possibility that *clpL2* is harbored by a plasmid that escaped our plasmid purification protocol. We did not find increased sensitivity of GRL1056 against any stressors studied compared to E-97800, its parental strain. Thus, the conditions, if any, in which ClpL2 confers selective advantage to E-97800 remains to be found. Several facts support the view that the transposing event of *clpL2* into E-97800 is relatively recent, at least when compared to the transposition of *clpL* into *L. lactis* (Huang *et al.*, 1993). The G+C content of *clpL2*, 40%, is divergent from those of *clpL1* and all the *L. rhamnosus* entries at GenBank, which are 49% and 48%, respectively (data not shown). The codon usage of the *clp*-like genes and the IS element in a lactococcal plasmid (Huang *et al.* 1993) suggests that the transposition event may have occurred many generations ago and that they both confer a phenotypic advantage to their host.

The generation times of GRL1056 and E-97800 calculated from six parallel cultures growing at 37 °C were 98 (± 4) and 121 (± 6) min, respectively (data not shown), while the generation times for GRL1056 and E-97800 growing at 44 °C were 120 (± 6) and 131 (± 2) min, respectively (data not shown), indicating that GRL1056 is well-adapted to grow both at 37 and 44 °C. The idea that *clpL2* mobilization in E-97800 is a controlled phenomenon rather than a random process is well supported by the growth kinetics of E-97800 and GRL1056. If the mobilization of the *clpL2* region was simply a random process leading to reduced genome size and therefore a shorter generation time of GRL1056, *clpL2* mobilization should also be detected after cultivation at 37 °C,

since the generation time of GRL1056 was shortened even more (~19%) at 37 °C than at 44 °C (~8%).

Another example of at least some degree of controlled genetic reorganization in LAB was reported by Strøman *et al.* (2003). They showed that a *Lactobacillus crispatus* strain can lose its erythromycin resistance (em^r) phenotype spontaneously but the frequency of the event is increased by heat shock. Further, they demonstrated that the loss was due the transposition of an IS element carrying the em^r trait (Strøman *et al.*, 2003).

5.1.3 Phenotypes of *clpL* deficient *L. rhamnosus* and *L. gasseri* strains

Since *clpL2* mobilization was detected only at suboptimal growth conditions we sought to determine whether the stress tolerance of GRL1056 had been altered. However, GRL1056 was not sensitive against stressors studied when compared to its parental strain indicating that ClpL2 is not essential for *L. rhamnosus* E-97800 during growth under the tested conditions. We aimed to study ClpL function *in vivo* with a genetic background devoid of another *clpL* gene. However, it was not possible to examine the *clpL1* function of GRL1056 with mutational studies, since appropriate tools are not available. We generated a *clpL* mutant of *L. gasseri* ATCC 33323 which possesses only one *clpL* gene by applying an allelic gene replacement technique based on the use of a thermosensitive vector developed for *L. gasseri* NCK102 by Neu and Henrich (2003).

Strain GRL1064, a *clpL* deletion mutant derivative of ATCC 33323, showed substantially reduced survival at lethal temperature (60°C), and inability to induce thermotolerance compared to the parental strain (Figure 5 of IV). Increased sensitivity of GRL1064 against other stressors, such as an acidic pH, could not be detected. The ClpL of *S. pneumoniae* possesses chaperone activity *in vitro* (Kwon *et al.*, 2003). Beltramo *et al.* (2004) speculated that ClpL might act with ClpP as a proteolytic complex while Kwon *et al.* (2003) suggested that ClpL acts as a chaperone. The tripeptide known to mediate functional association with ClpP is present in ClpC, ClpE, ClpX, but not in ClpL (Kim *et al.*, 2001; Frees *et al.*, 2007). ClpB, also lacking the ClpP recognition tripeptide is known to function synergistically with DnaK chaperone machinery in *E. coli* (Doyle *et al.*, 2007). Taken together, it remains to be determined whether ClpL functions as a chaperone and/or as a subunit in a protease. The putative proteinaceous partners of ClpL also remain to be studied. Recently, the expression of *clpL* in *L. reuteri* was shown to increase at a low pH (Wall *et al.*, 2007). Moreover, an *L. reuteri* strain carrying an inserted plasmid vector in its *clpL* gene showed slightly decreased survival at a low pH (Wall *et al.*, 2007) when compared to its parental strain.

