Survival strategies of *Listeria monocytogenes*

roles of regulators and transporters

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Survival strategies of *Listeria monocytogenes*

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General introduction

Introduction

The consumers' demand for fresh food products with extended shelf life that require a minimum effort for preparation has lead to the introduction and increased consumption of ready-to-eat, convenience foods. Many of these foods contain raw or minimally processed components, and therefore a risk exists of pathogenic bacteria surviving in these products. The increased popularity of ready-to-eat products throughout the world leads to larger production and consumption volumes and thus to a greater chance for health hazardous situations to occur.

Ready-to-eat foods rely heavily on refrigeration. Also, novel processing techniques, such as high hydrostatic pressure (HHP) treatment, are emerging. The application of combinations of mild preservation methods to foods (hurdle technology) is becoming more prevalent. It introduces sub-lethal stresses to bacteria which must be overcome to survive or grow in food products. However, the physiological mechanisms of micro-organisms adapting to sub-lethal stresses is not well understood. The food-borne human pathogen *Listeria monocytogenes* is known for its ability to survive a variety of environmental stresses, such as high osmotic pressures (NaCl concentrations up to 10%) and low temperatures, as low as -0.1°C (75). Many outbreaks caused by this organism have been associated with ready-to-eat foods, which may have undergone some form of minimal processing, or have been contaminated after processing.

The objective of the present research was to gain insight in the survival strategies of *L. monocytogenes* in relation to environmental conditions encountered during minimal processing. The literature survey below gives an introduction to *L. monocytogenes* and background information on the stress response of this organism relevant to food safety.

Listeria monocytogenes

L. monocytogenes is a gram-positive, non-sporeforming, facultative anaerobic bacterium, motile by means of flagella. It was first described by Murray *et al.* (48), who named it *Bacterium monocytogenes* because it infected the monocytes (white cells) of the blood. Later, in 1927, Pirie isolated the organism and named it *Listerella hepatolytica*, after the surgeon Joseph Lister. Since the name *Listerella* was already in use for a group of slime moulds, the name *Listeria monocytogenes* was agreed on (57). The genus Listeria comprises six species: *L. monocytogenes, L. innocua, L. seeligeri, L. ivanovii, L. welshimeri* and *L. grayi*. All of these species are widespread in the environment, and though both *L. monocytogenes* and *L. ivanovii* are animal pathogens, only *L. monocytogenes* is considered to be a significant human pathogen, and thus most important for food safety.

Food-borne transmission

Although Murray *et al.* (48) already described the oral route of infection for *L. monocytogenes* in 1926, it was not until 1983 with a description of an outbreak of listeriosis in Halifax, Canada, that *L. monocytogenes* was recognized as a food-borne pathogen. In this outbreak listeriosis was caused by contaminated coleslaw (Table 1). Food-relatedness of listeria infections was confirmed in the 1980s by the occurrence of several other outbreaks in the USA and Europe (Table 1, first six references). *L. monocytogenes* can often be isolated from soil, silage and other environmental sources and in food processing plants. Because of the ubiquitous nature of *L. monocytogenes*, the presence of the pathogen on raw food items seems unavoidable. Indeed, it has been isolated from all main categories of food, including milk and dairy products, meat and meat products, vegetables and seafood (21). The foods implicated in listeriosis cases are generally designed to be consumed without further cooking (i.e. ready-to-eat), and have extended shelf lives at refrigeration temperature, enabling *L. monocytogenes* to grow (Table 1).

Table 1. Examples of food-borne outbreaks due to *L. monocytogenes*.

Country	Year	No. of cases (deaths)	Food implicated	Reference
Canada	1981	41 (17)	Coleslaw	(63)
USA	1983	49 (14)	Pasteurized milk[a]	(24)
USA	1985	142 (48)	Mexican cheese	(45)
Switzerland	1983-1987	122 (31)	Soft cheese	(13)
USA	1986-1987	36 (16)	Ice cream, salami[a]	(64)
UK	1987-1989	> 300 (?)	Pâté	(46)
France	1992	279 (85)	Jellied pork tongue	(60)
Italy	1993	39 (0)	Rice salad	(62)
France	1995	20 (6)	Raw-milk soft cheese	(28)
Sweden	1994-1995	9 (2)	Rainbow trout	(71)
USA	2000	12 (5 stillbirths)	Mexican style fresh cheese	(15)
USA	2002	46 (10)	Turkey deli meat	(6)

[a] Indicates an epidemiological association only, without recovery of the implicated strain from the specific food item.

The dose response relation of *L. monocytogenes* for humans is not well known. The infective dose of food-borne pathogens depends on a number of variables including the condition of the host, the virulence of the strain, the type and amount of food consumed, and the concentration of the pathogen in the food. Most susceptible to listeriosis infection are the very young and elderly, pregnant women, people with other underlying conditions (e.g. diabetis, alcoholism, chronic liver disease, etc.), and people with a compromised immune system, e.g. patients receiving immunosuppressive therapy (after organ transplantation) and

AIDS patients (60). Also, the virulence (and thus the minimal infective dose) of *L. monocytogenes* varies among its serovars. Serovars 1/2a, 1/2b and 1/2c are predominantly recovered from food, and serovars 1/2a, 1/2b and 4b account for more than 90% of human and animal cases of listeriosis (74). Because of uncertainty in minimal infective dose of *L. monocytogenes*, the food laws of many countries have adopted a zero tolerance for *L. monocytogenes* in foods (not detectable in 25 g of food product). However, in The Netherlands and in some other European countries *L. monocytogenes* should not be detectable in 0.01 g of certain food products (fewer than 100 colony forming units per gram) up till the use-by date. This tolerance has an important impact for industry on *L. monocytogenes* detection; it allows the use of direct plating methods, delivering results within one or two days, compared to four to five days with enrichment procedures (10).

Pathogenesis

L. monocytogenes is able to invade numerous eukaryotic cells in different tissues, including normally non-phagocytic cells. The intracellular life cycle of *L. monocytogenes* has been worked out in detail (19). Following invasion of the eukaryotic cell and incorporation into a membrane-bound vacuole (using internalin A and B), the bacterium lyses this intracellular vacuolar compartment and escapes into the cytoplasm where it can multiply (Fig. 1).

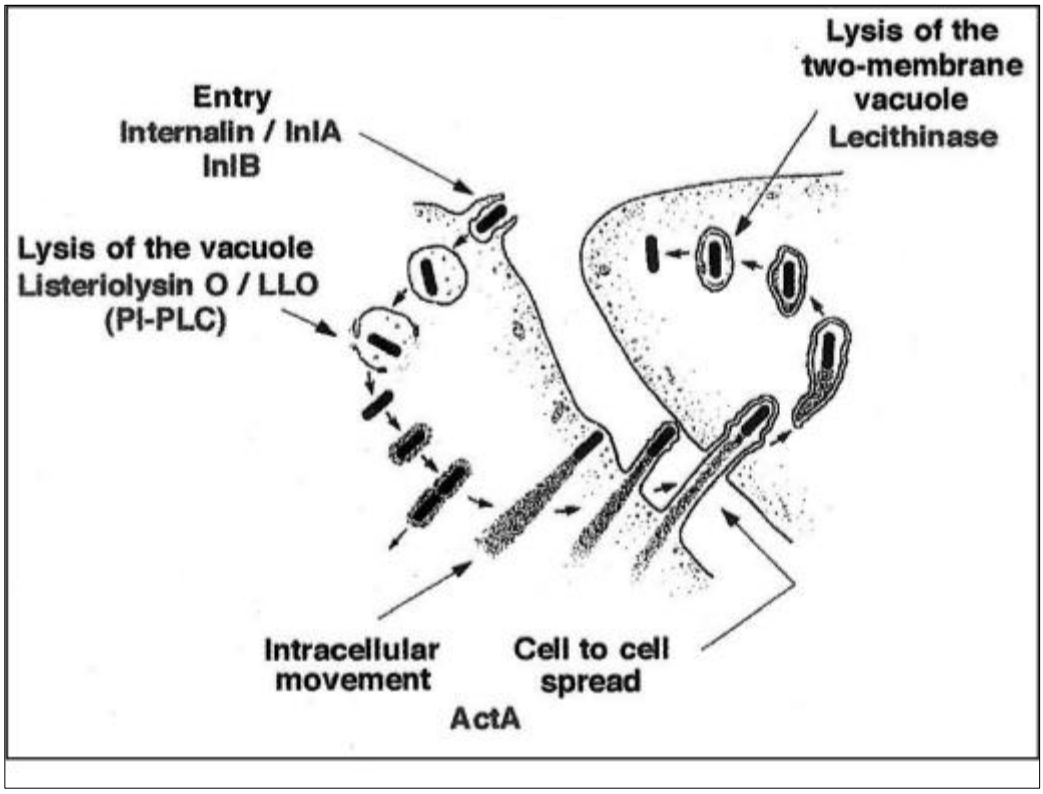

Figure 1. Schematic representation of cell invasion by *L. monocytogenes* and the function of virulence factors, adapted from Cossart *et al*. (19).

The escape from the vacuole is affected by two secreted bacterial proteins, listeriolysin O (LLO, encoded by *hly*) and a phophatidylinositol-specific phospholipase C (PI-PLC, the product of the *plcA* gene). This haemolytic activity is the cause of the halo's in blood-agar plates, often used in identification of *L. monocytogenes*. Then, actin filaments are accumulated (by means of ActA) and the bacterium is able to move within the cell and from cell to cell. After intrusion into a second cell, the resulting two membranes are lysed by LLO and a lecithinase (the phosphatidylcholine-specific phospholipase C (the product of the *plcB* gene)), resulting again in free, intracellular proliferating *L. monocytogenes*. The genes encoding these virulence factors are all clustered on a pathogenicity island (17). This island is not (completely) present in the genome of non-pathogenic species of Listeria (Fig. 2) (74).

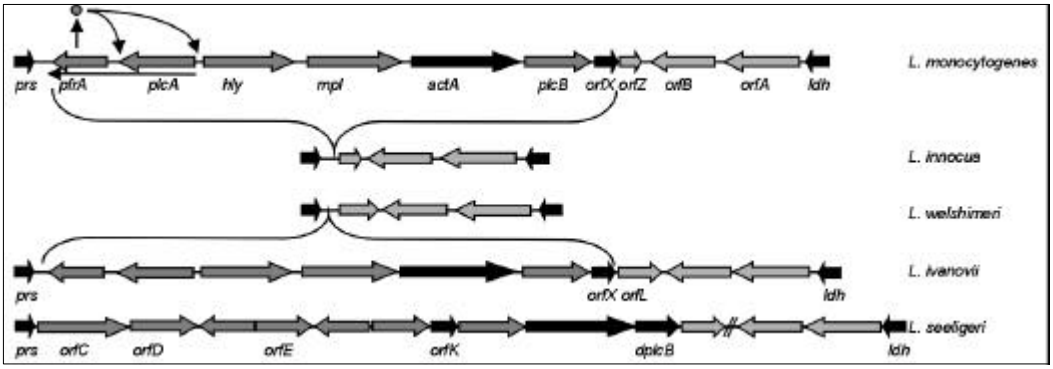

Figure 2. Genetic structure of the chromosomal region of the *hly* virulence gene cluster (LIPI-1) in *Listeria* spp (74).

As mentioned above, most susceptible to a listeriosis infection are the very young and elderly, pregnant women, people with a compromised immune system, and people with other underlying conditions (60). As *L. monocytogenes* can invade all kinds of cells, the symptoms do not only include gastrointestinal illness (found in healthy people), but also meningitis, septicaemia and meningo-encephalitis. For pregnant women, listeriosis can cause spontaneous abortion or stillbirth of their child, while they can experience only mild, flew-like symptoms.

Food safety and minimal processing

As consumers demand fresh food products with extended shelf life, food processing industry is using several preservation methods to satisfy this demand. Cold storage, mild heating, acidification, and increasing osmolarity of foods is widely used and relatively new processing techniques, such as high hydrostatic pressure (HHP) treatment, modified atmosphere and vacuum packaging, and the use of natural antimicrobial systems (bacteriocins), are emerging. The application of combinations of mild preservation methods to foods (hurdle technology (44)) is becoming more prevalent. It introduces several sub-lethal stresses to achieve a similar bacteriocidal effect as traditionally achieved by a single harsher

method. However, upon exposure to a specific sub-lethal stress micro-organisms might adapt, rendering less-susceptible bacteria for an additional insult of the same stress. Moreover, this stress adaptation may also include resistance to other stresses than that used to induce the response. To design optimal minimal processing methods, in which maximum safety is combined with the consumers' demand for fresh, wholesome food, it is essential to understand how bacteria respond to mild stresses (1, 37). A general response is the induction of the RNA-polymerase sigma factor σ^B, while specific responses include the uptake of osmolytes in high osmolarity environments and during low temperature growth, induction of amino acid decarboxylases (e.g. glutamate decarboxylase) and F_0F_1 ATPase upon acidification of the growth medium, and induction of sets of specific proteins, e.g. heat-shock proteins (HSPs) and cold-shock proteins (CSPs), upon high or low temperature, respectively. In the next paragraphs an overview is given of some important mechanisms used by *L. monocytogenes* to adapt to stresses imposed during food processing, including high hydrostatic pressure treatment (HHP). The adaptation mechanisms include the induction of the alternative sigma factor σ^B, the accumulation of osmolytes, and the induction of CSPs.

General stress response factor RNA-polymerase σ^B

Bacteria have evolved several strategies to survive or even adapt to adverse conditions in the environment, e.g. as encountered during food processing. The survival strategy of most bacteria involves entry into stationary phase. Stationary phase *Escherichia coli* cells show significant physiological changes conferring protection to several environmental insults (51). Additionally, in some gram-positive micro-organisms such as *Bacillus subtilis*, the survival strategy involves differentiation to spores.

For all bacteria adaptation to sudden adverse conditions in the environment requires the ability to respond rapidly. This involves activation of existing enzymes and transcription of genes resulting in enhanced levels of (stress-related) proteins. Initiation of transcription of mRNA from DNA is mediated by the holo-enzyme RNA-polymerase. The holo-enzyme consists of a core-enzyme and a sigma factor, in which the sigma factor is primarily responsible for transcription initiation; the recognition and binding of the polymerase to the promoter sequence in front of a gene. The main, or housekeeping sigma factor (σ^A in *B. subtilis* and σ^{70} in *E. coli*) is responsible for the recognition of the majority of promoters, transcribed during normal growth conditions. Alternative sigma factors recognize different promoter sequences and redirect transcription to, for example in *B. subtilis*, genes involved in heat-shock response, chemotaxis, sporulation, and general stress response (34).

In the genome sequence of *L. monocytogenes* EGD-e, five sigma factors have been found; *rpoD*, encoding σ^A, the housekeeping sigma factor, *sigH* and *sigL* encoding sigma-factors with unknown specific function, *sigV* of which the enzyme regulates ECF-genes

(extra-cytoplasmic function genes) and the alternative sigma factor, *sigB*, encoding σ^B which controls the transcription of genes involved in general stress response (79).

Using *sigB* null-mutation *L. monocytogenes* strains, a role for σ^B has been proposed in survival and growth during acid (23, 81), heat, oxidative (22) and low-temperature stress (9). Also, σ^B-regulated proteins seem to be involved in survival at HHP, as differences in survival clearly appear after pre-adaptation of wild type and *sigB* null mutation cells to low pH (79).

L. monocytogenes σ^B probably binds to promoters of genes with a –35 promoter sequence of GTTT and a –10 region of GGGTAA (9). In *L. monocytogenes* only few specific genes regulated by σ^B have been described. These include *opuCA*, the gene encoding the compatible solute uptake system OpuC, *Lmo1421*, encoding a putative osmolyte uptake system (26), and *gadB*, *C*, and *D*, encoding a glutamate/GABA antiporter (*gadC*) and two glutamate decarboxylase enzymes (*gadB* and *D*), involved in internal pH homeostasis by converting glutamate into γ–aminobutyrate (GABA) upon consumption of a proton (79). Using 2D-electrophoresis and a Δ*sigB* strain 9 proteins were identified in enhanced levels after exposure for 1 h to pH 4.5 in the wild type whereas they were not induced in the *sigB* mutant strain. These σ^B-regulated proteins involved in acid adaptation include Pfk (6-phosphofructokinase), GalE (UDP-glucose-4-epimerase), ClpP (the proteolytic subunit of the ATP-dependent Clp protease) and Lmo1580 (similar to unknown protein) (79).

Identification of σ^B-regulated proteins is of importance, as insight into σ^B-dependent proteins may elucidate a way to more effective hurdle technology. This is, as σ^B induces a specific set of proteins under one kind of stress, and, subsequently, cells are exposed to another, non-σ^B related stress, maximum reduction of viable cells can be achieved. Therefore, it is also important to know which environmental factors induce the transcription and translation of σ^B and subsequently induce σ^B-related genes. This has been examined for different stresses, the transcription of σ^B is induced upon exposure to e.g. low temperature, high osmolarity, heat and low pH (9), indicating a role for σ^B in cells adapting to these stresses. However, the regulation of the activity of the σ^B protein is complex. The *L. monocytogenes* σ^B operon comprises *rsbR, S, T, U, V, W, sigB* itself and *rsbX*, the encoded Rsb proteins are all involved in regulation of σ^B activity. Under normal growth conditions, RsbW binds with σ^B to form an inactive complex. However, upon exposure to stress, RsbU dephosphorylates RsbV, which is then able to bind to RsbW, rendering free σ^B. The other Rsb proteins, RsbR, S, T, and X presumably have an indirect effect (positive or negative) on the σ^B activity or gene expression.

The full impact of σ^B mode of action is not fully understood, though it is clear that this factor has an important role in regulation of the production of proteins involved in stress resistance of *L. monocytogenes*, and thus is a factor playing a major role in survival of *L. monocytogenes* in minimal processed foods. Next to more effective hurdle technology, insight into the mechanism of action of σ^B might also render possibilities to block stress sensing, regulation and the adaptation pathway.

Lowering water activity and osmoregulation

Lowering water activity (a_w) of foods by adding salt or sugar is one of the oldest preservation techniques in use. To control the growth of pathogens like *L. monocytogenes* in low and medium water activity foods it is of great importance to understand the underlying osmotic adaptation processes.

In bacteria, the intracellular concentration of solutes is high, resulting in an outward pressure on the cytoplasmic membrane. The cell wall prevents the membrane from rupturing, resulting in an outward pressure (acting on the cell wall), often referred to as turgor. Maintenance of turgor above a certain critical level is essential as it provides the mechanical force for expansion of the cell. Upon a hyperosmotic shock (an increase in medium osmolarity), the bacterium is faced with dehydration and a decrease in turgor. To restore turgor, bacteria raise the intracellular osmolarity via the accumulation of compatible solutes. In general compatible solutes are small, highly soluble molecules that carry no net charge at physiological pH. They are accumulated in the cell to maintain cell volume at elevated osmolarity, and may even stabilize protein and membrane structure and function (58, 61, 87).

In *L. monocytogenes*, Patchett *et al.* (53) identified increased concentrations of a number of potentially osmoprotective compounds in the cytoplasm following an osmotic upshock. Upon the addition of 7.5% NaCl, the internal potassium (K^+) concentration doubled (0.16 to 0.32 mM) and the concentration of glutamate was found to increase fivefold (52 to 281 mM). These data suggest that, as is the situation for the majority of bacteria, the accumulation of K^+ (and its counterion, glutamate) may constitute the primary response to hyperosmotic shock in *Listeria*. As these two components are no compatible solutes, i.e. they carry a net charge at physiological pH, the secondary response of *L. monocytogenes* to osmotic upshock is the accumulation of compatible osmoprotectants. The accumulation of the compatible solutes might be induced from the increase in charge within the cytoplasm, resulting from both the loss of water due to the hyperosmotic condition and the accumulated ions, as has been proposed for the gram-positive *Lactococcus lactis* (36).

The major accumulated components in salt-stressed *L. monocytogenes* are glycine betaine (trimethylglycine), L-carnitine (γ-*N*-trimethyl aminobutyrate), and the amino acid proline (Fig. 2) (11, 41, 53). In addition, certain peptides, specifically the proline-containing di- and tripeptides prolyl-hydroxy-proline (PHP) and prolyl-glycyl-glycine (PGG), can also function as osmoprotectants (3). Other organisms, like *E. coli*, *B. subtilis* and *Staphylococcus aureus*, are able to synthesize the major osmoprotectant betaine from exogenously supplied choline. The intracellular choline is converted to betaine by two enzymes, an alcohol dehydrogenase and a betaine aldehyde dehydrogenase. However, in *L. monocytogenes* no accumulated betaine has been found upon exogenously provided choline (Chapter 6).

The compatible solute glycine betaine is a plant metabolite and is therefore particularly present in food products of plant origin. Relatively high concentrations were found in sugar beets and other foods of plant origin (40 to 400 μmol/g [dry weight] under

natural or experimental saline or dry conditions) (59). However, the presence of betaine has also been demonstrated in meat products, in concentrations of about 0.4 nmol/mg (70). The compatible solute carnitine has a role in the beta-oxidation in mitochondria and is therefore ubiquitous in biological material (12). Its concentration varies from 0.2 nmol/mg for bologna to almost 1 nmol/mg in salami (70). Since the osmolarity of the gastrointestinal tract (equivalent to 0.3 M NaCl) is approximately twice that of the bloodstream, the presence of carnitine in mammals makes it the most readily available and thus possibly the most important osmolyte contributing to the survival of *L. monocytogenes* during infection (68, 80).

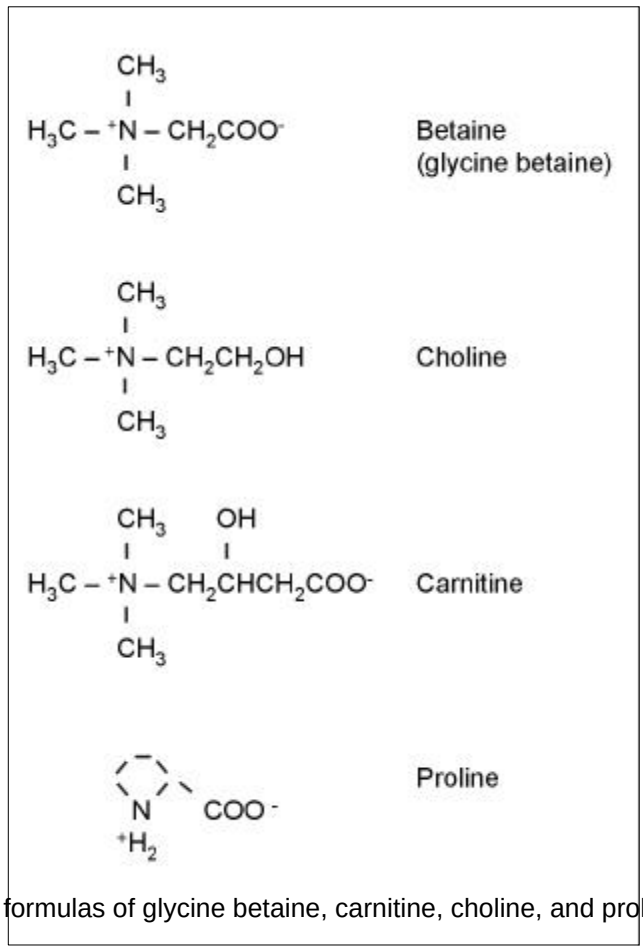

Figure 2. Structural formulas of glycine betaine, carnitine, choline, and proline.

In *L. monocytogenes*, the uptake of glycine betaine and carnitine is thought to be mediated *via* at least three osmolyte transporters: BetL, Gbu and OpuC (Fig. 3). BetL is a betaine transporter homologous to OpuD of *B. subtilis* and BetP of *Corynebacterium glutamicum* (66). These systems belong to a family of secondary transporters transporting an ion in symport with the osmolyte (27). The second transport system identified, Gbu, encoded by the *gbuABC* operon, belongs to the binding-protein-dependent ATP-binding cassette (ABC) superfamily of transporters and is homologous to OpuA in *B. subtilis* (40). An ATPase is encoded by *gbuA, gbuB* encodes a permease and *gbuC* a substrate binding protein. Membrane translocation of an osmolyte *via* Gbu is dependent on ATP hydrolysis. The third transporter, OpuC, is also a member of the ABC superfamily and is dedicated mainly to

carnitine uptake (25, 68). The *opuC* operon, encoding the OpuC transporter, is homologous to *opuC* and *opuB* of *B. subtilis*. As with the Gbu system, osmolyte uptake by OpuC is coupled to ATP hydrolysis.

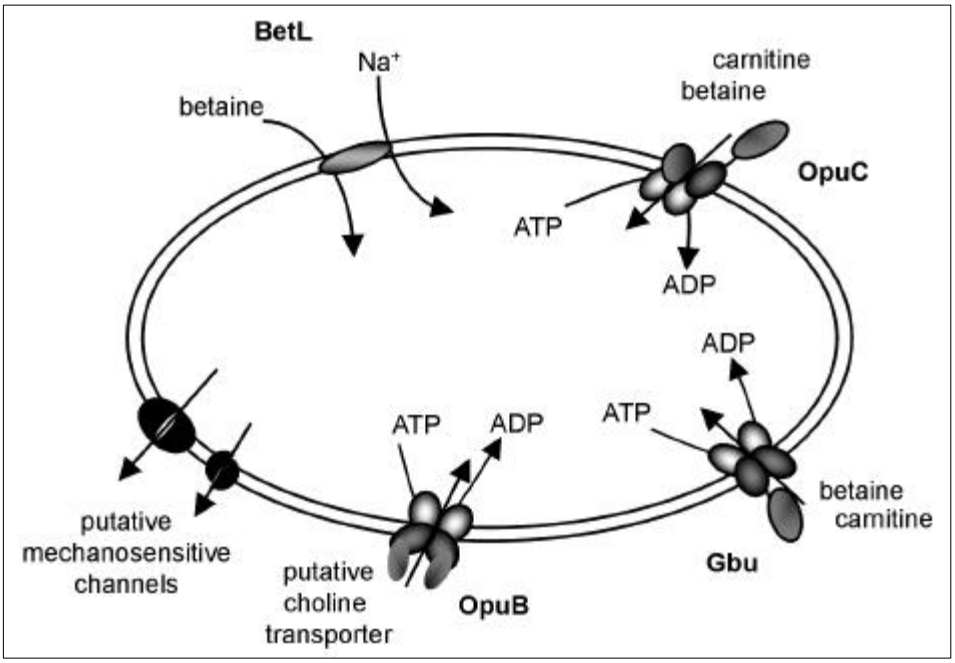

Figure 3. Schematic representation of transporters involved in the transport of osmolytes in *L. monocytogenes*.

Recently, a fourth system has been identified (67, 80, 78). This transporter, named OpuB, has homology to OpuB of *B. subtilis* (OpuBA shows 58% identity with OpuBA of *B. subtilis*, the permease and substrate-binding sub-units show 46% and 33% identity with OpuBB and OpuBC, respectively) and has the same unique protein structure as OpuA from *L. lactis* (36, Chapter 6). In *B. subtilis*, OpuB is a choline transporter. After transport into the *B. subtilis* cell, choline is subsequently converted into glycine betaine by two enzymes, an alcohol dehydrogenase GbsB and a glycine betaine aldehyde dehydrogenase GbsA. This transport of choline and subsequent conversion to betaine affords considerable osmoprotection to the cells (14). *B. subtilis* shares this ability to oxidize choline to glycine betaine for osmoprotective purposes with a number of gram-negative and gram-positive bacteria 20, 29, 42). Unlike in other organisms, choline is not converted into betaine in *L. monocytogenes*, though several genes exhibiting significant sequence similarities to *gbsA*: *lmo0913*, *lmo0383*, *lmo1179*, and with *gbsB*: *pduQ*, *lmo1634*, *lmo1166*, *lmo0554* were found in the genome (67). However, none of these genes are as tightly organized as *gbsA* and *gbsB* (separated by 44 basepairs) in *B. subtilis* (14). Using an *opuB* deletion mutant it was found that OpuB appears to play a minor role in low temperature and high osmolarity growth. Interestingly, it does seem to play a crucial role in virulence of *L. monocytogenes* LO28 (Chapter 6).

Mechanisms that allow low-temperature growth

Together with a large variety of foods, ready-to-eat foods rely heavily on refrigeration as preservation technique. A disadvantage of this method is that certain pathogens, like *L. monocytogenes, Yersinia enterocolitica, Bacillus cereus* and non-proteolytic *Clostridium botulinum*, are able to proliferate at refrigeration temperatures. Insight into the mechanisms allowing low-temperature growth is essential to understand and possibly control growth at low temperature of psychrotrophic pathogens and spoilage organisms. These mechanisms involve the maintenance of the cellular membrane fluidity, the uptake or synthesis of compatible solutes, and structure stabilization of macromolecules such as ribosomes, necessary for continued protein synthesis (1, 37). For *L. monocytogenes*, the adaptation of membranes to low temperature is accomplished by altering the branching in the methyl end of the fatty acid from iso to anteiso, and by shortening of the fatty acid chain length, resulting mainly in an increase of anteiso C15:0 fatty acids (5). These adaptations result in a more fluid membrane, compensating for the effects of low temperature, in which the membrane forms a crystalline structure.

Transport of the principal osmolytes glycine betaine and carnitine upon low temperature stress was found to be increased 15- and 4.5-fold, respectively (4, 41). In high salt environments, compatible solutes serve to balance the osmotic disturbance. However, their function at low temperature is largely unknown, although some functions have been proposed: they may serve as stabilizers of enzyme function and membrane bilayers (87).

Upon exposure of bacteria to low temperature, the synthesis of most proteins is blocked, possibly due to a cold-sensitive block in initiation of translation, resulting in a decrease in polysomes and an increase in 70S monosomes and ribosomal subunits. It is therefore proposed that the ribosome might be the temperature sensor in bacteria (73). Also in *L. monocytogenes* an instable 70S ribosomal particle structure is found upon cold shock (8), which must be restored to allow normal protein synthesis. Though upon cold shock the synthesis of most proteins is blocked, transcription of a specific set of genes is induced. By using proteomics a lot of these proteins have been identified. They are called cold-induced proteins (CIPs) and may vary from approximately 10 for *L. monocytogenes*, 18 for *E. coli*, 22 for *L. lactis* to 37 for *B. subtilis* (1 [review]). Among the CIPs a specific group of proteins is induced, called the cold-shock proteins (CSPs). CSPs are small (around 7 kDa), mainly acidic proteins highly induced upon a cold shock. They share a high degree of sequence similarity (>45%) in a variety of gram-positive and gram-negative bacteria (85). For most bacterial species, families of CSPs consisting of two to nine members have been found. The translation of *csp* mRNA upon a cold shock is possible because of the presence of a DB (downstream box) element on the mRNA ensuring additional binding to the impaired ribosome (47).

In the genome of *L. monocytogenes* EGD-e, the strain of which the DNA has been sequenced, three *csp* genes can be recovered, *cspB*, *D* and *L*. However, in *L. monocytogenes* LO28 four putative cold-shock proteins have been identified using western blotting, two were

shown to be produced in increased amounts following a cold shock from 30 to 10°C (77). The most extensively studied CSPs are $CspA^E$ of *E. coli* and $CspB^B$ of *B. subtilis* (30, 86). Several functions for CSPs have been postulated, mainly based on research on these two CSPs. Firstly, CSPs contain regions that are highly homologous to the cold-shock domain of eukaryotic DNA-binding proteins that are known to act as transcription factors. Both $CspA^E$ and $CspB^B$ are able to bind to single-stranded DNA with a specific motif, thereby regulating gene expression (33, 49). Interestingly, it has been shown that $CspA^E$ acts as a transcriptional activator of at least two genes encoding CIPs, possibly by stabilization of the open complex formation by RNA polymerase (16). Both proteins (GyrA and H-NS) are involved in DNA supercoiling, which is significantly modified at low temperature. Another finding supporting the theory that $CspB^B$ functions as a regulatory protein is that heterologous expression of $CspB^B$ in *E. coli* induced a protein pattern that strongly resembled the protein pattern upon cold shock (32). Secondly, CSPs are considered to be RNA-binding proteins (38). They can act as RNA chaperones, thereby minimizing the secondary folding of mRNA at low temperature. By this action, CSPs facilitate the initiation of translation in which the RNA should be in a linear form. Thirdly, CSPs may function as transcription anti-terminators. At cold-shock of *E. coli*, expression of the promoter distal genes of the *metY-rpsO* operon, *nusA*, *infB*, *rbfA*, and *pnp*, is increased. This implies that the efficiency of the transcription termination on the internal ρ-independent terminator is reduced by $CspE^E$ by preventing the formation of the stem-loop structure. The proteins encoded by these genes (NusA, IF2, RbfA, and PNP, respectively) are known to be induced at cold-shock and presumably enable the cells to adapt to low temperature (7, 55, 56). Finally, CSPs have been suggested to act as anti-freeze proteins. For both *B. subtilis* and *L. lactis* a freeze-sensitive phenotype was found upon deletion of *csp* genes (82, 83). Moreover, increased survival against freezing was found in a *L. lactis* strain overproducing CSPs (84). Stabilization of the RNA and DNA during the freezing process might be the mechanism through which the CSPs perform this function.

High hydrostatic pressure (HHP) treatment of foods

At the surface of the earth the ambient pressure is 0.1 MPa, while at the deepest point in the oceans the pressure is about 100 MPa, and at the center of the earth about 360 GPa (52). The term “high hydrostatic pressure” (HHP) in food applications represents pressures in the range of 50 to 1000 MPa. HHP may be used as a non-thermal processing technique to destroy food-borne micro-organisms and enhance the safety and shelf life of food. HHP treated foods have been commercially available since the ’90s in Japan and since 1996 in Europe and the USA. It is an emerging technology that is being developed and applied as a minimal process for the production of safe and nutritious foods. In general, HHP processing does not have adverse effects on small molecules in a food, such as vitamins, minerals, flavours and colours. In many cases, HHP can replace more conventional methods such as additives and/or high

temperature treatments. Products that can be treated with HHP include packed liquid or solid food in water suspension and unpacked liquid food in a closed HHP treatment system. HHP treatment exposes the product to a pressure between 100 MPa and 800 MPa for a certain period of time, at a desirable temperature. A phenomenon involved in HHP treatment is a decrease in pH, since the dissociation constants (pK_a values) of water and weak acids or weak bases depend on the absolute value of pressure. Because the pK_a values of phosphate and carboxylic acids are decreased by pressure, the pH decreases during high-pressure treatment. This may influence the survival of the bacteria in the processed foods. Growth of micro-organisms is generally inhibited at pressures in the range of 20 to 130 MPa, while higher pressures of between 130 and 800 MPa may result in cell death. HHP affects a combination of processes that take place in the cell, and does not inhibit or disrupt only one specific function. In general it disrupts the ionic, hydrophobic and H-bonds of the molecules without affecting the covalent bonds.

Several mechanisms are suggested to play a role in microbial inactivation by HHP. The cell membrane plays an important role in survival upon HHP treatment. Hydrostatic pressure induces a phase transition of the cytoplasmic membrane from the physiological liquid-crystalline to the gel phase, characterized by an increased rigidity and reduced conformational degrees of freedom for the acyl chains. This increases the membrane permeability and induces leakage of sodium and calcium ions (69, 72). In general, less fluid membranes are more sensitive to HHP. Differential scanning calorimetry allowed the detection of in vivo changes in ribosome conformation upon pressurization (50). Indeed, cell death upon exposure to high pressure was associated with ribosome damage. Additionally, Graumann and Marahiel (31) proposed that the partial inactivation of ribosomes at low temperature is the main trigger for the induction of CSPs. Indeed, enhanced levels of CSPs were not only found at low temperature, also upon pressurization (76, 77). This indicates that CSPs might have a role in adaptation to both stresses. Hydrostatic pressure may also affect the intracellular pH of microorganisms by enhancing the dissociation of weak organic acids, increasing the permeability of the cytoplasmic membrane and inactivation of enzymes required for pH homeostasis. Furthermore, proteins can be irreversibly denatured above a certain pressure threshold, mainly because of distortion of their tertiary and quaternary structure. Upon decompression the molecules refold, but in a manner different from the original configuration (18).

The effectiveness of HHP treatment on the inactivation of vegetative bacteria (spores are highly pressure-resistant) is dependent on the organism tested, growth phase, pressurization time, temperature and pH (a. o. 2, 43, 54). As a whole, gram-positive bacteria are more piezotolerant than gram-negative bacteria, probably as a result of their more robust cell envelope (containing a high percentage of peptidoglycan and teichoic acids). The variation in pressure resistance of some pathogenic bacterial strains to HHP could be the reason for variation in results obtained by researchers using different strains of the same species. For example, *L. monocytogenes* CA was more pressure resistant than *L.*

monocytogenes ScottA and *L. monocytogenes* NCTC 11994 was relatively the most pressure resistant of the three strains tested (65). Moreover, variance of pressure resistance can even exist within a strain (35, 39).

The variability in HHP resistance of bacteria of different species, strains, after pre-treatment, etc., makes the task of designing HHP treatments in minimally processing that cause adequate reductions of bacteria challenging, and insight in the underlying resistance mechanisms is therefore desirable.

Thesis outline

As minimally processed foods are becoming more and more popular, it is essential to understand the physiology of pathogens such as *L. monocytogenes*. With this knowledge, minimal processing regimes that result in the production of safe food products can be designed. The aim of the work described in this thesis was to gain insight in the survival strategies of *L. monocytogenes* in relation to environmental conditions encountered during minimal processing. Research focussed on two types of regulators; the alternative sigma factor σ^B, whose role has been assessed in acid adaptation, and the so-called cold-shock proteins (CSPs), of which the expression is enhanced at low temperature and during high pressure exposure. Also, the uptake of osmolytes via the osmolyte transporters BetL, Gbu, OpuC, and the putative choline transporter OpuB, is analysed in adaptation processes in high osmolarity and low temperature environments.