We examined whether *clpL* deletion caused an increase in nonnative proteins which would be expected to be counterbalanced by increased HSP expression in the GRL1064 strain. GroEL or HSP60 is a well-known HSP and a good marker of the folding status of cellular proteins. If the relative amount of nonnative proteins increases, the cell should respond to this change by adjusting the cellular GroEL chaperone system. As demonstrated by Western blot analysis the abundance of GroEL is not changed (Figure

5) in a *clpL* deficient background indicating that the protein folding status is essentially the same in both strains.

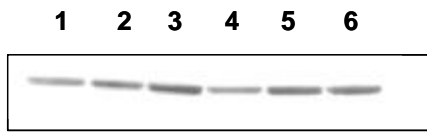


Figure 5. The abundance of GroEL is not affected by the absence of ClpL in *L. gasseri* ATCC 33323. Western blot analysis of GroEL expression in *L. gasseri* ATCC 33323 (lanes 1 to 3) and GRL1064 (lanes 4 to 6) during heat stress. Five micrograms of total soluble protein extracted from cells grown in MRS before (lanes 1 and 4) and 15 min (2 and 5) or 30 min (3 and 6) after the application of heat stress at 49 °C was separated per lane and detected by immunoblotting with GroEL-specific antibodies. The results shown are a representative of two independent experiment.

Recently, it was shown that ClpE, the most closest ClpL paralog in *B. subtilis*, autoregulates its own expression (Miethke *et al.*, 2006). Therefore, we studied whether *clpL* expression has been altered in *L. gasseri* with a *clpL* deficient background. However, according to Northern blot analysis, the amounts of *clpL*-specific transcripts were essentially equals in both genetic backgrounds and ClpL therefore probably does not have autoregulative activity in *L. gasseri* (Figure 6).

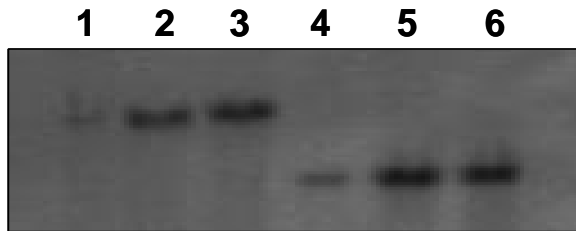


Figure 6. The expression of *clpL* is not autoregulated in *L. gasseri* ATCC 33323. Northern blot analysis of *clpL* expression in ATCC 33323 (lanes 1 to 3) and GRL1064 (lanes 4 to 6) before and after heat stress. Total RNA extracted from cells grown in MRS before (lanes 1 and 4) and 10 min (2 and 5) or 20 min (3 and 6) after application of heat stress at 49 °C was separated per lane and detected by a *clpL*-specific DNA probe.

5.1.4 Regulation of *clp* gene expression is organized differently among LAB

An HrcA binding element, called CIRCE, but no CtsR binding site was found within the promoter sequence of *clpL1* in E-97800 (Figure 5 of I) and *clpL* of ATCC 33323 (Figure 1 of IV). In the LAB prototype, *L. lactis*, *clpB*, *clpC* and *clpE* are regulated by CtsR (Varmanen *et al.*, 2000). In *L. rhamnosus* E-97800, upstream of the *clpP* gene both the CtsR binding site and CIRCE element resembling sequences can be found indicating that *clpP* expression is regulated by both CtsR and HrcA (unpublished data), as is the case in *Streptococcus salivarius* (Chastanet & Msadek, 2003). Interestingly,

neither the CtsR nor the HrcA regulon from the genus *Lactobacillus* has been characterized to date. Recently, it was shown that the expression of most genes encoding molecular chaperones, including *dnaK*, *groEL*, and *clp* genes, are under exclusive control of CtsR in *Oenococcus oeni* IOB 8413 (Grandvalet *et al.*, 2005). Moreover, neither genes encoding alternative sigma factors nor other known regulators of the heat-shock response appear to be encoded by the genome of *O. oeni* IOB 8413 (Grandvalet *et al.*, 2005 and citation (33.) in this reference), indicating that CtsR is the master regulator of the heat-shock response in this bacterium. The extensive variation in these specific repressor-operator systems among low G+C content Gram-positive bacteria might reflect the ecological niches they have adapted to. This diversification is highlighted in Table 3.