Chapter 2 reports the identification of four CSPs in *L. monocytogenes* LO28. Protein levels of two (Csp1 and Csp2) are shown to be enhanced upon a cold shock. Also, levels of two CSPs (Csp1 and Csp3) are induced after HHP treatment. Strikingly, survival after pressurization of cold-shocked cells was 100-fold higher compared to that of exponentially growing cells. These data imply cross protection of cold shocked cells to HHP exposure, which may affect the efficiency of combined preservation techniques**.**

Chapter 3 focuses on the role of the alternative sigma factor σ^B in acid adaptation, HHP and freeze-thaw survival. Using two-dimensional gel-electrophoresis and Maldi-TOF σ^B-dependent proteins are identified.

Chapter 4 describes the role of the osmolyte transporters BetL, Gbu, and OpuC in growth at high osmolarity using single, double and triple mutants. Of these three systems Gbu is the major osmolyte transporter during growth in hyperosmotic conditions, whereas the carnitine transporter OpuC is most important during infection.

Chapter 5 focuses on the role of the osmolyte transporters during growth at low temperature.

Chapter 6 describes the fourth osmolyte transporter, OpuB. Using an *opuB* deletion mutant and a quadruple mutant (ΔbetL, ΔopuC, Δgbu, ΔopuB), it was found that OpuB plays

no role in low temperature and high osmolarity growth. Additionally, the crucial role of this transporter in virulence of *L. monocytogenes* LO28 is discussed.

Chapter 7 describes and characterizes the di- and tripeptide transporter DtpT and assesses its role in growth in several environments and environmental conditions as well as its role in the pathogenesis of *L. monocytogenes*.

In Chapter 8 the findings presented in this work in relation to the role of the regulators and transporters in the growth and survival of *L. monocytogenes* under stress conditions are integrated and discussed.

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Chapter 2

Enhanced levels of cold-shock proteins in *Listeria monocytogenes* LO28 upon exposure to low temperature and high hydrostatic pressure

Summary

Listeria monocytogenes is a psychrotrophic food-borne pathogen that is problematic for the food industry because of its ubiquitous distribution in nature and its ability to grow at low temperatures and in the presence of high salt concentrations. Here we demonstrate that the process of adaptation to low temperature after cold shock includes elevated levels of cold-shock proteins (CSPs) and that the levels of CSPs are also elevated after treatment with high hydrostatic pressure (HHP). Two-dimensional gel electrophoresis combined with Western-blotting performed with anti-CspB of *Bacillus subtilis* was used to identify four 7-kDa proteins, designated Csp1, Csp2, Csp3 and Csp4. In addition, Southern blotting revealed four chromosomal DNA fragments that reacted with a *csp* probe, which also indicated that a CSP family is present in *L. monocytogenes* LO28. After a cold shock in which the temperature was decreased from 37°C to 10°C the levels of Csp1 and Csp3 increased 10- and 3.5-fold, respectively, but the levels of Csp2 and Csp4 were not elevated. Pressurization of *L. monocytogenes* LO28 cells resulted in 3.5- and 2-fold increases in the levels of Csp1 and Csp2, respectively. Strikingly, the level of survival after pressurization of cold-shocked cells was 100-fold higher than that of cells growing exponentially at 37°C. These findings imply that cold-shocked cells are protected from HHP treatment, which may affect the efficiency of combined preservation techniques.

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Introduction

As the demand for fresh food products with extended shelf lives is increasing, the food processing industry is using novel preservation methods to satisfy this demand. Cold storage is widely used and new processing techniques, such as high hydrostatic pressure (HHP) treatment, are emerging. The application of combinations of mild preservation methods to foods (hurdle technology) is becoming more prevalent. It introduces sub-lethal stresses to bacteria which must be overcome to survive or grow in food products. However, the physiological mechanism of micro-organisms adapting to sub-lethal stresses is not well understood. The food-borne human pathogen *Listeria monocytogenes* is known for its ability to survive a variety of environmental stresses, such as high osmotic pressures (NaCl concentrations up to 10%) and low temperatures, as low as -0.1°C (30). It can cause listeriosis in immuno-compromised individuals and accounts for almost 35% of all deaths in the USA due to known foodborne bacterial pathogens (19). Many outbreaks have been associated with ready-to-eat foods which may have undergone some form of minimal processing.

Mechanisms that allow low-temperature growth of micro-organisms involve the maintenance of the cellular membrane fluidity, the uptake or synthesis of compatible solutes, the maintenance of the structure of macromolecules, such as ribosomes, and maintenance of protein synthesis. For *L. monocytogenes*, the adaptation of membranes to low temperature is accomplished by altering the branching in the methyl end of the fatty acid from iso to anteiso and by shortening of the fatty acid chain length, mainly resulting in an increase of anteiso C15:0 fatty acids (1). The osmolytes betaine and carnitine are accumulated and stimulate growth at low temperature (5, 13, 17). In *L. monocytogenes* instability in the 70S ribosomal particle structure is found upon cold shock (4), which must be overcome for normal protein synthesis. In *Escherichia coli*, protein synthesis after cold shock is related to the synthesis of so-called cold-shock proteins (CSPs) (23). However, for *L. monocytogenes* production of these proteins has never been monitored, although *csp* genes have been described in two strains. Two *csp* genes were identified in the *L. monocytogenes* ATCC23074 genome using universal *csp* gene primers (11). For *L. monocytogenes* EGD two *csp* genes have been sequenced (E. M. Busch and T. Chakraborty, unpublished. Accession numbers LMO012349 and LMO012350).

CSPs are 7 kDa proteins of which families have been found in nearly all bacteria (10, 14, 23, 34). Several functions have been postulated for CSPs. They can act as RNA chaperones by which they minimize secondary folding (14, 15). They may also act as transcription activators or anti-terminators, thereby stimulating the production of non 7-kDa cold-induced proteins (CIPs) (2, 8, 18). Furthermore, a role of CSPs in protection to freezing has been suggested (33). CSPs are not only induced at low temperature but they are also induced by other environmental stresses. CspA, the major cold-shock protein in *E. coli*, was also found to be expressed on exit from stationary phase (9). Furthermore, of the nine CSPs found in *E. coli*, five were not found to be induced at low temperature. CspD, for example, is

induced upon nutritional stress and at stationary growth phase (35). *Bacillus subtilis* possesses three CSPs, CspB, C, and D, and upon cold shock all three CSPs are induced. However, CspB and CspC are also induced during stationary phase conditions (16).

Currently, the possible application of high pressure technology for the mild preservation of food products is being explored. The main advantage of this technology, which is proposed as a valuable alternative to conventional heat treatments, is that it preserves the characteristics of the initial product (vitamins and flavor). The inactivation of *L. monocytogenes* by high pressure has been described in a number of communications (22, 28). However, the physiological response of microorganisms to sub-lethal pressures is not well understood. It is proposed that HHP treatments target cellular membranes and ribosomes, the latter adopting a less stable conformation upon pressurization (20, 25). In *E. coli* the production of CspA is induced after elevated hydrostatic pressure treatment, as well as the production of certain CIPs and heat-shock proteins (31). Whenever mild preservation methods are combined, it is essential to know if bacterial adaptive responses to a stress condition (e.g. low temperature) can affect the susceptibility of these bacteria to other stress conditions. Here we demonstrate that the adaptation process of *L. monocytogenes* LO28 to low temperature includes the induction of CSPs and that the induction of CSPs is also apparent at HHP treatment. We found that the survival after pressurization of cold-shocked cells was higher than the survival of pressurized exponentially growing cells at 37°C, which may have implications for the efficiency of combined preservation techniques.

Materials and methods

Bacterial strains and growth conditions. *Listeria monocytogenes* LO28 (Serotype 1/2c) was grown in brain heart infusion broth (BHI) at 37°C. Cell growth was monitored spectrophotometrically by measuring the optical density at 620 nm (OD_{620}). At OD_{620} = 0.4 a cold shock to 10°C was performed.

Cold adaptation and freeze survival. To study the effect of cold shock on freeze survival of *L. monocytogenes* a freeze-thaw challenge with aliquots taken before and 1, 2, 3, 4 and 20 h after cold shock was performed. Samples (10 ml in 50 ml tubes) were withdrawn, concentrated using a centrifuge (15 min at 3000 g), suspended in fresh medium and the number of colony forming units was determined after which the samples were frozen at exactly -20°C for 20 h. After this freezing period the samples were thawed for 4 min at 37°C in a water bath and the number of colony forming units was determined. Furthermore, after thawing, growth at 37°C was monitored by measuring OD_{620}.

Protein extraction. Samples of 10 ml bacterial suspension were taken, centrifuged and suspended in water to an OD_{620} of 10. Total cellular proteins were extracted from the cells with a MSK cell homogenizer (B. Braun Biotech International, Germany) and zirconium beads (0.1 mm, Biospec Products, USA) by 8 treatments for 1 min (cooled on ice between

treatments). Thereafter the zirconium beads were allowed to sediment by gravity and subsequently the supernatant, containing the cellular proteins, was analyzed by two-dimensional gel-electrophoresis.

Protein analysis using two-dimensional gel electrophoresis (2D-E) and one-dimensional SDS-PAGE. 2D-E was essentially performed as described previously (21, 33) using a Pharmacia 2D-E system (Pharmacia Biotech, Sweden). Prior to loading the samples on the iso-electric focusing (IEF) gel, 12.5 µl of protein solution (40 µg protein) was treated with 12.5 µl lysis solution (9 M urea, 2% 2-mercaptoethanol, 2% IPG buffer 3-10L (Pharmacia Biotech, Sweden), 2% Triton X-100 and 6 mM pefabloc® SC (Merck, Germany)). This was put on ice and 25 µl of sample solution (8 M urea, 2% 2-mercaptoethanol, 2% IPG buffer 3-10L, 0.5% Triton X-100, few grains of bromophenolblue) was added. The total volume (50 µl) was loaded on the acidic end of the first dimensional IEF gel with iso-electric point (pI) ranging from 3 to 10 (Immobiline Dry strips, Pharmacia Biotech, Sweden). IEF gels with pI ranging from 4.0 to 5.0 were loaded with 100 µg protein and the lysis buffer and the sample solution were made with IPG buffer 3.5-5.0L. Both IEF gels were linear according to the instructions by the manufacturer (Pharmacia Biotech, Sweden). For the second dimension, 15% homogeneous SDS-PAGE gels or 12–14% SDS-PAGE gels (ExcelGel, Pharmacia Biotech, Sweden) were used. A high molecular weight marker (Pharmacia Biotech, Sweden) was used with band sizes of 97, 66, 45, 30, 20.1 and 14.4 kDa and a low molecular weight marker was used with band sizes of 16.9, 14.4, 10.7, 8.2, 6.2 and 2.5 kDa (Pharmacia Biotech, Sweden). The gels were silver stained according to Blum *et al*. (7) and were analyzed using PDQuest software (BioRad, Richmond, USA). Representative gels of duplo experiments are depicted in the figures. Standardization of the gels was achieved by calculating the intensity of each spot as a percentage of the total intensity of the spots visualized on a gel and, subsequently, induction factors were calculated. One-dimensional tricine-SDS-PAGE for the separation of low molecular weight proteins was performed as described by Schägger and Von Jagow (27).

Immunoblotting with anti-CspB. 500 µg of total protein was loaded on a 2D-E gel (and 30 µg on a one-dimensional gel) for immunoblotting with rabbit anti-CspB antibody of *B. subtilis*, kindly provided by Dr. P. Graumann (Philipps-Universität Marburg, Germany). The proteins were blotted on a nitrocellulose membrane (BioRad) using a transfer buffer (25mM Tris, 192 mM glycine, 20% methanol, pH 8.3) and incubated overnight with the anti-CspB antibody. The membrane was treated with goat-anti-rabbit solution (BioRad) and colored with DAB/H_2O_2.

Southern blot hybridization. Chromosomal DNA of *L. monocytogenes* was isolated as described previously (29). Restriction enzyme *Pst*I was purchased from GIBCO/BRL Life Technologies, New England Biolabs. Radiolabeling of PCR products, agarose gel electrophoresis and Southern-blot hybridizations were performed according to established procedures (26). PCR reagents (Taq polymerase and deoxynucleoside triphosphates dNTPs) were purchased from Boehringer GmbH (Mannheim, FRG) and used according to the

manufacturer's instruction. Using universal *csp* gene primers (12) and PCR a product carrying a partial *csp* gene was obtained. This product was sequenced with an ALF automatic sequencer (Pharmacia Biotech) in combination with an AutoRead sequencing kit (Pharmacia Biotech) with fluorescein-labeled primers. Radiolabeled PCR product was used as a probe in Southern hybridization. Stringency conditions were uses as follows: 4 min at 65°C with 5 x SSC with 0.1% SDS, followed by washing with 2 x SSC with 0.1% SDS (4 min at 65°C) and 0.5 x SSC with 0.1% SDS (3 min at 65°C).

High hydrostatic pressure treatment. 30 ml suspensions of exponentially growing *L. monocytogenes* (OD_{620} = 0.4) were packed in plastic bags. Samples were pressurized for 10 min at 50, 100 or 200 MPa at 30°C in an HHP apparatus (Resato, Roden, The Netherlands). Depressurization was achieved within seconds and total cellular proteins were extracted and analysed by 2D-E. Growth of the cells after the pressurization was examined by measuring OD_{620} at 37°C. The survival after high pressure treatments, with pressurization for 20 minutes at 200, 250, 300 and 350 MPa, was determined for exponentially growing cells and cells cold shocked to 10°C.

Results

Growth after cold shock and freeze survival. Growth of *L. monocytogenes* LO28 was determined after cold shock from 37°C to 10°C. *L. monocytogenes* LO28 grew in BHI at 37°C at a growth rate of $\mu = 0.6\ h^{-1}$. When exponentially growing cells were cold shocked to 10°C, an acclimation phase of about 3 h occurred before growth resumed with a growth rate of about $0.2\ h^{-1}$ (Fig. 1).

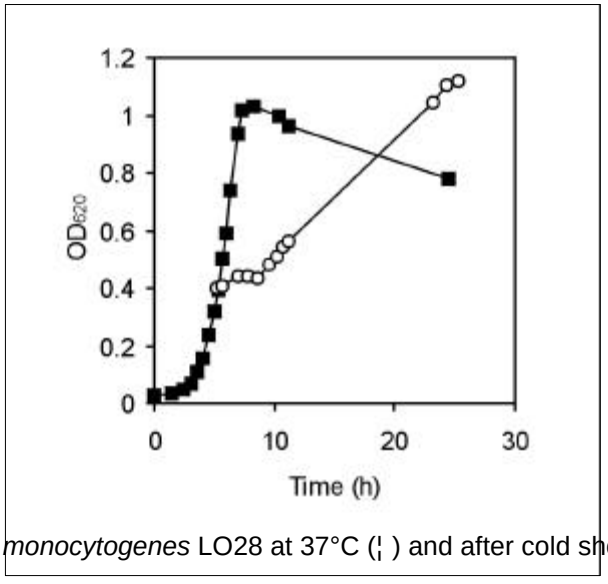

Figure 1. Growth of *L. monocytogenes* LO28 at 37°C (¦) and after cold shock to 10°C (?).

Since low temperature processing of foods is frequently followed by freezing, 4 h cold-shocked cells (10°C) were subjected to freezing. Exponentially growing *L.*

monocytogenes LO28 cells (37°C) showed about 50% survival after freezing. However, when cells were cold shocked for 4 h at 10°C before freezing, the survival after freezing increased to 90%. Growth at 37°C after thawing of *L. monocytogenes* cells resumed after a lag period of about two hours. However, after thawing of cold-shocked cells growth at 37°C resumed without a lag period (data not shown). This indicates that cell-history is an important parameter for the survival after freezing and subsequent growth.

Identification and production of CSPs. To study the production of CSPs in *L. monocytogenes* one-dimensional gel electrophoresis was performed upon cold shock. Two hours after cold shock enhanced levels of 7 kDa proteins in *L. monocytogenes* were found (Fig. 2A), increasing even further upon growth at 10°C. A Western immunoblot, using anti-CspB of *B. subtilis*, indicated the increased presence of CSPs upon cold shock. The level was increased 1.5-fold 2 h after cold shock, increasing to almost 4-fold after 20 h at 10°C. In stationary phase at 37°C CSP levels were not elevated.

To study the presence of CSPs in more detail, we performed 2D-E with proteins extracted from cells in exponential phase and from cells cold shocked for 4 h (cells in early exponential phase). For the first dimension, a pI range of 3 to 10 was used. Increased production levels of 7 kDa proteins were observed in the acidic region of the gel (Fig. 2B) and a Western immunoblot indicated the presence of 4 putative CSPs in this region (data not shown). Six other proteins were found to have at least 3-fold increased levels upon cold shock (Fig. 2B). To further characterize CSPs from *L. monocytogenes* LO28, 2D-E with a pI range of 4 to 5 was performed (Fig. 3). This was followed by Western immunoblotting, using anti-CspB of *B. subtilis* which is known to react with all CSPs in *B. subtilis* (15). In this way four spots were visualized, that were named Csp1, Csp2, Csp3 and Csp4 (Fig. 3). Based on the migration on 2D-E gels, the pI values for Csp1, 2, 3 and 4 are 4.32, 4.35, 4.43 and 4.51 and their molecular weights are 7.4, 8.0, 7.0 and 7.6 kDa, respectively. The derived proteins encoded by the *cspL* and *cspLB* genes from *L. monocytogenes* EGD (E. M. Busch and T. Chakraborty, unpublished. Accession numbers LMO012349 and LMO012350, respectively) reveal calculated iso-electric points of 4.32 and 4.35 and molecular weights of 7.3 and 7.5, respectively. These calculated pI-values and molecular weights match with the observed values for Csp1 and Csp2 of LO28.

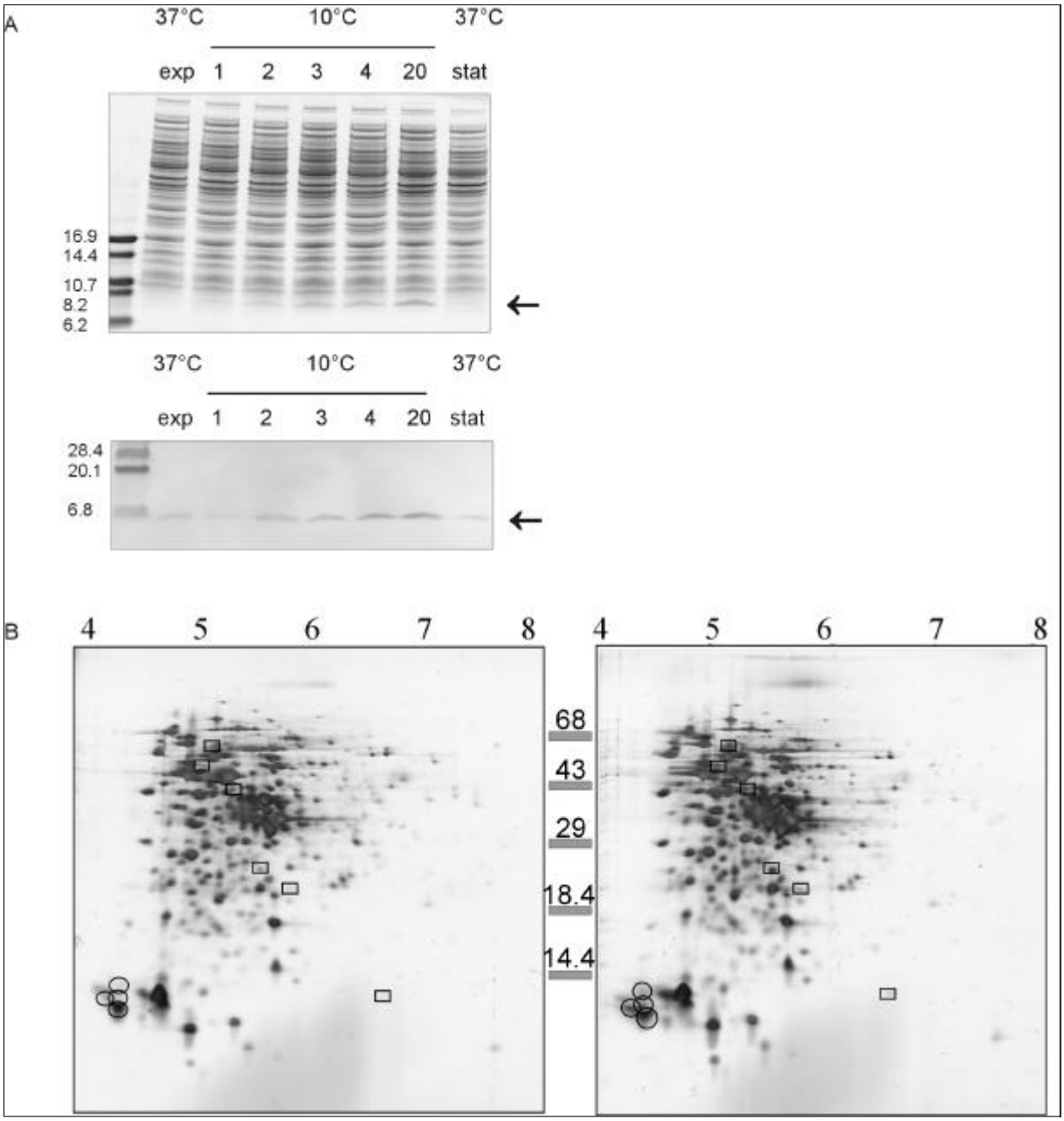

Figure 2. Protein analysis of *L. monocytogenes* LO28. (A) Upper panel: One-dimensional gel electrophoresis of mid-exponential cells (37°C), cells cold shocked to 10°C for 1, 2, 3, 4 and 20 h and cells in stationary phase at 37°C. Lower panel: Western blot of an identical 1D gel using anti-CspB from B. subtilis. (B) Two-dimensional gel electrophoresis of cell-free extracts of *L. monocytogenes* LO28, using a pI ranging from 3 to 10. Proteins induced at least three-fold are boxed, the CSPs are circled. Left panel: Exponential phase cells at 37°C. Right panel: Cells 4 h after cold shock to 10°C.

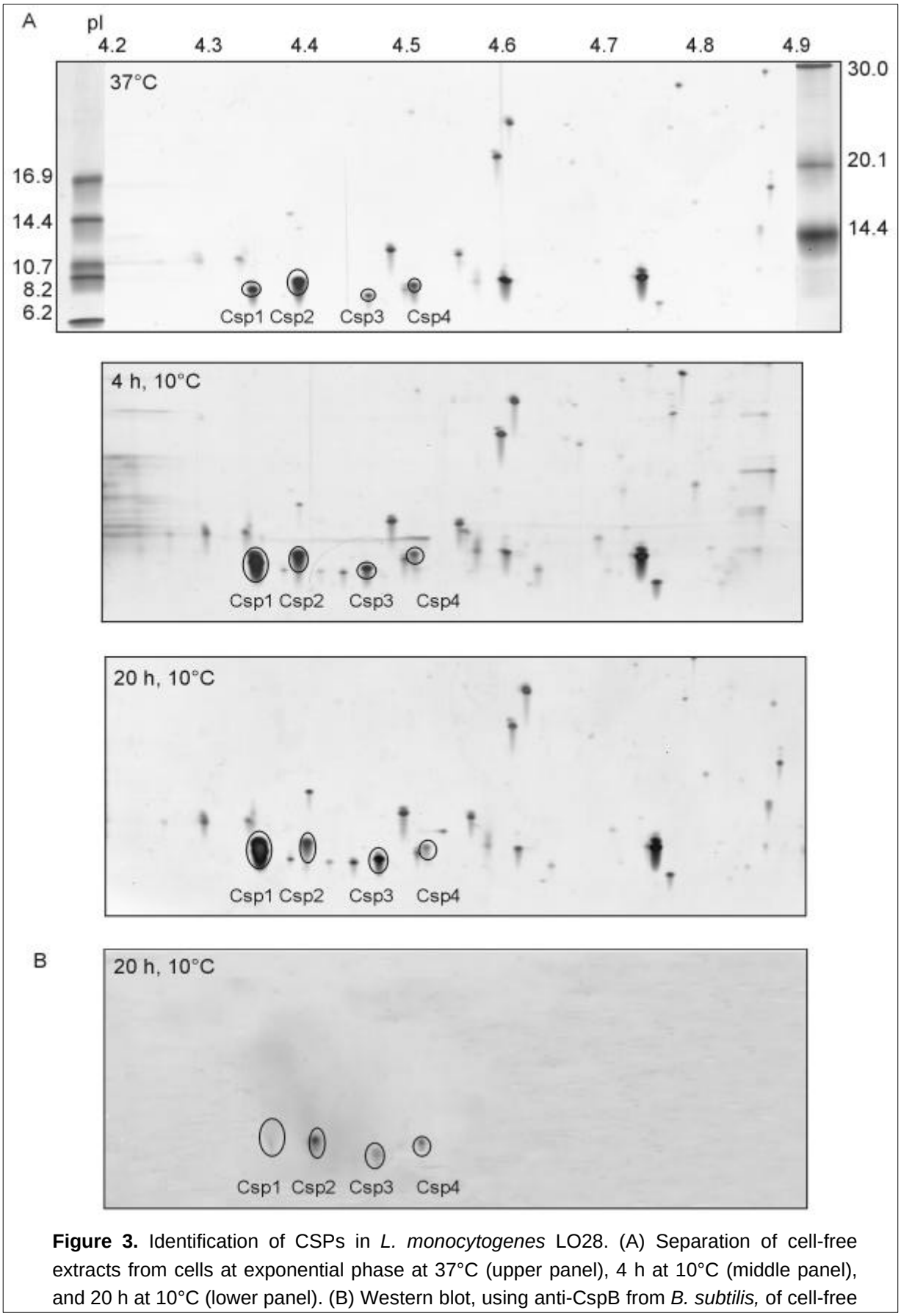

Figure 3. Identification of CSPs in *L. monocytogenes* LO28. (A) Separation of cell-free extracts from cells at exponential phase at 37°C (upper panel), 4 h at 10°C (middle panel), and 20 h at 10°C (lower panel). (B) Western blot, using anti-CspB from *B. subtilis,* of cell-free extract of *L. monocytogenes* LO28 cells 20 h after cold shock to 10°C, separated at identical conditions.

By using universal *csp* gene primers with *L. monocytogenes* LO28 total DNA as a template, a PCR product carrying a partial *csp* gene was obtained. The nucleic acid sequence is 100% identical to a partial *cspL* gene sequence of *L. monocytogenes* ATCC 23074 (11) and with *cspL* of *L. monocytogenes* EGD (E. M. Busch and T. Chakraborty, unpublished). Southern blotting of *L. monocytogenes* LO28 genomic DNA *Pst*I fragments, by using the PCR product as a probe, revealed four hybridizing bands (Fig. 4); one major band and three less intense hybridizing fragments. This would indicate the presence of four *csp* genes on the *L. monocytogenes* LO28 chromosome, corresponding to the four cold-shock proteins found on 2D-E gels.

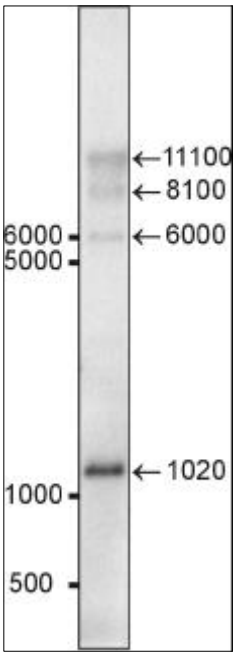

Figure 4. *csp* genes in *L. monocytogenes* LO28. Southern blot of *L. monocytogenes* LO28 *Pst*I fragments in wich the *csp* PCR product was used as the probe. The sizes of markers (in base pairs) are indicated on the left and the sizes of the hybridizing fragments (in base pairs) are indicated on the right.

CSP production has never been monitored in *L. monocytogenes*. Here we show the increased production of Csp1 and Csp3 after cold shock. Four hours after cold shock their levels were elevated 6,5 and 2-fold, respectively (Table 1).

Table 1. CSPs in *L. monocytogenes* LO28 and induction of CSPs after cold shock.

	Mw* (kDa)	pI*	exp, 37°C**	4 h, 10°C	20 h, 10°C
Csp1	7.4	4.32	1	6.6	9.8
Csp2	8.0	4.35	1	0.5	0.3
Csp3	7.0	4.43	1	2.1	3.4
Csp4	7.6	4.51	1	0.7	0.3

* Based on migration in a 2D-E gel

** Level of CSPs at 37°C is set at one

20 h post cold shock, enhanced levels of 10 and 3.5-fold were observed for Csp1 and Csp3, respectively. Csp2 and Csp4 levels were not elevated upon cold shock. We found no increased levels of CSPs for *L. monocytogenes* LO28 cells in stationary phase at 37°C (data not shown).

Response of *L. monocytogenes* to high hydrostatic pressure treatment. In pressurized *E. coli* cells, an induction of CspA was found (31). This indicates that CSPs may have a function in adaptation to pressurization. Here we analyze the response of *L. monocytogenes* LO28 to hydrostatic pressure treatment, by monitoring growth of pressurized cells and performing 2D-E with cell-free extracts from pressurized cells. After 10 min of pressurization to 50 or 100 Mpa, growth was hardly affected. However, cells pressurized to 200 MPa did not show an increase in OD_{620} for about 1 h (Fig 5a). 2D-E analysis of cell-free extracts from cells extracted after 10 min of pressurization to 200 MPa, revealed an increase in Csp1 and Csp2 compared to the levels in control cells (Fig. 5b). The Csp1 level was increased 3.5-fold and the amount of Csp2 was increased 2-fold. Since cold shock and high pressure treatment both increased CSP levels, we examined a possible cross-protection. Upon pressurization of cold-shocked cells, the survival was indeed higher than the survival of pressurized exponential growing cells. For cells pressurized for 20 min at 300 MPa the survival was 100-fold higher for cold-shocked cells compared to that of exponential growing cells at 37°C (Fig. 6).

Discussion

The adaptation process of *L. monocytogenes* to low temperature includes the maintenance of the membrane fluidity (1), the uptake of compatible solutes (17, 13, 5), the maintenance of ribosomal structure (4) and the induction of several proteins (3, 24). In the current paper we focus on the identification of 7 kDa CSPs in *L. monocytogenes* LO28. CSPs have not been identified before in 2D-gelelectrophoresis studies focussing on adaptation of *L. monocytogenes* to low temperature (3, 24). In these previous studies the low molecular weight proteins were not visualized due to experimental set-up. We describe here the identification of four CSPs in *L. monocytogenes* LO28, named Csp1, 2, 3 and 4. Csp1 and Csp3 levels were elevated upon cold shock, respectively 6.5 and 2-fold after 4 h at 10°C, whereas Csp2 and Csp4 were not. The levels of Csp1 and Csp3 increased even further after cell growth at low temperature had resumed. The production was highest at stationary phase after cold shock. Non-transient induction upon cold shock was also observed for CapA, a CSP homologue, of *Arthrobacter globiformis*, a psychrotrophic bacterium (6). The non-transient increased production of Csp1 and Csp3 indicates that CSPs are not only required for acclimation, but also for growth at low temperature. Next, we observed that all four CSPs were present in substantial levels at 37°C in *L. monocytogenes* LO28, which would imply that these proteins

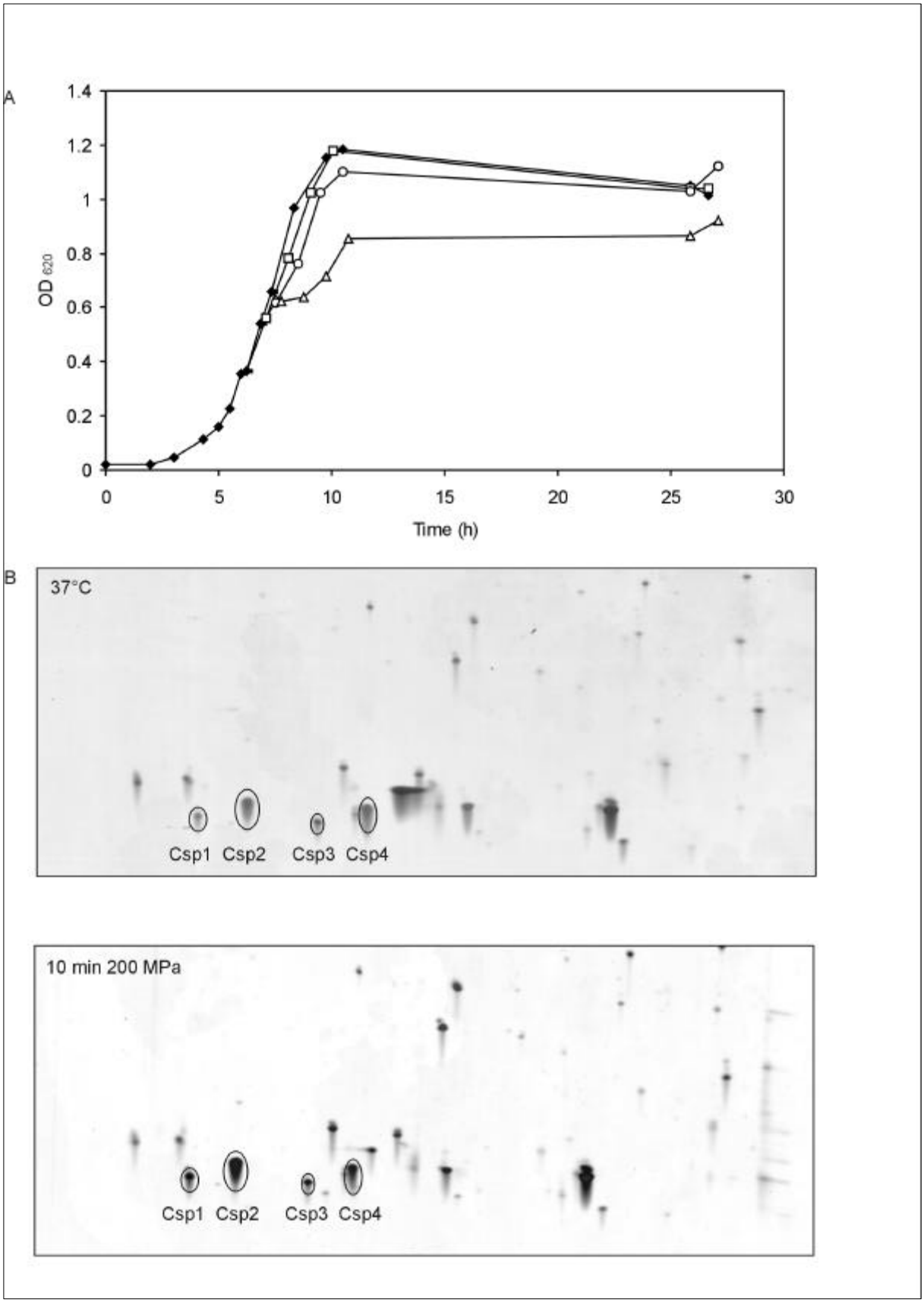

Figure 5. Response of *L. monocytogenes* LO28 to high hydrostatic pressure treatment. (A) Growth (OD_{620}) at 37°C (?), growth after 10 min exposure to 50 MPa (?), 100 MPa (?) and 200 MPa (Δ). (B) 2D-E with pI range 4-5 of cell-free extracts from untreated cells (upper panel) and of cell-free extracts from cells treated for 10 min at 200 MPa (lower panel).

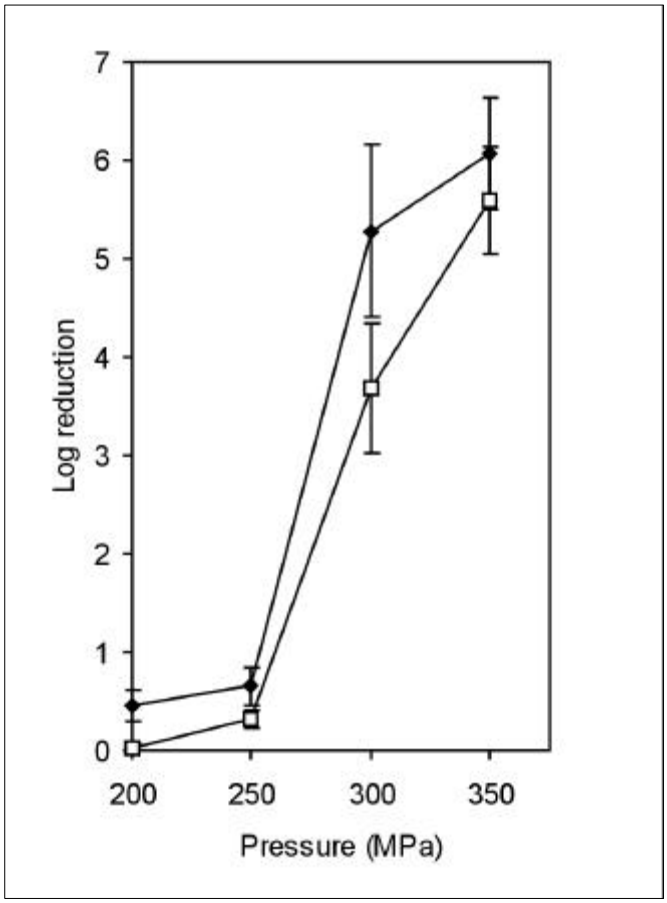

Figure 6. Reduction of viable cells of *L. monocytogenes* after exposure for 20 min to 200, 250, 300 and 350 MPa of exponentially growing cells at 37°C (?) and cells exposed to 10°C for 4 h (?).

have a function during normal growth. For *B. subtilis*, it was shown that the presence of at least one CSP is required for growth at low, and at normal temperature (15).

The survival of *L. monocytogenes* cells after freezing may impose a danger on public health. Therefore, it is of importance to monitor the survival of *L. monocytogenes* cells after freezing at different growth stages. The survival after freezing of exponentially growing *L. monocytogenes* LO28 was approximately 50%. However, when cells were cold shocked before freezing, the survival increased to 90%. This phenomenon of adaptation to freezing by pre-exposure to low temperature has been described for a number of bacteria, such as *B. subtilis* (32) and *L. lactis* (33). In these organisms CSPs play a role in freeze protection, shown by CSP overproducing variants, which showed higher survival after freezing (33), and by *csp* deletion mutants, which showed decreased freeze survival (32). Notably, after a 20 h freezing period of a suspension of mid-exponential *L. monocytogenes* LO28 cells, growth started only after a 2 h lag period, whereas the growth of cold-shocked cells resumed immediately. These data indicate that in order to design safe food processing methods, it is of utmost importance to take into account the effects that cell history might impart on bacterial survival potential, as is shown here for the freeze survival of *L. monocytogenes* after low temperature exposure.