Table 3. Regulation of *clp* gene expression in selected Gram-positive bacteria

organism	gene			reference(s)
	<i>clpP</i>	<i>clpL</i>	<i>clpL2</i>	
<i>Lactococcus lactis</i>	CtsR	-	-	Varmanen <i>et al.</i> , 2000
<i>Streptococcus salivarius</i> ^a	CtsR + HrcA	-	-	Kwon <i>et al.</i> , 2003
<i>Staphylococcus aureus</i> ^b	CtsR + HrcA	SigB (σ^B)	-	Chastanet and Msadek, 2003
<i>Lactobacillus acidophilus</i> group ^c	HrcA	HrcA	-	Study IV
<i>Lactobacillus rhamnosus</i>	CtsR+HrcA	HrcA	unknown	Study I; data not shown
Bifidobacteria ^d	ClgR/HspR	-	-	Ventura <i>et al.</i> , 2005b; 2005c
<i>Oenococcus oeni</i> ^e	CtsR	CtsR+unknown	unknown	Grandvalet <i>et al.</i> , 2005

-, not reported to be carried by a chromosome from this species thus far

^a CtsR and HrcA regulons partially overlap in streptococci

^b HrcA regulon is fully embedded within the CtsR regulon in staphylococci

^c HrcA is the master regulator of stress responses as judged by sequence, northern and EMSA analyses

^d *clpP1*, *clpP2* and *clpC* are regulated by ClgR and *clpB* is regulated by HspR in *B. breve*

^e CtsR is the master regulator of stress responses

Several observations point towards HrcA being the master regulator of heat-shock response in the *L. acidophilus* complex. HrcA was demonstrated to bind specifically to the DNA fragment carrying a CIRCE element from the *clpL* region (Figures 1 and 6 of IV). Moreover, HrcA was shown to specifically bind to the fragment containing the promoter region of the *dnaK* operon with a CIRCE element (Figure 7). The northern hybridization (Figure 4 of IV) and EMSA results (Figure 7) indicate that HrcA is involved in autoregulation of its expression in *L. gasseri*. The genetic constellation of putative *dnaK* operon (*hrcA-grpE-dnaK-dnaJ*) in *L. gasseri* is typical of that widely

conserved among bacteria (Weng *et al.*, 2001), including lactobacilli (Schmidt *et al.*, 1999; Zink *et al.*, 2000). GroEL is negatively autoregulated via interaction with HrcA in a number of bacteria (Mogk *et al.*, 1997; Lemos *et al.*, 2001; Wilson *et al.*, 2005). When nonnative proteins arise in the cytosol, GroEL is no longer free to fold HrcA leading to an increased proportion of nonnative HrcA and subsequent derepression of the CIRCE regulon. When the stress situation is over, GroEL is free to bind to HrcA again leading to an increased proportion of functional HrcA and the CIRCE regulon returns to its repressed state as prior to the stress. No CtsR encoding gene has been found from currently completed and published genomes of the *L. acidophilus* complex (van de Guchte *et al.*, 2006; Altermann *et al.*, 2005; Berger *et al.*, 2006; Pridmore *et al.*, 2004) while it can be found in *L. sakei* (data not shown), *L. casei* (data not shown), most likely from *L. rhamnosus* E-97800 (data not shown), and *L. plantarum* (Van de Guchte *et al.*, 2006). Moreover, van de Guchte and co-workers (2006) recently reported that in *L. delbrückii* subsp. *bulgaricus*, *L. acidophilus* and *L. johnsonii*, *clpP* and *clpE* are preceded by CIRCE boxes, suggesti that HrcA-dependent regulation of *clp* gene expression might be conserved among the *L. acidophilus* complex.

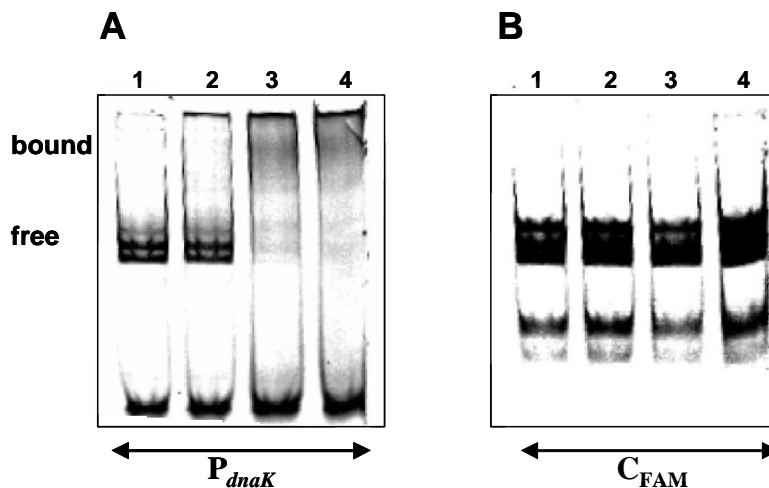


Figure 7. HrcA specifically binds to the promoter region of the *dnaK* operon in *L. gasseri* ATCC 33323. Multilabel EMSA of HrcA binding to the putative promoter region of the ATCC 33323 *dnaK* operon. Reactions containing 50 ng of the PCR-derived 217 bp P_{dnaK} (-227 to +10) fragment labelled with TAMRA dye and the C_{FAM} (-335 to -81) fragment labelled with FAM representing the control DNA were mixed with 0 ng (lane 1), 500 ng (lane 2), 750 (lane 3) or 1000 ng (lane 4) of rHrcA. Reactions were separated in a 5% PAGE followed by scanning with a Fuji FLA-5100 Scanner (Fuji Photo Film Co., Ltd, Japan) using an excitation laser at 490 nm and the following settings: TAMRA, output voltage 400 V, emission filter 585 nm (panel A); FAM, output voltage 400 V, emission filter 515 nm (B). The positions of the bound and free probe are indicated on the left.

In *B. subtilis*, the model of low G+C content Gram-positive bacteria, all the proteins encoded in the CtsR regulon affect CtsR stability and thus their own expression and stability, comprising a complex regulatory network by which it can adapt to its altered milieu (Kirstein *et al.*, 2005; Miethke *et al.*, 2006; Kirstein *et al.*, 2007). The *clpL*- and *clpP*-specific transcripts were detected at 3-fold and >20-fold levels, respectively, after heat stress (Figure 4 of IV and Figure 8). Almost a 7-fold difference between the induction folds within the regulon might indicate the existence of additional regulative mechanism(s) in the HrcA/CIRCE-regulon. A post-transcriptional regulative potential of CIRCE was demonstrated in *Rhodobacter capsulatus* *GroEL* operon preceded by a CIRCE element was constitutively expressed, but the stability of the *GroEL* mRNA was increased after heat shock (Jager *et al.*, 2004). Notably, an HrcA-encoding gene is apparently missing in *R. capsulatus* (Jager *et al.*, 2004) indicating that the CIRCE element can withstand selective pressure even without HrcA. However, the fine-tuning mechanisms within the CIRCE regulon in LAB are not known and need to be studied in the future.

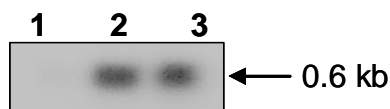


Figure 8. The *clpP* expression is derepressed during heat stress in *L. gasseri* ATCC 33323. Northern blot analysis of *clpP* gene expression in *L. gasseri* ATCC 33323 during heat stress with a *clpP*-specific DNA probe. Total RNA samples were isolated from cells grown in MRS prior to (lane 1) and 10 min (lane 2) or 20 min (lane 3) after heat stress (49 °C) was applied. The size of mRNA was estimated according to an RNA molecular weight marker (Promega). Representative results of two independent experiments are shown.