The use of high hydrostatic pressure treatment may be a powerful new preservation strategy to reduce the risk of food-borne infections. Here we show that pressurization for 10 min at 200 MPa has a severe effect on cellular growth of *L. monocytogenes*. To analyze the cellular response of *L. monocytogenes* to this treatment, we performed 2D-E with a focus on the production of 7 kDa CSPs. For *E. coli* it is reported that CspA levels increase upon pressurization of the cell (31). We found an increase of Csp1 and Csp2 levels in *L. monocytogenes* LO28 upon pressurization to 200 MPa. Csp1 levels were elevated up to 10-fold after cold shock, and 3.5-fold after high pressure treatment. Strikingly, the level of Csp2 was not increased upon cold shock, while its level was increased 2-fold after pressurization. Graumann and Marahiel (16) proposed that the partial inactivation of ribosomes is the main trigger for the induction of CSPs at low temperature. A similar effect on the ribosomal structure as was found after cold shock, was observed upon high pressure treatment (20), suggesting an overlap in the response to these two stress conditions. Indeed, we found an induction of CSPs both at low temperature as well as upon pressurization of *L. monocytogenes* LO28. This indicates that CSPs might have a role in adaptation to both stresses. Combining cold shock with pressurization led to an increased survival after pressurization. The induced levels of CSPs after cold shock might confer protection to the cell at high pressure. It is conceivable that the cold-adapted ribosomes are less sensitive to the ribosome-deteriorating effects of pressure, resulting in higher survival after pressurization. This protection to pressurization supplied by low-temperature treatment can be of importance to processing technology, e.g. if cold storage of food products is combined with a pressurization treatment.

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Chapter 3

Identification of sigma factor σ^B controlled genes and their impact on acid stress, high hydrostatic pressure and freeze survival in *Listeria monocytogenes* EGD-e

Summary

The gene encoding the alternative sigma factor σ^B in *Listeria monocytogenes* is induced upon exposure of cells to several stresses. In this study, we investigated the impact of a *sigB* null mutation on the survival of *L. monocytogenes* EGD-e at low pH, during high hydrostatic pressure treatment and freezing. Survival of Δ*sigB* mutant exponential phase cells at pH 2.5 was 10,000-fold lower than survival of EGD-e wild-type cells. Moreover, the Δ*sigB* mutant failed to show an acid tolerance response. Upon pre-exposure for 1h to pH 4.5, survival at pH 2.5 was 100,000-fold lower for the Δ*sigB* mutant compared to the wild type. Important in survival and adaptation of *L. monocytogenes* in acidic conditions is the glutamate decarboxylase (GAD) acid resistance system. The σ^B-dependence of the *gad* genes (*gadA*, *B*, *C*, *D*, and *E*), was analysed *in silico*. Putative σ^B-dependent promoter sites were found in front of the operon *gadCB* (encoding a glutamate/γ-aminobutyrate antiporter and a glutamate decarboxylase enzyme, respectively) and the gene *lmo2434* (*gadD*, encoding a putative glutamate decarboxylase enzyme). Reverse-transcriptase PCR revealed that the expression of the operon *gadCB* and *gadD* are indeed σ^B-dependent. In addition, a proteomics approach was used to analyse the protein expression profiles upon acid exposure. Although the GAD proteins were not recovered, nine proteins were enhanced in the wild type but not in the Δ*sigB* strain. These proteins include Pfk, GalE, ClpP and Lmo1580. Exposure to pH 4.5, in order to preload cells with σ^B, and consequently with σ^B-dependent general stress proteins, conferred also considerable protection against high hydrostatic pressure treatments and freezing. These combined data argue that the expression of σ^B-dependent genes provides *L. monocytogenes* with a nonspecific multiple stress resistance that may be relevant for survival in the natural environment as well as during food processing.

H. H. Wemekamp-Kamphuis, J. A. Wouters, P. P. L. A. de Leeuw, T. Hain, T. Chakraborty, and T. Abee. Identification of sigma factor σ^B controlled genes and their impact on acid stress, high hydrostatic pressure and freeze survival in *Listeria monocytogenes* EGD-e. Submitted for publication.

Introduction

Listeria monocytogenes is a gram-positive, non-sporulating, facultative anaerobic micro-organism. It is the causative agent of listeriosis, a serious illness for which especially the young, elderly and immunocompromised are at risk (30). *L. monocytogenes* is of particular concern for the food industry due to the severity of the illness and the wide distribution of the pathogen in the environment and consequently its presence on raw and minimally processed foods. Moreover, *L. monocytogenes* is known for its ability to survive and proliferate in adverse environmental conditions, e.g. acidic conditions, refrigeration temperatures and high osmolarity (up to 10%) (4, 36). These characteristics of *L. monocytogenes* make it a food-borne pathogen problematic for certain kind of foods, especially minimally processed, ready-to-eat foods.

Adaptation to (sudden) adverse conditions in the environment of the bacterium requires the ability to respond rapidly. Such a response of bacteria to environmental changes involves activation of existing enzymes and the onset of the transcription of genes resulting in enhanced levels of (defensive) proteins. Initiation of transcription of mRNA from DNA is mediated by the holo-enzyme RNA-polymerase. The holo-enzyme consists of a core-enzyme and a sigma-factor, in which the sigma-factor is primarily responsible for recognition and binding of the polymerase to the promoter sequence in front of a gene. In the genome sequence of *L. monocytogenes* EGD-e, five sigma factors are present (14) (recovered using Ergo™ integrated genomics database). These include *rpoD*, encoding σ^A that is regulating housekeeping genes, *sigH* encoding a sigma factor with unknown specific function, *rpoN* encoding σ^{54} (SigL) having in *L. monocytogenes* a role in resistance to the antibacterial peptide mesentericin Y105 (24), *sigV* (*lmo0423*) of which the enzyme regulates ECF-genes (extra-cytoplasmic function genes) and the alternative sigma factor, *sigB*, encoding σ^B which controls the transcription of genes involved in stress-adaptation.

The role of σ^B in stress response of gram-positives, including *Bacillus subtilis*, *Staphylococcus aureus*, and *L. monocytogenes*, has gained considerable attention in recent years. The best-studied system is that in *B. subtilis*. The activity of the alternative sigma factor σ^B protein is regulated by a posttranslational mechanism. The *B. subtilis* σ^B operon comprises *rsbR, S, T, U, V, W, sigB* and *rsbX*, the encoded Rsb proteins are all involved in regulation of σ^B activity. RsbW binds under normal growth conditions with σ^B to form an inactive complex. However, the affinity of RsbW for its antagonists σ^B and RsbV can alter via two independent processes that promote the binding of RsbW to RsbV and thus leaving free σ^B, which is then capable of forming holoenzyme complexes with core RNA polymerase. Carbon limitation and entry into stationary phase correlate with a drop in the intracellular levels of ATP, which may have a direct effect of the binding preference of RsbW; shifting from σ^B to RsbV. Secondly, upon exposure to a number of environmental insults such as ethanol treatment, salt or acid shock, RsbU dephosphorylates RsbV, which is then able to bind to

RsbW, rendering free σ^B (31). The other Rsb proteins presumably have an indirect effect (positive or negative) on the σ^B activity or gene expression. Activation of σ^B in *L. monocytogenes* might involve a similar process, as the structure and organization of the *rsbU, V, W and X* genes and the *sigB* gene show high similarity with their *B. subtilis* counterparts, the encoding proteins show predicted identities of 53, 45, 47, 29 and 66%, respectively (2, 39).

In *B. subtilis*, the alternative sigma factor σ^B regulates expression of a large general stress operon contributing to transcription of about 150 genes involved in heat, acid, ethanol, salt, and freezing stress resistance (23, 33, 34). These genes include the *katE* gene, encoding a catalase (8), the *opuE* gene, encoding a transporter dedicated to the transport of the osmoprotectant proline (35), the *clpC* gene, which is similar to stress-induced ATPase subunits of ClpP-type proteases (17), and the *gtaB* gene, encoding an UDP-glucose pyrophosphorylase believed to participate in trehalose biosynthesis (29).

In *L. monocytogenes*, a role for σ^B has been ascribed in the response to several stresses, e.g. in acid resistance of stationary phase cells, in oxidative stress resistance, during carbon starvation, and in growth at low temperature (3, 9, 39). *L. monocytogenes* displays an active acid tolerance response upon exposure to low, non-lethal pH and subsequent exposure to lethal pH. Recently, the contribution of σ^B to growth phase-dependent acid resistance and to the adaptive acid tolerance response in *L. monocytogenes* 10403S was analyzed by Ferreira *et al.* (10). Survival of the Δ*sigB* strain was consistently lower upon exposure to BHI of pH 2.5 (with and without prior acid adaptation) than that of the wild-type strain throughout all phases of growth. However, σ^B-mediated contributions to acquired acid tolerance appeared to be greatest in early logarithmic growth. The acid tolerance response has high impact on food processing and virulence of the bacterium, since exposure to acidic conditions does not only enhance survival at lethal pH, it also confers protection to other challenges, such as heat, ethanol, oxidative, and osmotic stresses (11, 18).

Here, we present the role of σ^B in acid stress adaptation of exponentially growing *L. monocytogenes* EGD-e cells. Since the glutamate decarboxylase (GAD) acid resistance system is important in survival and adaptation of *L. monocytogenes* in acidic conditions (6), we tested whether this system plays a role in the acid adaptation of the wild type and the σ^B-deleted strain. The putative σ^B-dependence of the *gad*-genes (*gadA, B, C, D (lmo2434)*, and *E (lmo0449*)) was analysed *in silico*, and subsequently reverse-transcriptase PCR was performed to analyse the transcription of the *gad*-genes. In addition, a proteomics approach was used to analyse the protein expression profiles upon acid exposure of the wild type and the Δ*sigB* strain. Additionally, the role of σ^B in the industrially important processes high hydrostatic pressure treatment and freezing was assessed, also in combination with a pre-treatment of low-pH exposure to assess the protective effect of the adaptation to these food-processing methods.

Materials and methods

Bacterial strains and generation of mutant. *L. monocytogenes* EGD-e and its Δ*sigB* mutant were used throughout this study. The Δ*sigB* mutant was constructed using the temperature-sensitive suicide plasmid pAUL-A (32).

Survival during acidic conditions and freeze-thaw cycles. Cells were grown in BHI at 30°C to an OD_{620} of 0.4, centrifuged and resuspended in fresh BHI with pH 2.5 (adjusted with HCl). Colony forming units were determined during a 3h incubation at 30°C. Survival in this acidic environment was also measured after adaptation for 1h to BHI with a pH of 4.5 (adjusted with HCl). Survival of *L. monocytogenes* and its Δ*sigB* mutant were also measured in freeze-thaw cycles. Exponential phase cells, grown in BHI at 30°C, were centrifuged, resuspended in fresh BHI, and subsequently frozen for 24h at –20°C. Colony forming units were determined after 2 min thawing at 30°C for 5 freeze-thaw cycles. Freeze-thaw survival was also measured after exposure of the mid-exponential phase cells to BHI of pH 4.5 for 1h and to BHI of 7°C for 4h.

Analysis of protein extracts using two-dimensional gel electrophoresis (2D-E) and Maldi-TOF. Samples of 10 ml bacterial suspension (OD_{620} = 0.4) were taken, centrifuged and suspended in water to an OD_{620} of 10. Total cellular proteins were extracted from the cells with a bead-beater (B. Braun Biotech International, Germany) and zirconium beads (0.1 mm, Biospec Products, USA) by 3 treatments for 1 min (cooled on ice between treatments). Thereafter the zirconium beads were allowed to sediment by gravity and subsequently the supernatant, containing the cellular proteins, was analyzed by 2D-E using a pI range of 4 to 7 and a 12-14% gel for the second dimension. 2D-E was essentially performed as described previously (37). Protein spots were made visible by silver staining and the gels were analyzed with PDQuest software (BioRad, Richmond, USA). Differentially expressed proteins (>3-fold) were boxed (increased expression upon acid exposure) or circled (decreased expression upon acid exposure). For identification of protein spots gels were loaded with 200 µg protein and coloured with Coomassie blue. Differentially expressed proteins were cut out of the gel, and analyzed in the Proteomics Center, Department of Human Biology, University of Maastricht, The Netherlands (Dr. J. Renes and F. Bouwman, B.Sc.) by digestion in a MassPrep station (Micromass, Almere, The Netherlands) and subsequent analysis with a Maldi-TOF LR mass spectrometer (Micromass). Identification of the proteins was performed with PeptIdent.

Transcriptional analysis of the *gad* genes. *L. monocytogenes* EGD-e and the Δ*sigB* mutant were grown to mid-exponential phase at 30°C. A portion of 10 ml was centrifuged and resuspended in 1 ml of BHI (with and without an adjusted pH of 4.5). After 1h incubation, cells were centrifuged and RNA was extracted using the RNeasy (Qiagen, Hilden, Germany) RNA extraction kit. cDNA was synthesized by adding 2 µl of total RNA to 1 µl of reverse-primer mix (10 pmol/µl), 1 µl of 5 times first strand buffer and 1 µl of water. This mixture

was incubated for 2 min at 75°C, after which 3 µl of first strand buffer, 2 µl of DTT (0.1 M), 4 µl of dNTPs (2.5 mM), 0.4 µl of Superscripts II reverse transcriptase enzyme, and 5.6 µl water was added. An incubation for 1h at 48°C followed and 3.55 µl water, 0.2 µl of RNAse H, and 1 µl of first strand buffer were added for cleavage of the RNA in RNA:DNA hybrids during an incubation for 10 min at room temperature.
PCR was carried out using the primers listed in Table 1, and 20, 25 and 30 cycles were performed to allow optimal quantification of PCR products. Template cDNA was used in the reactions at levels that gave similar band intensities for 16S RNA reactions.

Table 1. Primers used in this study.

Gene	Primer
gadA (glutamate decarboxylase, *lmo0447*)	
Forward	5'-cgg tgt ttg gct ctt tt ga-3'
Reverse	5'-ctc cga ttc atc cac att cc-3'
gadB (glutamate decarboxylase, *lmo2363*)	
Forward	5'-ggc atg cac cta agg acc aaa aat-3'
Reverse	5'-gat acc gag gat gcc gac cac ac-3'
gadC (antiporter, *lmo2362*)	
Forward	5'-aaa tgg cga cgg tgg atg gt-3'
Reverse	5'-ttt tgc gat ttt agc cgt gtt tt-3'
gadD (glutamate decarboxylase, *lmo2434*)	
Forward	5'-act tgg caa aaa ctg tag aaa a-3'
Reverse	5'-tag tgc gta aat ccg tat gaa-3'
gadE (antiporter, *lmo0448*)	
Forward	5'-att cgg cgg cgg tgg ta-3'
Reverse	5'-aaa acg gaa tta aaa tag tga cga-3'
16S RNA	
Forward	5'-tta gct agt tgg tag ggt-3'
Reverse	5'-aat ccg gac aac gct tgc-3'

High hydrostatic pressure (HHP) treatment. Cells were harvested by centrifugation, resuspended in 50 mM N-[2-acetamido]-2-aminoethanesulfonic acid (ACES buffer; Sigma-Aldrich, Steinheim, Germany), pH 7.0 and subsequently packed in plastic bags. The survival after high pressure treatment, with pressurization for 20 minutes at 150, 200, 250, 300, 350, and 400 MPa, was determined for exponentially growing cells, cells exposed to pH 4.5 for 1h, and cells exposed to 10°C for 4h. Depressurization of the high hydrostatic pressure apparatus (Resato, Roden, The Netherlands) was achieved within seconds. The pressurization was performed at 20°C, while temperatures during pressurization did not exceed 32°C. In addition,

L. monocytogenes wild type and Δ*sigB* mutant cells, pre-exposed to pH 4.5, were exposed to 350 MPa for 3, 8, 14, 18, 23, and 28 min at 20°C.

Results

Survival of *L. monocytogenes* EGD-e at low pH is s^B-dependent. Survival of exponential phase cells *L. monocytogenes* EGD-e and the σ^B null mutant in BHI of pH 2.5 was determined in order to analyze its σ^B-dependence. About 5% of wild-type *L. monocytogenes* cells survived a 1h-exposure to the acidic conditions (Fig. 1).

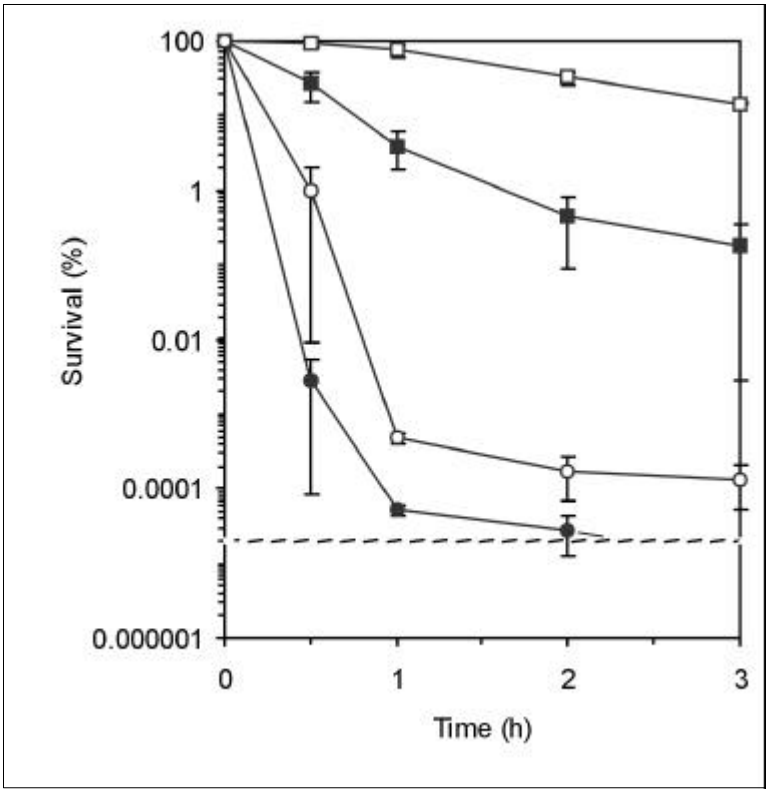

Figure 1. Survival of *L. monocytogenes* EGD-e (squares) and the Δ*sigB* mutant (circles) during incubation at pH 2.5, with (?, ?) and without (¦ , ?) pre-exposure for 1h to pH 4.5.