5.2 Effect of stress on protein synthesis and abundance in bacteria studied by [³⁵S]methionine labelling and DIGE

Using 2D-PAGE and mass spectrometric methods it is possible to identify cellular proteins that are differentially expressed soon after heat stress is applied and even measure their relative synthesis rate if specific radioactive amino acids are used. These techniques are extremely powerful when used simultaneously. A comparative study in which silver staining and [³⁵S]methionine labelling was applied showed a reasonable correlation of protein synthesis with the amount of protein in *B. subtilis* during the exponential growth phase (Bernhardt *et al.*, 2003). However, after imposition of the stress stimulus this correlation no longer exists (Bernhardt *et al.*, 1999; 2003). The use of a metabolic label such as [³⁵S]methionine requires a chemically defined medium (CDM), where the amount of unlabelled methionine is adjustable in order to obtain an efficiently-labelled protein sample. It is typical that during stress conditions the increased expression of single (stress) regulons occurs in a sequential manner. This type of reprogramming of gene expression can be revealed by a kinetic study. Kinetic studies with a radiolabel also have the potential to reveal unstable proteins and allocate proteins to distinct groups, like stimulons and regulons, in order to predict the functions of unknown proteins.

Two-dimensional difference gel electrophoresis (DIGE) utilizes at least two fluorescent dyes to label two different protein samples *in vitro* prior to 2D-PAGE. Compared to 2D-PAGE with other staining methods, DIGE has the major advantage that both the control and experimental sample can be run in the same gel, reducing gel-to-gel variation and thus the number of technical replicate samples needed. DIGE was applied in Study IV and [³⁵S]methionine labelling in Studies II and III.

5.2.1 Efficient protein radiolabelling and 2D-PAGE for *Bifidobacterium longum*

Bifidobacteria have adapted to ecological niches rich in nutrients and they have been isolated from the intestine, the oral cavity, food, the insect gut, and sewage. Bifidobacteria need complex media to support their growth. The available semi-defined medium (SDM) supporting the growth of *B. infantis* (Perrin *et al.*, 2001) is rich in unlabelled methionine which would reduce the protein labelling efficiency with [³⁵S]methionine. A commercial methionine assay medium (MAM; Difco laboratories) containing 42 constituents proved to be a poor medium for *B. longum* when supplemented with 200 µg/ml of methionine. MAM was optimized with increased concentration of certain constituents and the presence of some new factors to make radiolabelling-based proteomic studies possible for *B. longum* 3A. The labelling efficiency of *B. longum* proteins was assessed by liquid scintillation counting and SDS-PAGE. The radiolabelled proteins could be detected after less than 4 hours of exposure to the imaging screen, indicating the applicability of the developed approaches to study the *de novo* protein synthesis rate in the *B. longum* 3A. The proteins whose synthesis rate was most strongly induced after heat increment of 10 °C (from 37°C to 47°C) were HtrA and DnaK (Figures 1 and 2 of II). The induction ratios of the synthesis rate of these proteins were over 70. The synthesis rate of eleven other proteins were also induced at least 2-fold by heat stress. HtrA synthesis rate was also induced over two-fold after bile salt stress, indicating that this protein is part of the defence mechanism against bile in *B. longum*.

Overall, while knowledge of the heat shock stimulon and its control in bifidobacteria is far from complete, several recent studies applying transcriptional and proteomic approaches have suggested that the global heat-shock response in bifidobacteria is organized into two classes, one responding to mild or moderate heat stress, and another responding to severe, almost lethal heat stress. These results indicate that the GroEL chaperonin machine and protease ClpCP are needed after a moderate heat increment of 5-12 °C (Rezzonico *et al.*, 2007; Ventura *et al.*, 2005a) while the DnaK system and ClpB chaperone are essential after a larger heat increment (≥ 13 °C) (Ventura *et al.*, AEM2004). This at least partial specialization of the HSPs according to the degree or the type of the stress has also been observed in other bacteria (Tomoyasu *et al.*, 2001; Frees *et al.*, 2004; Susin *et al.*, 2006).

5.2.2 Efficient protein radiolabelling and 2D-PAGE for several strains of the genus *Lactobacillus*

Media optimization for LAB is laborious and time-consuming, since LAB have adapted to habitats rich in protein and sugars with extensive loss of superfluous metabolic pathways and functions. Chemically-defined media were optimized for *Lactobacillus brevis* ATCC 8287, *Lactobacillus reuteri* SD 2112 and *Lactobacillus rhamnosus* E-97800 strains in order to develop experimental conditions for efficient protein radiolabelling and 2D-PAGE. The heat shock proteome of *Lactococcus lactis* (a control), *Lactobacillus brevis*, *Lactobacillus reuteri* and *Lactobacillus rhamnosus* strains was studied. As shown by 2-DE protein, several spots induced at least 10-fold from all of these LAB strains after a moderate heat shock demonstrating the efficiency of media optimization and the labelling procedure (Figure 9). These protein spots were identified to represent GroEL, DnaK and ClpATPase proteins by Western blotting using antibodies specific to these proteins (Figure 9). Previous studies on *L. lactis* have shown that the synthesis rates of DnaK and GroEL are induced 30- to 40-fold and the synthesis rates of ClpE and ClpB ATPases are induced 10- to 40-fold under heat-shock conditions in this bacterium (Kilstrup *et al.*, 1997; Ingmer *et al.*, 1999). In repeated 2D-PAGE analyses of *L. rhamnosus* and *L. reuteri*, some of the probable ClpATPases migrating with the pI and M_r values ranging from 5.2–6.4 to 70–85 kDa, respectively, constantly appeared as horizontal strings of spots in 2D-gels after applying heat stress indicating that these proteins undergo post-translational modifications. Future studies are needed to confirm these modifications and assess the putative biological relevance.

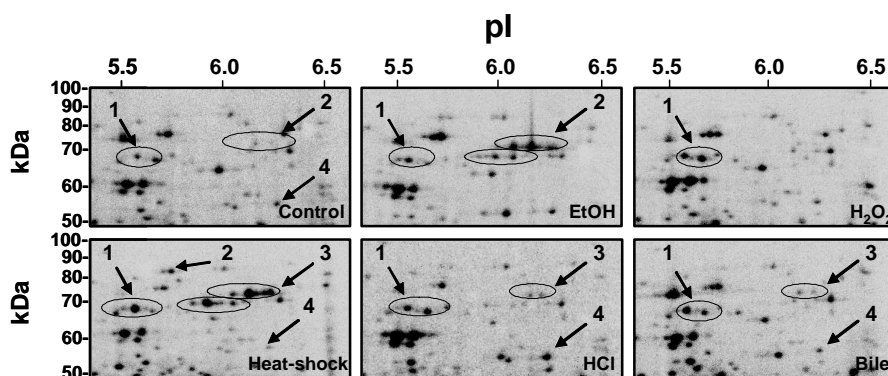


Figure 9. The relative synthesis rates of putative ClpATPases is increased in *L. rhamnosus* E-97800 during stress. Portions of miniscale 2D-gels of pulse-chase labelled proteins extracted from cells before and 20 min after a heat-stress, or 35 min (15 min preincubation with the chemical reagent followed by pulse-chase labelling of 20 min) after the addition of ethanol (7.5%), H₂O₂ (0.003%), HCl (50 mM) and bile (0.3%). Proteins circled and marked with an arrowhead refer to ClpATPase proteins. All 2D-gels were calibrated using 2-D SDS-PAGE standards (Bio-Rad).

5.2.3 DIGE analysis of *L. gasseri* ATCC 33323 heat shock proteome

One of the biggest disadvantages of 2-DE based methods is gel-to-gel variation. However, an elegant solution to this problem was developed by Unlu *et al.* (1997); which involves the detection of paired samples in the same gel to avoid gel-to-gel variation. At present, using different fluorescent dyes it is possible to identify and quantify the amounts of proteins before and after a stress condition. 2D-DIGE approach was applied to study heat shock response in *L. gasseri* ATCC 33323. A total of 20 protein spots showing increased levels after 30 min heat-shock were identified in *L. gasseri* ATCC 33323 (Table 4 and Figure 3 of IV). The most strongly induced proteins during heat shock according to 2D-DIGE in the pH range of 3-10 were the classical chaperones DnaK (4.4-fold induction) and GroEL (3.8-fold), HflX GTPase (4.4-fold), a pyrimidine operon attenuation protein (2.5-fold), an ATPase of the ABC-type polar amino acid transport system (2.1-fold), and ClpL ATPase (4.4-fold). The relative amounts of ClpE, ClpC, and ClpL in *L. gasseri* cells were notably (> 1.5-fold) increased under heat stress conditions most likely indicating their important role under these conditions. In contrast, our results revealed that the expression of ClpX is only slightly (< 1.5-fold) increased during heat shock. Published reports indicate that there are differences in the physiological role of ClpX among Firmicutes, since the deletion of *clpX* lowered the heat tolerance of *B. subtilis* (Gerth *et al.*, 1998), while *clpX* inactivation increased the heat tolerance of *S. aureus* (Frees *et al.*, 2003; 2004). The physiological role of ClpX in *L. gasseri* remains to be studied.