For the σ^B-mutant cells this was about 0.00005%, i.e. a log-5 difference. This clearly shows that the survival of *L. monocytogenes* at pH 2.5 is σ^B-dependent. In addition, we determined the σ^B-dependence of the ATR (acid tolerance response) in *L. monocytogenes* EGD-e. Cells were pre-exposed to a non-lethal pH (pH 4.5) prior to exposure to pH 2.5. After this pre-exposure to pH 4.5, almost 100% of EGD-e wild-type cells survived an exposure of 1h to pH 2.5. However, only about 0.0005% pre-exposed cells with a σ^B-null mutation survived, indicating the importance of the induction and/or activation of σ^B during adaptation to low pH for subsequent survival at lethal pH (Fig. 1).

σ^B-dependent expression of *gad* genes. Adaptation of bacteria to acidic conditions involves the maintenance of a relative high intracellular pH (pH_i) (20). A specific mechanism for acid adaptation in *L. monocytogenes* aimed at the maintenance of pH_i is the glutamate decarboxylase (GAD) acid resistance system. It involves an antiporter, transporting glutamate into the cell, and a glutamate decarboxylase enzyme, converting glutamate into γ–aminobutyrate (GABA) upon consumption of a proton. The glutamate/GABA antiporter subsequently excludes the GABA from the cell. Cotter *et al.* (6) identified 3 genes involved in this system, *gadA* (*lmo447*), encoding a glutamate decarboxylase enzyme, *gadB* (*lmo2363*), coding for another glutamate decarboxylase enzyme, and *gadC* (*lmo2362*), encoding the associated glutamate/GABA antiporter.

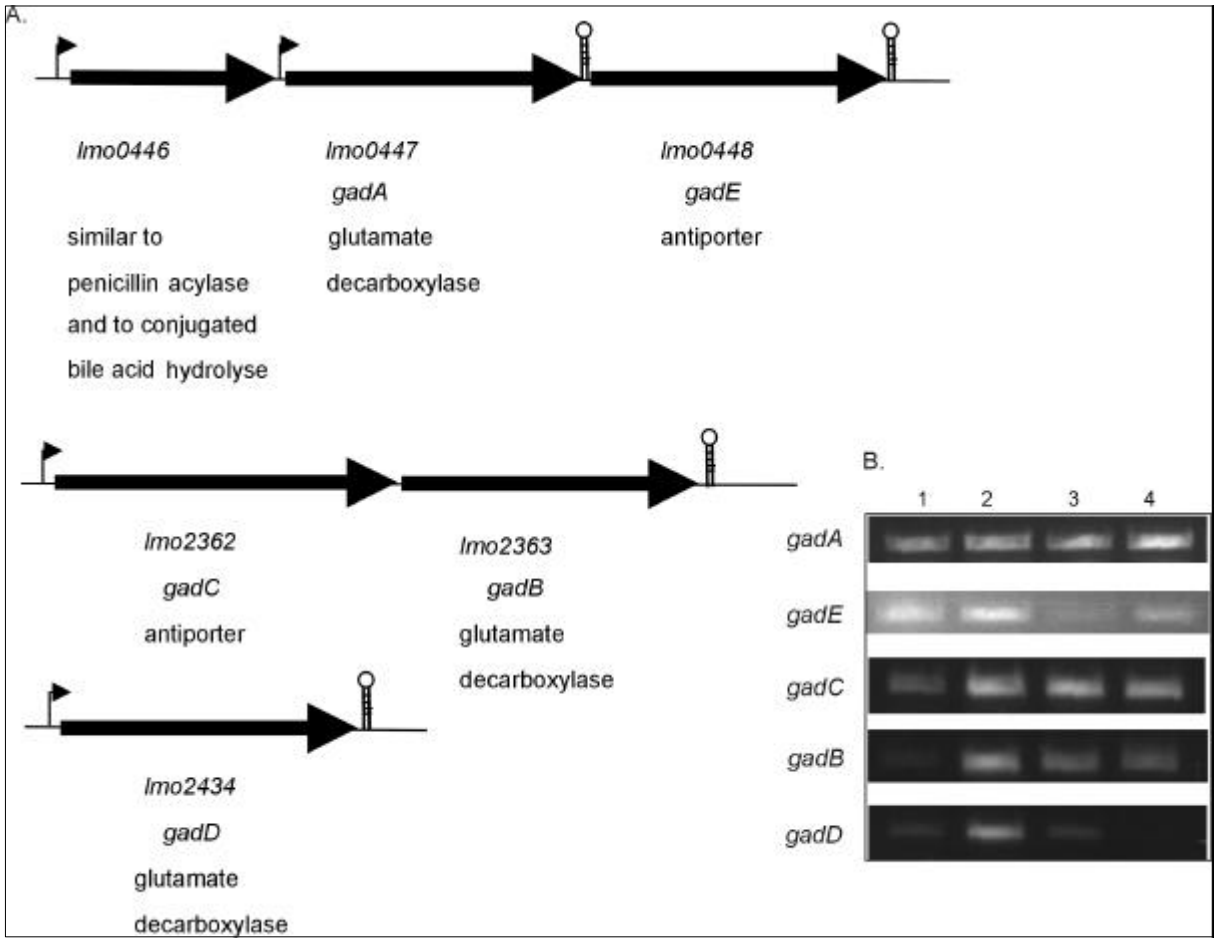

Figure 2. (A) Organization of *gad* genes in *L. monocytogenes* EGD-e. The hairpin structures indicate putative terminators. The numbering of the ORFs follows the numbering of the *L. monocytogenes* EGD-e genome sequence (14). (B) Transcriptional analysis of *gad* genes in *L. monocytogenes* EGD-e (lanes 1 and 2) and its Δ*sigB* mutant (lanes 3 and 4), in exponential phase cells (lanes 1 and 3) and cells exposed for 1h to pH 4.5 (lanes 2 and 4).

Conte *et al.* (5) identified from the genome data of *L. monocytogenes* EGD-e (http://genolist.pasteur.fr/ListiList/) two other genes with high homology to the *gad* genes.

Coding for another glutamate decarboxylase is *lmo2434* (here designated as *gadD*), and *lmo448* (here designated as *gadE*) encoding an additional antiporter. Spaced close together, *gadA* and *gadE* are co-transcribed. However, a stem-loop-like structure within the intergenic spacer downstream of *gadA* stops readthrough transcription, resulting in single transcription of *gadA* (or in co-transcription with *lmo0446*) (5). Cotter *et al.* (6) already described the co-transcription of *gadC* and *gadB*, this observation, together with the genome data, suggests that these two genes form an operon. Oppositely oriented and further downstream of the operon is *gadD* (Fig. 2A). Considering the low survival of a Δ*sigB* mutant at low pH, the putative σ^B-dependence of the *gad* genes was analysed *in silico*. Comparison of the *L. monocytogenes* σ^B promoter sequence (GTTTTA <14> GGGTAA, taken from Becker *et al.* (2)) revealed only 3 and 2 mismatches in front of the operon *gadCB* and *gadD*, respectively. Using reverse-transcriptase PCR, we analysed the transcription of the *gad* genes (*gadA, B, C, D*, and *E*) upon exposure to pH 4.5 for 1h for both the wild-type and the Δ*sigB* strain. For the wild-type EGD-e all *gad* genes except *gadA* were induced after exposure to low pH (Fig. 2B). This is an indication that GadA plays a minor role in acid adaptation, supported by acid survival experiments from Cotter *et al.* (6) using *gad* deletion-mutant strains. Of the four induced transcripts in the wild type, only 1 was induced in the Δ*sigB* mutant strain, namely *gadE*. This indicates that transcription of the other three genes, *gadB, C*, and *D*, is, at least partly, σ^B-regulated under the conditions tested. The apparent constitutive expression of the *gadCB* operon in the Δ*sigB* strain suggests that additional regulators are involved. A slight induction of *gadA* transcripts could be detected upon acid exposure of the Δ*sigB* strain. This might be a compensation for the reduced transcription of the other genes encoding for glutamate decarboxylase enzymes, e.g. *gadD*.

2D-electrophoresis and identification of proteins involved in acid adaptation. Previous research showed that the acid tolerance response of *L. monocytogenes* includes the induction of several proteins (7, 21, 22). In this study the differentially expressed proteins in wild type and Δ*sigB* strains after exposure to pH 4.5 were analyzed using 2D-E (Fig. 3). After exposure to pH 4.5 wild-type cells showed a decreased production of 5 proteins, including Lmo1704; a protein similar to conserved hypothetical proteins, FbaA; a protein similar to fructose-1,6-biphosphate aldolase, and HPr; PTS phosphocarrier protein HPr (Table 2). However, 16 proteins were present in enhanced levels after low-pH exposure, including ValS; valyl-tRNA-synthetase, Pfk; 6-phosphofructokinase, FlaA; flagellin protein, Lmo1709; similar to methionine aminopeptidases, GalE; UDP-glucose-4-epimerase, ClpP; the proteolytic subunit of the ATP-dependent Clp protease, and Lmo1580; similar to unknown protein. In the Δ*sigB* strain the production of 14 proteins was reduced after exposure to low pH, whereas 8 proteins were induced. Of the 16 proteins induced in the wild type, 7 were also present in enhanced levels in the Δ*sigB* mutant, indicating that these are not strictly σ^B-regulated. Of the 16 proteins, 9 were present in enhanced levels in the wild type (Fig. 3)

whereas they were not induced in the Δ*sigB* mutant strain. These putatively σ^B-regulated proteins, which seem to be involved in acid adaptation, include Pfk, GalE, ClpP and Lmo1580.

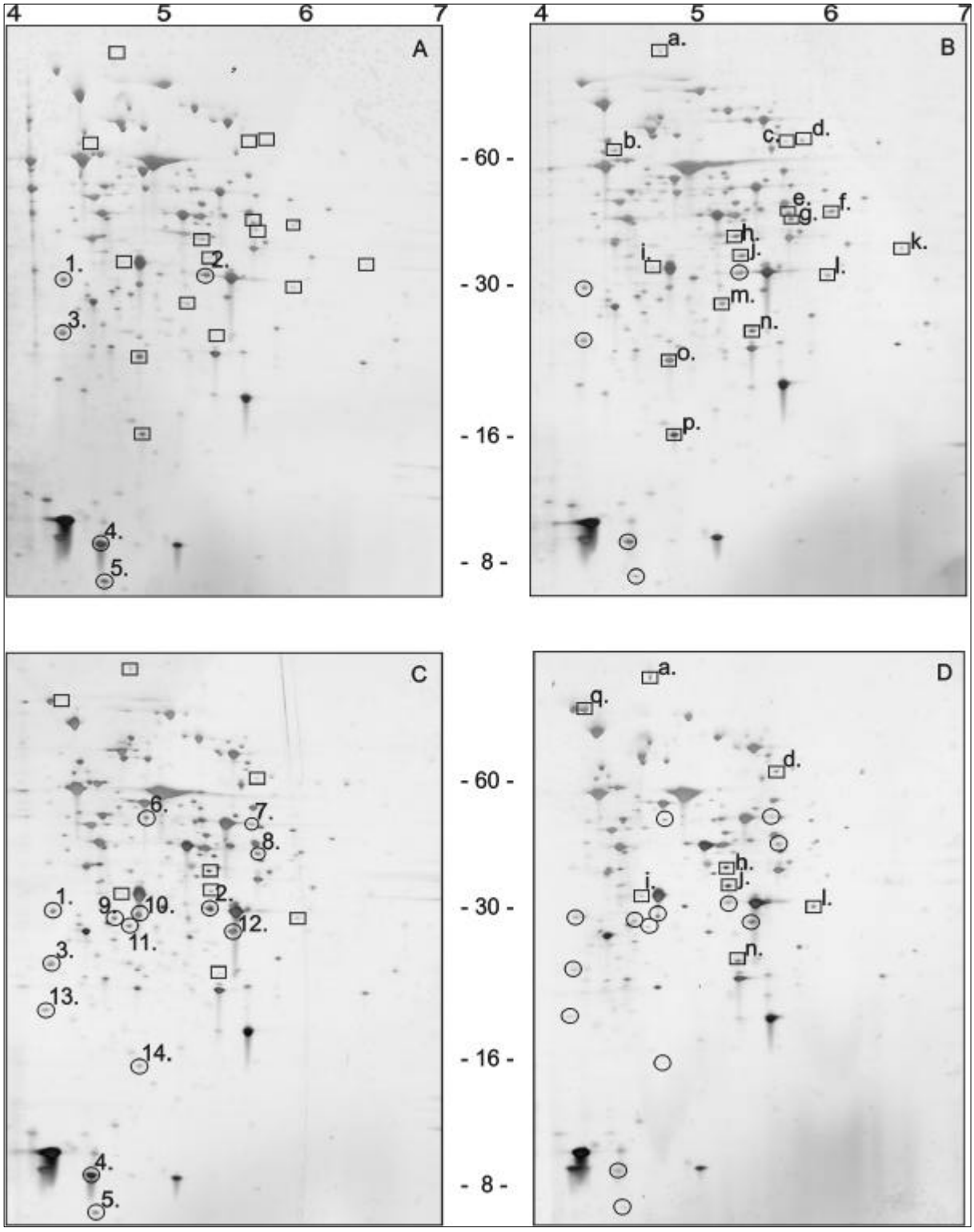

Figure 3. Analysis of protein production in *L. monocytogenes* EGD-e and the Δ*sigB* mutant exponential phase cells (A and C, respectively) and cells exposed for 1h to pH 4.5 (B and D,

respectively) using 2D-E. Differentially expressed proteins are indicated, circled spots are reduced, boxed spots are induced upon low pH exposure.

Table 2. Differentially produced proteins upon acid shock of *L. monocytogenes* EGD-e and Δ*sigB* mutant.

Spot	Protein	Description	pI	Mw (kDa)
A. *Reduced protein production upon acid shock of EGD-e wild type*				
1.	N.D.[a]			
2.	FbaA	Similar to fructose-1,6-biphosphate aldolase	5.1	30.0
3.	N.D.			
4.	Hpr	PTS phosphocarrier protein Hpr	4.5	9.4
5.	Lmo1704	Similar to conserved hypothetical proteins	5.8	14.4
B. *Increased protein production upon acid shock of EGD-e wild type*				
a.	ValS[b]	Valyl-tRNA-synthetase	4.7	102
b.	N.D.			
c.	N.D.			
d.	N.D.			
e. (8)	N.D.			
f.	Pfk	6-phosphofructokinase	5.4	34.4
g.	N.D.			
h.	N.D.			
i.	FlaA	Flagellin protein	4.7	30.4
j.	N.D.			
k.	N.D.			
l.	Lmo1709	Similar to methionine aminopeptidases	5.4	27.9
m.	GalE	UDP-glucose-4-epimerase	4.9	36.2
n.	N.D.			
o.	ClpP	ATP-dependent Clp protease proteolytic subunit	4.7	21.6
p. (14.)	Lmo1580	Similar to unknown protein	4.7	16.9
C. *Reduced protein production upon acid shock of EGD-e DsigB*				
1.	N.D.			
2.	FbaA	Similar to fructose-1,6-biphosphate aldolase	5.1	30.0
3.	N.D.			
4.	Hpr	PTS phosphocarrier protein Hpr	4.5	9.4
5.	Lmo1704	Similar to conserved hypothetical proteins	5.8	14.4
6.	N.D.			
7.	N.D.			
8.	N.D.			
9.	N.D.			
10.	Lmo1011	Hypothetical protein, similar to tetrahydrodipicolinate succinylase	4.7	24.8
11.	N.D.			
12.	ThiD	Phosphomethyl pyrimidine kinase	5.0	28.8
13.	N.D.			

14.	Lmo1580	Similar to unknown protein	4.7	16.9
D. *Increased protein production upon acid shock of EGD-e DsigB*				
a.	ValS[b]	Valyl-tRNA-synthetase	4.7	102
d.	N.D.			
h.	N.D.			
i.	FlaA	Flagellin protein	4.7	30.4
j.	N.D.			
l.	Lmo1709	Similar to methionine aminopeptidases	5.4	27.9
n.	N.D.			
q.	N.D.			
E. *Putative s^B-regulated proteins involved in acid adaptation*				
b.	N.D.			
c.	N.D.			
e. (8.)	N.D.			
f.	Pfk	6-phosphofructokinase	5.4	34.4
g.	N.D.			
k.	N.D.			
m.	GalE	UDP-glucose-4-epimerase	4.9	36.2
o.	ClpP	ATP-dependent Clp protease proteolytic subunit	4.7	21.6
p. (14.)	Lmo1580	Similar to unknown protein	4.7	16.9

[a] N.D.: Not determined

[b] Only 6.2% of sequence covered

Using the *L. monocytogenes* σ^B-promoter sequence for putative σ^B-regulated promoter site recognition (2), we matched the putative promoter sequences of the genes encoding these proteins *in silico*. Three mismatches were found in the promoter regions of *pfk*, *clpP* and *lmo1580*, and 4 mismatches for *galE* (Table 3).

Table 3. Alignment of σ^B-dependent promoters in *L. monocytogenes*.

	Promoter sequence			
Gene	-35	spacing	-10	# mismatches
sigB	GTTTTA	-N14-	GGGTAA	
pfk	GTTTTG	-N11-	GTTTAA	3
clpP	GTTTGA	-N16-	GTGTAT	3
lmo1580	GGTTCT	-N13-	GGGTAA	3
galE	ATAGAT	-N14-	GGGTCT	4
gadD	TTTTTA	-N12-	CGGTAA	2
gadC	GTTTGT	-N14-	GGGTAT	3
pykA	GTTTTA	-N12-	TGGTAA	1

lmo1339	GTTTAA	-N16-	GGAGAA	3

Role of s^B in HHP survival and freeze-thaw cycles. To identify the role of σ^B in other important food manufacturing processes, we assessed the survival of the parent strain and the mutant after high hydrostatic pressure treatment and freeze-thaw cycles. The parent strain is about a 100-fold less susceptible to 20 min pressurization at 300 MPa as the Δ*sigB* mutant strain (Fig. 4a).