Very little is known about the HflX GTPase. HflX has been not identified as an HSP before. HflX belongs to the GTPases related Obg (orthologues characterized from Gram-negative bacteria are sometimes named CgtA) (Leipe *et al.*, 2002), and is characterized by a glycine-rich region near the N-terminus of its GTPase domain (Leipe *et al.*, 2002). Although the HflX family is almost universally conserved in all three superkingdoms (Caldon and March, 2003), its role in the regulation of cellular functions is largely unknown. Nine GTPases of the Era/Obg family are present in the *B. subtilis* genome, six of these being indispensable for the growth of the bacterium (Morimoto *et al.*, 2002). Moreover, an Obg is needed for stress activation of σ^B in *B. subtilis* (Scott and Haldenwang, 1999). Induction data of the present study might indicate that HflX is an essential regulator during stress in ATCC 33323. Indeed, the role(s) of HflX in cellular processes of a probiotic bacteria merits future work.

The pyrimidine synthesis regulator, PyrR, was one of the proteins found to be up-regulated during heat shock. The increased (2.5-fold) amount of PyrR could be part of the mechanism by which the cell slows down pyrimidine biosynthesis in response to the reduced replication rate under heat-shock conditions. The relative amount of protein annotated as the ATPase component (YP_814363) of the ABC-type polar amino acid transport system, was shown to increase (2.1-fold) during heat stress. The biological relevance of the increased expression of the ABC-type polar amino acid transporter by heat shock is not clear. However, it has been demonstrated in several intestinal microbes, including *E. coli* (De Biase *et al.* 1999) and *L. monocytogenes* (Cotter *et al.* 2001), that the accumulation of intracellular glutamate, a compatible solute, enhances the survival of these microbes during challenges such as like osmotic and acid stress.

The accumulation of compatible solutes has been reported to generate thermoprotection even without de novo protein synthesis (Caldas *et al.*, 1999). In addition, the relative amount of a betaine ABC transporter ATPase has been shown to increase during acid adaptation in *L. lactis* MG1363 (Budin-Verneuil *et al.*, 2005), and upon osmotic and heat stress in *L. rhamnosus* (Prasad *et al.*, 2003). Moreover, it has been shown that compatible solutes are able to stabilize proteins both *in vitro* (Lippert and Galinski, 1992) and *in vivo* (Diamant, *et al.*, 2001) against very low and high temperatures. Although compatible solute transporters have a physiological role during adaptation to stressfully high temperatures in *B. subtilis* (Holtmann and Bremer, 2004), it has been shown that the *hrcA* locus in *Bifidobacterium breve* UCC2003, which is a well-known gut inhabitant, is increasingly transcribed briefly after osmotic shock but not upon heat stress (Ventura *et al.*, 2005c). This possibly reflects the ecological niche of *B. breve* UCC2003, the mammalian gut, where the temperature remains constant but osmotic conditions fluctuate. Taken together, it is tempting to speculate that there is a link between osmotic and heat-shock responses in *L. gasseri* reflecting a need to adapt simultaneously to both changing osmotic conditions and temperature when it is ingested by an animal. Future studies will reveal whether the osmotic and heat-shock responses are somehow coordinated in ATCC 33323 and other LAB.

6 CONCLUSIONS AND FUTURE PROSPECTS

The huge potential of the probiotic bacteria is widely accepted, however, very little is known about the molecular mechanisms underlying the probiotic traits. Whilst virulence and stress responses are closely related in several Gram-positive bacteria, extremely little is known about the possible overlap of stress defence mechanisms and the probiotic nature of bacteria.

Ubiquitous HSP/100 Clp AAA+ ATPases are conserved in all kingdoms of life, and act as a substrate selector, thus playing a crucial regulative role in controlled proteolysis when they constitute a bipartite protease with ClpP peptidase. ClpATPases are known to regulate several vital biological processes in Gram-positive bacteria with a low G+C content including the starting of developmental programmes such as sporulation in *B. subtilis*. ClpATPases are also known as virulence factors in several pathogens. However, it is not known whether ClpATPases regulate adherence or other essential phenotypes in probiotic bacteria. Recently, it was shown that ClpC ATPase in *L. plantarum* is essential to the probiotic features in a mouse model. ClpC acts as a substrate selector in cellular protease with ClpP. However, some ClpATPases, like ClpL, are not known to take part of the proteolytic complex together with ClpP. Neither biological substrate(s) nor putative proteinaceous cofactor(s) of ClpL are known.

In the first part of this thesis, genes encoding *clpL* ATPase and their protein products were characterized in two probiotic lactobacilli, *L. rhamnosus* E-97800 and *L. gasseri* ATCC 33323. Practically nothing is known about ClpL's putative contribution to the probiotic character of bacteria. The ClpL encoding gene is not carried in the genomes of well-studied model bacteria such as *Escherichia coli*, *Bacillus subtilis*, and *Lactococcus lactis*, while it is essential for the virulence of *Streptococcus pneumoniae* and the survival of *Staphylococcus aureus* during severe heat stress.

It was observed that a *clpL* gene in *L. gasseri* is needed for the development of thermotolerance and that some LAB possess an extra copy of the *clpL* gene, assigned as *clpL2*. Expression of both *clpL1* and *clpL2* in *L. rhamnosus* E-97800 was induced after heat stress and *clpL2* was found to be mobilized in a stress-specific manner. While it was found that the *clpL2* gene has been a subject of horizontal gene transfer in LAB, the putative selective advantage of this gene to host bacteria remains to be studied. Gene products essential during stress, like ClpL, might have potential as genetic markers especially when antibiotic resistance encoding genetic markers cannot be used, i.e. with food supplies and functional food.

Recently, heat adaption was shown to improve the technological characteristics of *L. helveticus* including proteinase and peptidase activities during its propagation in cheese whey. It was demonstrated for *L. paracasei* that heat adapted cells showed increased tolerance against spray-drying, which otherwise cause a substantial loss of viability. However, Desmond and co-workers (2004) found only moderately increased stress tolerance after overproduction of the GroEL chaperone (up to 20% of the total cellular protein) during heat stress in *L. paracasei* and *L. lactis*. This fact might reflect the involvement of players other than the GroEL machinery during heat stress or the

cellular stress caused by a plasmid itself. Several hundred substrates of GroEL have been identified and moreover, GroEL modulates other stress-response regulators, as is the case for the HrcA regulator in *B. subtilis*, in *Helicobacter pylori* and most likely also in *Clostridium acetobutylicum*. Taken together, ClpL might be a more promising candidate than GroEL for improving the stress tolerance of an industrially relevant strain. Future studies are needed to investigate whether gene encoding the ClpL when transformed into a technologically-relevant lactobacilli is able to develop increased tolerance against the various stresses that the bacteria have to withstand. Moreover, the expression level of ClpL in a probiotic strain might be a good indicator whether the adaptation to technologically relevant conditions has been successful.

In the second part of this work, proteomic tools were applied to the investigation of probiotic bacteria. Probiotic bacteria have adapted to rich media and thus the optimization of growth in defined conditions that allow efficient metabolic labelling is laborious and time-consuming. 2-DE-based proteomics studies are extremely well suited to studying the stress responses and most likely adaption to GIT models or technologically relevant conditions. The growth media for these studies have been developed in this work for both probiotic bifidobacteria and lactobacilli. Moreover, these methods proved to have potential to study the stress responses of probiotic bacteria. In the future, the proteomic approaches will provide new insights into the probiotic nature of bacteria. Comparative functional genomic studies of phylogenically unrelated lactobacilli and bifidobacteria could possibly reveal whether there is a set of specific “probiotic factors”, shared between virtually all probiotic bacteria, analogous to virulence factors in pathogenic bacteria.

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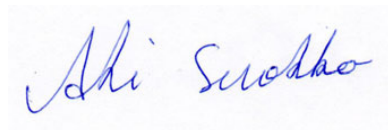
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