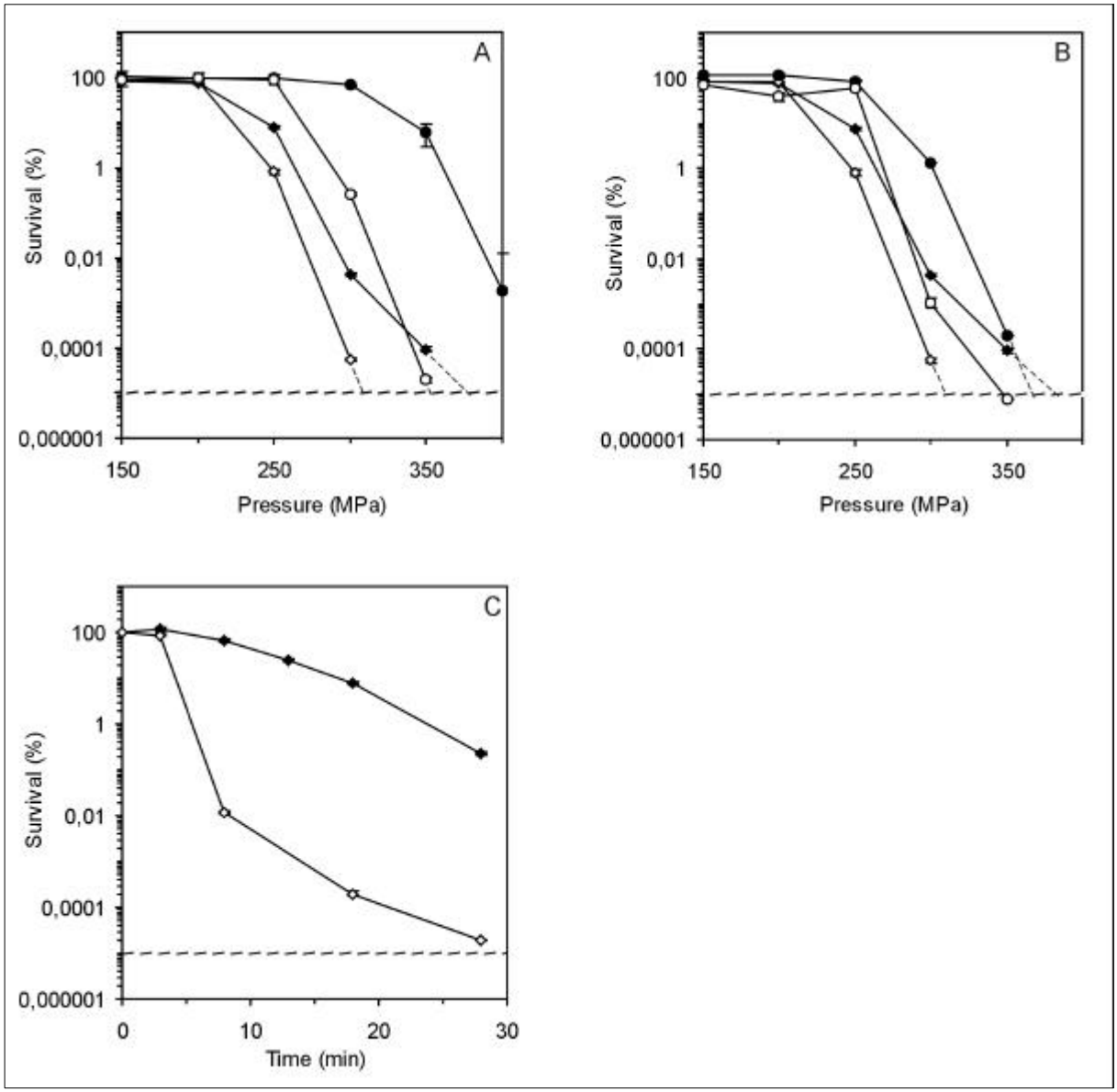

Figure 4. High hydrostatic pressure survival of *L. monocytogenes* EGD-e (closed symbols) and its Δ*sigB* mutant (open symbols). (A) Survival of exponential phase cells (?, ?) and cells exposed for 1h to pH 4.5 (?, ?). (B) Survival of exponential phase cells (?, ?) and cells exposed for 4h to 10°C (?, ?). (C) Survival of pre-exposed (1h pH 4.5) *L. monocytogenes* EGD-e (?) and the Δ*sigB* mutant (?) upon exposure to 350 MPa.

Exposure to pH 4.5 for 1h increases the survival of the parent strain in subsequent HHP exposure dramatically, i.e. pressurization at 300 MPa does not significantly decrease

viable cell count. Notably, even pressurization at 350 MPa only decreases viable cell count to 10% of the initial count, whereas cell counts of the Δ*sigB* mutant strain (pre-exposed to pH 4.5) are only just above the detection limit after pressurization at 350 MPa, i.e. more than a 5-log difference. Additionally, we analysed this difference in greater detail by varying the exposure duration (Fig. 4c). There is a clear difference in the survival of EGD-e wild-type cells and the Δ*sigB* mutant, e.g. after 28 min of exposure to 350 MPa, there is a 4-log difference. These results indicate that acid-induced proteins encoded by σ^B-dependent genes are conferring considerable protection to cells exposed to HHP. Pre-exposure of the cells to low temperature also resulted in increased pressure survival (more than 100-fold at 300 MPa) (Fig. 4b), as was found previously for *L. monocytogenes* LO28 cells (38). Again the Δ*sigB* mutant shows lower resistance to pressurization than the wild-type, indicating that the σ^B-dependent proteins formed during low-temperature adaptation are involved in high-pressure tolerance. However, survival of both wild-type and Δ*sigB* mutant acid-exposed cells is higher than survival of refrigerated cells.

Freeze-thaw survival of cells increased after pre-exposure to another insult, with 4h at 7°C as effective as low pH exposure (Fig. 5). The protection of a pre-exposure before freezing is partially σ^B-dependent, as the percentage of survivals of wild-type EGD-e is 3-fold higher after 5 freeze-thaw cycles compared to the Δ*sigB* mutant.

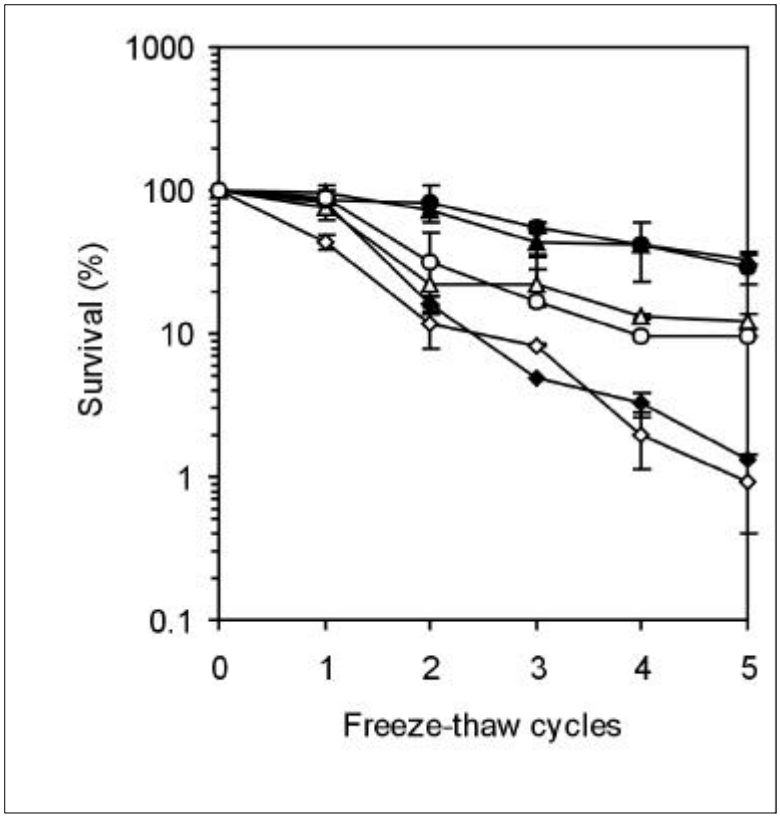

Figure 5. Survival of *L. monocytogenes* EGD-e (closed symbols) and the Δ*sigB* mutant (open symbols) after freeze-thaw cycles. Freeze-thaw cycles were performed on exponential

phase cells (?, ?), cells pre-exposed to pH 4.5 for 1h (? , ∆), and cells pre-exposed to 10°C for 4h (?, ?).

Discussion

Here we show that the survival upon exposure to lethal acidic conditions and the acid tolerance response of *L. monocytogenes* EGD-e exponential phase cells is highly σ^B-dependent. In *L. monocytogenes*, maintenance of a relative high intracellular pH is important in survival at low pH. A specific mechanism for acid adaptation in *L. monocytogenes* aimed at the maintenance of pH_i is the glutamate decarboxylase (GAD) acid resistance system. It involves an antiporter, transporting glutamate into the cell, and a glutamate decarboxylase enzyme, converting glutamate into γ–aminobutyrate (GABA) upon consumption of a proton. The glutamate/GABA antiporter subsequently excludes the GABA from the cell. As the Δ*sigB* mutant is significantly more acid sensitive than the wild type, the putative σ^B-dependence of the *gad* genes was analysed *in silico*. Putative σ^B-dependent promoter sites were found in front of the operon *gadCB* and *gadD*, displaying 3 and 2 mismatches, respectively. Additionally, upon transcription analysis using RT-PCR, we confirmed the σ^B-dependent transcription of *gadCB* and *gadD* upon exposure to pH 4.5 for 1h. This can partly explain the loss of acid tolerance of the Δ*sigB* mutant strain, as null mutations in the *gad*-genes (*gadA*, *B*, and *C*) show an important role in acid resistance and adaptation (6). Ferreira *et al*. (10) did not find a contribution of σ^B to the net proton movement across the cell membrane or by the GAD system. However, their experimental set-up included incubation at pH 2.5 for 1 hour of stationary phase cells that had not been pre-adapted to low pH, of which they showed that about 10% of wild-type cells and only about 0.1% of Δ*sigB* cells survived. Additionally, they analyzed the contribution of σ^B to growth phase-dependent acid resistance and to the adaptive acid tolerance response in *L. monocytogenes* 10403S. Survival of the Δ*sigB* strain was consistently lower upon exposure to BHI of pH 2.5 (with and without prior acid adaptation) than that of the wild-type strain throughout all phases of growth (10).

The acid tolerance response induces *de novo* protein synthesis as was shown with 2D-E (7, 21, 22). Moreover, treatment with chloramphenicol during acid adaptation annihilates the protective effect (21). Two-dimensional electrophoresis of proteins from cells exposed to acidic pH of the wild-type strain and the Δ*sigB* mutant revealed several proteins that were induced (16 and 8, respectively) or reduced (5 and 14, respectively) at least 3-fold. Proteins that are reduced in both the wild-type strain and the Δ*sigB* mutant are Lmo1704; a protein similar to conserved hypothetical proteins, FbaA; which is a protein similar to fructose-1,6-biphosphate aldolase, HPr; PTS phosphocarrier protein HPr, and 2 unidentified spots. Although not previously described in *L. monocytogenes*, HPr is also reduced in *Lactococcus lactis* upon acid exposure. HPr does not only have a role in the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS), it also has a regulatory role in cell metabolism (19, 40).

For the proteins ValS, FlaA, and Lmo1709, increased expression during low pH exposure was found in both wild type and mutant strain. FlaA, the structural protein of the

flagellum, might be induced for increased motility of the bacterium, and subsequent taxis away from growth inhibiting conditions.

σ^B-dependent proteins induced in the wild type after acid adaptation include Pfk, GalE, ClpP and Lmo1580. Pfk, 6-phosphofructokinase, and GalE are enzymes involved in glycolysis and sugar metabolism, respectively. Pfk is the key enzyme in the control of glycolysis, together with hexokinase (glucose kinase) and pyruvate kinase. Strikingly, comparison of the promoter site of pyruvate kinase (*pykA*) and glucose kinase (*lmo1339*) with the sequence recognized by σ^B, revealed only one and three mismatches (Table 3), respectively, suggesting that these enzymes might also be σ^B regulated. These data together may suggest that the rate of glycolysis is partly σ^B-regulated. ClpP is the proteolytic subunit of the ATP-dependent Clp protease. The ATPases ClpC and ClpE and the proteolytic subunit ClpP are all required for stress survival, growth at high temperature and virulence (12, 20, 25). Clp ATPases regulate ATP-dependent proteolysis, preventing the accumulation of misfolded proteins, and also play a role as molecular chaperones involved in protein folding and assembly (37). The *clp* genes in *L. monocytogenes* form part of the CtsR (class three stress gene repressor) stress response regulon, in which CtsR negatively regulates the *clpP*, *clpC* and *clpE* genes. In *B. subtilis*, two transcriptional start sites upstream of the *clpP* gene were identified, preceded by sequences resembling the consensus sequences of promoters recognized by σ^A and σ^B transcriptional factors of the *B. subtilis* RNA polymerase. Transcription initiation occurred predominantly at the putative σ^A-dependent promoter. However, after exposure to stress, initiation of transcription also increased at the σ^B-dependent promoter (13). *In silico* analysis of the promoter area of *clpP* of *L. monocytogenes* EGD-e revealed a putative promoter with 3 mismatches to the σ^B consensus promoter sequence. Supported by our proteomics findings this indicates that *clpP* in *L. monocytogenes* might also be, next to CtsR-regulated, σ^B-regulated.

Pre-exposure to low pH does not only confer protection to an otherwise lethal pH, it also enhances survival during other challenges, such as heat, ethanol, oxidative, and osmotic stresses (11, 18). Here we show the effect of acidic pre-exposure of cells on HHP survival and freezing. Cells exposed to low pH prior to HHP treatment are significantly more piezotolerant. This effect was found to be mainly conferred by σ^B-dependent proteins, as Δ*sigB* mutant cells pre-exposed to low pH were not as piezotolerant as wild-type cells. Piezotolerance was also observed for a mutant (AK01) naturally occurring in a *L. monocytogenes* Scott A culture (15, 16). In this strain 2D-E showed enhanced levels of ClpP, resulting from a single amino acid deletion in the highly conserved glycine-rich region of CtsR, a repressor of the *clp* genes. As we also found enhanced expression of ClpP in the wild type upon exposure to low pH, and not in the Δ*sigB* mutant, this protein may be an important factor in survival at high pressure. However, this could not be the single reason for increased pressure resistance, as survival of AK01 was only about 2-logs higher than that of the wild type over a broad range of pressures (15), whereas survival of acid-adapted wild-type EGD-e cells and Δ*sigB* mutant cells differ

more than 5-logs at 350 MPa. Low-temperature adaptation of *L. monocytogenes* EGD-e cells also increases HHP survival. This is in agreement with previous results for HHP survival of low-temperature adapted *L. monocytogenes* LO28 cells (38). Two cold shock proteins, Csp1 and Csp3, were found to be induced after a cold shock to 10°C. Strikingly, Csp1 was also induced after 10 min exposure to 200 MPa (38). Analysis of the promoter sequence of the presumable coding gene, *cspL*, revealed putative σ^B-regulated –10 and –35 promoter sequences. It can be speculated that the relative sensitivity of the Δ*sigB* mutant to high pressure after exposure to low temperature may be due to the reduced expression of Csp1. Additionally, other (σ^B-regulated) proteins and other adaptation processes (e.g. membrane adaptations) might influence the survival during high pressure. For *L. monocytogenes*, the adaptation of membranes to low temperature is accomplished by altering branching in the methyl end of the fatty acid from iso to anteiso, and by shortening of the fatty acid chain length, resulting mainly in an increase of anteiso C15:0 fatty acids (1). In acid-adapted *L. monocytogenes* cells, straight-chain fatty acids C14:0 and C16:0 were significantly increased while levels of C18:0 decreased (27). In analogy, the fatty acids of barophilic microorganisms become more poly-unsaturated with increase in growth pressure (28). These observations together indicate that membrane adaptations that are induced in mildly acidic conditions may provide cross-protection against high hydrostatic pressure. The influence of the membrane fluidity on piezotolerance may be exerted through the control of the ion pumps in the membrane that are essential for maintaining pH homeostasis (26). Based on the importance of the GAD-system during adaptation to acidic conditions and its σ^B-dependence, this system may also have a large impact on piezotolerance of acid adapted wild-type and Δ*sigB* strains.

The results from this study contribute to the understanding of acid survival and adaptation of *L. monocytogenes*. σ^B-dependent protective mechanisms involved in survival and adaptation to low pH have been identified, including 3 genes of the GAD system and 9 proteins induced at acidic pH involved in stress protection and metabolism. Moreover, it is clear that acid adaptation confers, mainly in a σ^B-dependent manner, protection to high hydrostatic pressure and freezing. The observed phenomenon of cross-adaptation can have a significant impact on food processing, e.g. if acification of food products is combined with a pressurization treatment. Moreover, exposure of *L. monocytogenes* to low pH also has a positive impact on subsequent challenge with macrophagic cells (5). Indicating that low pH exposure does not only render more resistant *L. monocytogenes* cells, it also increases virulence.

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Chapter 4

Multiple deletions of the osmolyte transporters BetL, Gbu and OpuC of *Listeria monocytogenes* affect virulence and growth at high osmolarity

Summary

The success of *Listeria monocytogenes* as a food-borne pathogen owes much to its ability to survive a variety of stresses, both in the food environment and, after ingestion, within the animal host. Growth at high salt concentrations is attributed mainly to the accumulation of organic solutes such as glycine betaine and carnitine. We characterized *L. monocytogenes* LO28 strains with single, double and triple deletions in the osmolyte transport systems BetL, Gbu and OpuC. Of the single mutants altering Gbu has the most drastic effect on growth rate in BHI with 6% added NaCl. The highest reduction in growth rate was found for the triple mutant LO28BCG (Δ*betL*Δ*opuC*Δ*gbu*), although it was still capable of growth under these adverse conditions. In addition, we analyzed the growth and survival of this triple mutant in an animal (murine) model. LO28BCG showed a significant reduction in its ability to cause systemic infection following peroral co-inoculation with the wild type parent. Altering OpuC alone resulted in similar effects (R. D. Sleator, J. Wouters, C. G. M. Gahan, T. Abee, and C. Hill, Appl. Environ. Microbiol. 67:2692-2698, 2001), leading to the assumption that OpuC may play an important role in listerial pathogenesis. Analysis of the accumulation of osmolytes revealed that betaine is accumulated up to 300 μmol/ g dry weight when grown in BHI plus 6% NaCl, whereas no carnitine accumulation could be detected. Radiolabelled betaine uptake studies revealed an inability of BGSOE (Δ*betL*Δ*gbu*) and LO28BCG to transport betaine. Indeed, for LO28BCG no accumulated betaine was found, however carnitine was accumulated in this strain up to 600 μmol/g dry weight cells, indicating the presence of a possible fourth osmolyte transporter.

H. H. Wemekamp-Kamphuis, J. A. Wouters, R. D. Sleator, G. C. M. Gahand, C. Hill, and T. Abee. 2002. Multiple deletions of the osmolyte transporters BetL, Gbu, and OpuC of *Listeria monocytogenes* affect virulence and growth at high osmolarity. Appl. Environ. Microbiol. 68, 4710-4716.

Introduction

Listeria monocytogenes is a gram-positive food-borne pathogen that is highly resistant to osmotic stress (NaCl concentrations of up to 10%) and can grow at refrigeration temperatures (31). This characteristic growth and survival under such adverse environmental conditions is attributed mainly to the accumulation of the organic compounds glycine betaine (*N,N,N*-trimethylglycine) (16, 18) and carnitine (β-hydroxy-γ-N-trimethyl aminobutyrate) (3). Accumulated to high internal concentrations without adversely affecting vital cellular processes, these compounds are often referred to as "compatible solutes" (32). In general compatible solutes are small, highly soluble molecules which carry no net charge at physiological pH and function to stabilize protein structure and function, while also maintaining cell volume at elevated osmolarity (14, 27). Next to glycine betaine and carnitine, other compounds have been shown to function as effective compatible solutes in bacterial cells, such as sugars, amino acids, amino acid derivatives, sulphate esters and small peptides (14). For *L. monocytogenes* cells grown in complex media with 7.5% added NaCl, increases in glycine betaine and carnitine were accompanied by elevated concentrations of potassium, glutamate, glycine, alanine and proline (18). However, while proline at extracellular concentrations of <10 mM is incapable of promoting growth at elevated osmolarity (1, 18), a recent study by Sleator *et al*. (2001) revealed that growth at high salt concentrations in complex media is significantly reduced in a strain lacking the proline biosynthesis pathway (25). In a defined medium with 4% added NaCl, betaine, carnitine, acetyl-carnitine, γ-butyrobetaine and proline betaine all acted as osmoprotectants resulting in significant growth stimulation (1). Of these, betaine and carnitine are found to be the most effective osmoprotectants in *L. monocytogenes* (18, 3). Transport of glycine betaine into the cell was found to be induced 200-fold upon salt stress (16), and in another study the duration of transport of betaine and carnitine was found to be directly related to the osmotic strength of the medium (30).

The uptake of glycine betaine and carnitine is thought to be mediated *via* three osmolyte transporters: BetL, Gbu and OpuC (7, 15, 24, 28). Firstly, BetL of *L. monocytogenes* LO28 is a betaine transporter homologous to OpuD of *Bacillus subtilis* and BetP of *Corynebacterium glutamicum* (24). These systems belong to a family of secondary transporters transporting an ion, (9), in symport with the osmolyte (9). Deletion of the *betL* gene resulted in a lower specific growth rate at high osmolarities (26). However no significant difference in virulence potential was observed in the absence of a functional BetL transporter (26). The second transport system identified, Gbu, encoded by the *gbuABC* operon, belongs to the binding-protein-dependent ATP-binding cassette (ABC) superfamily of transporters and is homologous to OpuA in *B. subtilis* (15). An ATPase is encoded by *gbuA, gbuB* encodes a permease and *gbuC* a substrate binding protein. Membrane translocation of an osmolyte *via* Gbu is dependent on ATP hydrolysis. Deletion of *gbu* in *L. monocytogenes* 10403S results in

a decreased growth rate both at high salt concentrations (15) and at reduced temperatures (15). Moreover, glycine betaine transport activity in a *gbu* mutant was about fourfold lower than in wild type cells in modified Pine's medium with 8% NaCl (15). The most recently identified transporter, OpuC, also a member of the ABC superfamily, is dedicated mainly to carnitine uptake (7, 28). The *opuCABCD* operon, encoding the transporter, is homologous to *opuC* and *opuB* of *B. subtilis*. As with the Gbu system osmolyte uptake by OpuC is coupled to ATP hydrolysis. Carnitine transport was severely reduced in an *opuC* mutant of *L. monocytogenes* EGD (7). In addition virulence studies revealed that an *opuC* deletion mutant of *Listeria* exhibits a reduced ability to colonize the upper small intestine and cause subsequent systemic infection following peroral inoculation (28).

With the identification and characterization of these three osmolyte transporters the mechanism of salt tolerance is becoming clearer. However, the role and impact of accumulated osmolytes, both betaine and carnitine, during high osmolarity growth remains to be clarified. Using multiple deletion mutants in the known osmolyte transporters, the aim of this study was to describe the role of betaine and carnitine during growth at high osmolarity and in virulence.

Materials and methods

Strains, chemicals and growth conditions. The *L. monocytogenes* LO28 wild-type strain used throughout this study was a gift from P. Cossart, Institut Pasteur, Paris, France. Strains disrupted in the BetL (BSOE (24, 26)), Gbu (GSOE) or OpuC (LO28C (28)) transport systems, were used, as well as double and triple mutants constructed against these single mutant backgrounds. As we encountered problems in growing the double mutant LO28BC (Δ*betL*Δ*opuC*) this strain was excluded from the experiments. Strains were grown either in brain heart infusion (BHI) broth, or in the defined medium (DM), described by Premaratne *et al.* (21). When required the media osmolarity was adjusted by the addition of NaCl (DM + 3% NaCl: DMS). Where indicated, carnitine and glycine betaine (Sigma Chemical Co., St. Louis, Mo.) were added to DM(S) as filter-sterilized solutions to a final concentration of 1 mM. For growth of the single, double and triple mutants LO28C (Δ*opuC*), LO28CG (Δ*opuC*Δ*gbu*) and LO28BCG (Δ*betL*Δ*opuC*Δ*gbu*), erythromycin (10 μg/ml) was used as a selection marker. Experiments were performed at least twice, cell growth was monitored spectrophotometrically by measuring the optical density at 620 nm (OD_{620}) and growth rates were calculated.

Generation of mutants. The bank of single, double and triple mutants used in this study was constructed using a combination of plasmid (pORI19) mediated insertional mutagenesis, and gene disruption based on the splicing by overlap extension (SOE) procedure described by Horton *et al.* (1990). While the construction of BSOE (Δ*betL*) and LO28C (Δ*opuC*) was described previously (24, 26, 28), deletions in *gbuABC* were obtained using the

SOEing technique to remove a 1 kb fragment from the center of the operon. Essentially two ~ 325 bp PCR products (amplified by *gbu* SOEA [5′ GAATTCGTTAATTTTGAAAAAGACG G 3′] and *gbu* SOEB [5′ CCAGCATCAATTCCTGTGATTTCCAGAAAGTGCGGCCAG 3′] and *gbu* SOEC [5′ ATCACAGGAATTGATGCTGG 3′] and *gbu* SOED [5′ TCTAGAAGAAATTATCTAACACTTG 3′] respectively) flanking the sequence to be deleted, were spliced giving a ~ 650 bp hybrid which was subsequently cloned into the temperature sensitive shuttle vector pKSV-7, and transformed into *E. coli* DH5. The resulting plasmid designated pCPL17 was subsequently transformed into the LO28 wild type and Δ*betL* strains and the resulting transformants were selected on BHI plates containing 10 μg/ml chloramphenicol (Cm). Selection at 42°C of cells with chromosomal integration of pCPL17, followed by sequential passaging in BHI broth at 30°C in the absence of Cm, facilitated recovery of cells in which allelic exchange between the intact *gbuABC* operon and the ~ 650 bp insert on pCPL17 had occurred, thus giving rise to GSOE (Δ*gbu*) and BGSOE (Δ*betL*Δ*gbu*). The double mutants LO28BC (Δ*betL*Δ*opuC*) and LO28CG (Δ*opuC*Δ*gbu*) together with triple mutant LO28BCG (Δ*betL*Δ*opuC*Δ*gbu*) were constructed using pORI19 mediated insertional mutagenesis. Essentially plasmid pCPL5 (pORI19 containing 1.1 kb of the listerial *opuC* operon (28)) was transformed into strains BSOE (Δ*betL*), GSOE (Δ*gbu*) and BGSOE (Δ*betL*Δ*gbu*) containing the $RepA^+$, temperature sensitive helper plasmid pVE6007 and transformants were selected on BHI plates containing 5 μg/ml erythromycin (Ery) at 30°C. Temperature upshift from 30 to the non-permissive 42°C resulted in loss of pVE6007 and targeted chromosomal integration of pCPL5, thus creating LO28CG (Δ*opuC*Δ*gbu*), LO28BC (Δ*betL*Δ*opuC*) and LO28BCG (Δ*betL*Δ*opuC*Δ*gbu*) respectively.

Virulence assays. Bacterial virulence was determined by peroral co-inoculation of 8- to 12-week-old BALB/c mice as described by Sleator *et al*. (28). Essentially, mutant and wild-type strains were suspended in buffered saline with gelatin (0.85% NaCl, 0.01% gelatin, 2.2 mM K_2HPO_4, and 4.2 mM Na_2HPO_4). Each animal was infected with approximately 5 x 10^9 cells using a micropipette tip placed behind the incisors. At 3 days postinfection mice were euthanized and the listerial numbers in the livers and spleens of infected animals were determined by spread plating homogenized samples onto BHI agar plates.

Uptake studies. *L. monocytogenes* LO28 wild type and mutants were grown in DMS until early exponential phase (OD_{620} ~ 0.25). Osmolyte uptake studies were carried out essentially as described by Verheul *et al*. (30). Cells were concentrated to an OD_{620} of 20 in 50 mM potassium phosphate buffer (pH 6.8) containing 5 mM $MgSO_4$, 3% NaCl and chloramphenicol (100 μg/ml) to inhibit protein synthesis. Cells were stored on ice until use. Cells (OD_{620} of 1) were preenergized at 37°C with 1% glucose for 5 min prior to the addition of radiolabelled osmolyte *N,N,N*-[1-^{14}C]trimethylglycine (final concentration of 19 μM), purchased from Campo Scientific (Veenendaal, The Netherlands). Samples were withdrawn after 0.5, 1, 2, 3 and 4 min and uptake was stopped by addition of 2 ml of 50 mM potassium

phosphate buffer (pH 6.8) containing 5 mM $MgSO_4$ and 3% NaCl. The cells were collected on 0.2-μm-pore-size cellulose nitrate filters (Schleicher and Schuell GmbH, Dassell, Germany) under vacuum; the filters were washed with another 2 ml buffer and the radioactivity trapped in the cells was measured using a scintillation counter (model 1600TR; Packard Instruments Co., Downers Grove, I11). Uptake of osmolytes by the cells was normalized to total cellular protein. The experiment was performed in triplicate, and the results of a typical experiment are displayed in the figure.

Accumulation studies. Cells were grown in BHI at 30°C with 3 or 6% added NaCl until mid-exponential growth-phase (OD_{620} ~ 0.4). 100 ml of culture was pelleted and washed twice in a potassium phosphate buffer (50 mM with 5 mM $MgSO_4$, pH 6.8) with 3 or 6% added NaCl (isotonic with the growth medium). The cells were resuspended in 750 μl water and freeze-dried for determination of dry weight. The cells were extracted by the procedure of Galinski and Herzog (8), using methanol and chloroform. Betaine and carnitine concentrations were determined as described by Verheul *et al*. (30). Betaine and carnitine were measured by refractive index after HPLC using a LiChrosphere 100-NH_2, 5 μm column (Merck, darmstadt, Germany) at a flow rate of 1 ml/min at 45°C with a mobile phase of 80:20 (vol/vol) acetonitril-20 mM potassium phosphate (pH 7.0). The concentrations of betaine and carnitine were calculated from the area under the respective peaks using calibration curves. The experiment was performed in duplicate, and the results of a typical experiment are displayed in the figure.

Results

Growth of the osmolyte transporter mutants. *L. monocytogenes* is known for its ability to grow under high-osmolarity conditions. To identify the role of osmolytes in salt stress, we monitored the growth of the osmolyte uptake mutants in BHI with 6% added NaCl (Fig. 1).

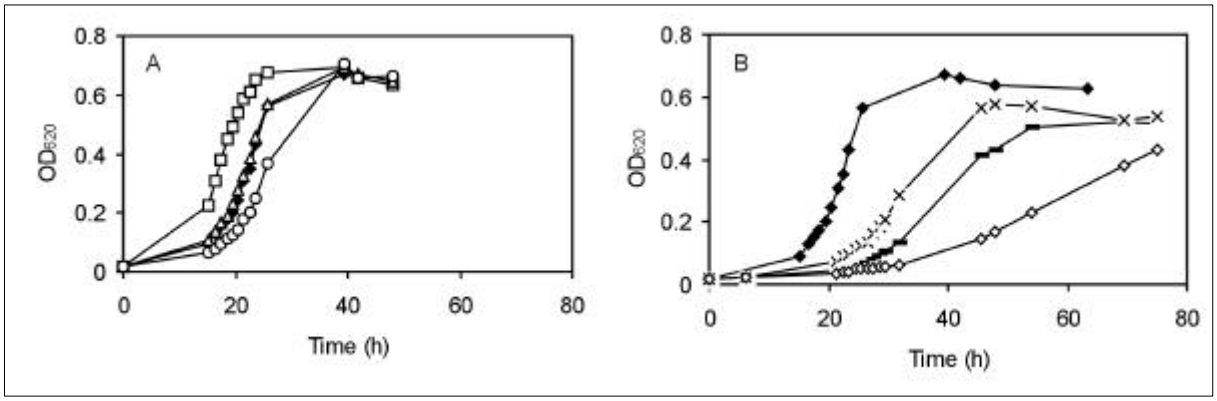

Figure 1. Growth of *L. monocytogenes* and osmolyte transporter mutants in BHI with 6% NaCl at 30°C. (A) LO28 (♦); BSOE (Δ*betL*) (?); GSOE (Δ*gbu*) (?); LO28C (Δ*opuC*) (Δ). (B)

LO28 (♦); BGSOE (Δ*betL*Δ*gbu*) (-); LO28CG (Δ*opuC*Δ*gbu*) (x); LO28BCG (Δ*betL*Δ*opuC*Δ*gbu*) (?).

Growth of wild-type LO28 and the mutants in BHI at 30°C was similar (data not shown). Upon addition of 6% NaCl the growth rate of wild type LO28 was 0.059 h^{-1} (Table 1). Similar growth rates were found for the single mutants BSOE (Δ*betL*) and LO28C (Δ*opuC*), while the growth rate of GSOE (Δ*gbu*) was reduced to 0.044 h^{-1}. Surprisingly, the lag time of the single mutant BSOE (Δ*betL*) was found to be shorter than that of the wild type. The growth rate of the double mutant BGSOE (Δ*betL*Δ*gbu)* was lower (0.022 h^{-1}) than that of LO28CG (Δ*opuC*Δ*gbu*) (0.029 h^{-1}), indicating the importance of the betaine transporters BetL and Gbu. Deletion of all three transporters resulted in a strain severely affected in its growth (0.010 h^{-1}). To identify the individual influence of betaine and carnitine, the strains were grown in DMS (3% NaCl) with added betaine and/or carnitine. Growth rate of *L. monocytogenes* in DMS increased by the addition of betaine or carnitine (Table 1).

Table 1. Growth rates (h^{-1} ± SD) of *L. monocytogenes* LO28 and mutants in BHI with 6% added NaCl, and DMS with or without additional compatible solutes at 30°C.

	LO28	BGSOE	LO28CG	LO28BCG
BHI + 6% NaCl	0.059 ± 0.003	0.022 ± 0.001	0.029 ± 0.001	0.010 ± 0.001
DMS	0.057 ± 0.007	0.031 ± 0.001	0.028 ± 0.002	0.028 ± 0.004
DMS + betaine	0.086 ± 0.005	0.034 ± 0.004	0.095 ± 0.008	0.038 ± 0.005
DMS + carnitine	0.074 ± 0.008	0.051 ± 0.007	0.041 ± 0.002	0.050 ± 0.008
DMS + betaine and carnitine	0.110 ± 0.017	0.038 ± 0.001	0.091 ± 0.008	0.041 ± 0.008

Relief of osmotic pressure was only partly achieved by addition of betaine to DMS for the double mutant BGSOE (Δ*betL*Δ*gbu*), while addition of carnitine resulted in significant growth stimulation (Fig. 2). While addition of betaine to LO28CG (Δ*opuC*Δ*gbu*) resulted in similar growth as observed for the wild type, the presence of carnitine also resulted in an increase in the growth of this strain. Surprisingly, the presence of carnitine resulted in a slight increase in the growth of the triple mutant too. Growth of this strain was promoted slightly upon addition of betaine and even more so by carnitine (Fig. 2), thus suggesting the presence of a fourth osmolyte transporter, transporting carnitine (and betaine).

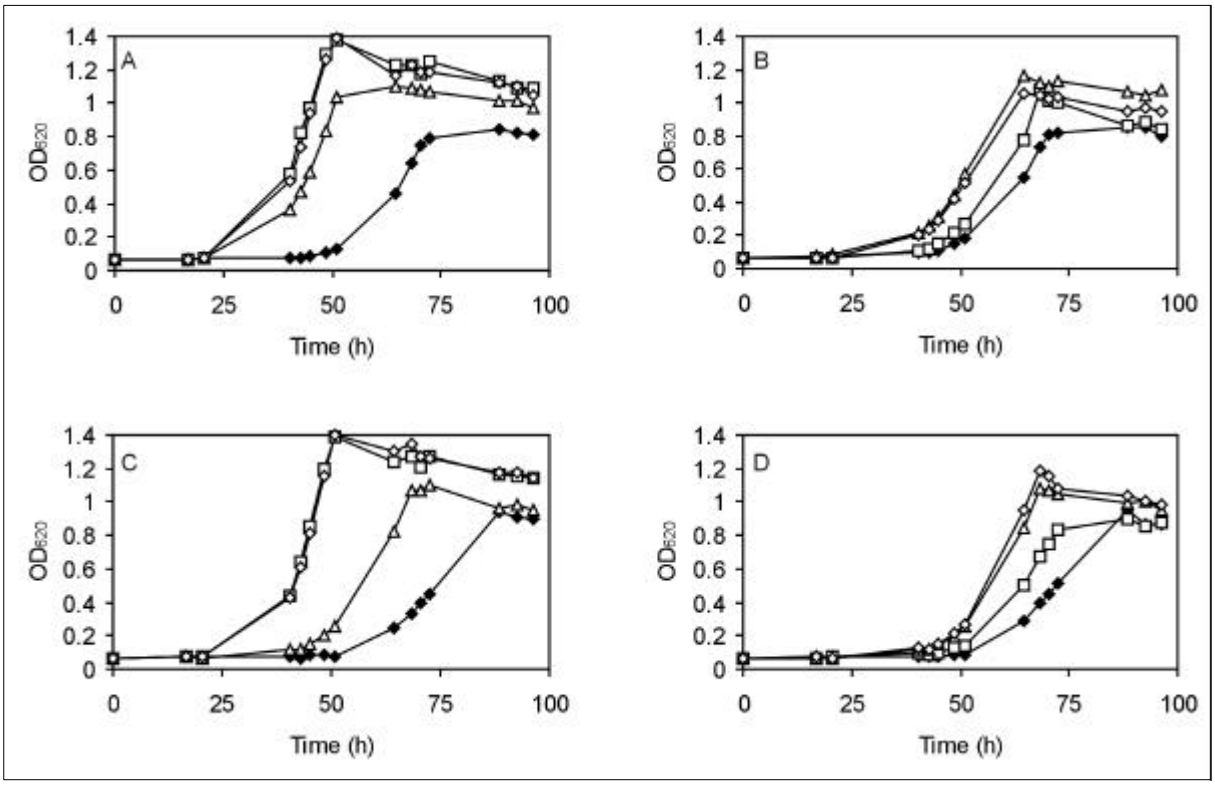

Figure 2. Growth at 30°C of *L. monocytogenes* LO28 (A), BGSOE (Δ*betL*Δ*gbu*) (B), LO28CG (Δ*opuC*Δ*gbu*) (C) and triple mutant (Δ*betL*Δ*opuC*Δ*gbu*) (D) in DMS without added osmolytes (♦), with 1 mM added betaine (?), with 1 mM added carnitine (?), with 1 mM betaine and 1 mM carnitine (?).

Virulence studies. Given that *Listeria* is a foodborne pathogen, the most common route of infection is *via* the oral route. Also since the osmolarity of the gastrointestinal tract (equivalent to 0.3 M NaCl) is approximately twice that of the blood stream, the ability of the mutants to reach and proliferate within the target organs (liver and spleen) was investigated following peroral co-inoculation with the wild-type parent. Consistent with previous investigations, only those mutants carrying deletions in the *opuC* operon appeared significantly affected in their ability to reach, and multiply within, the liver and spleens of infected animals (Fig. 3). These results suggest that OpuC is the most important osmolyte uptake system at least during infection of the animal (murine) model. This is not altogether surprising given that OpuC represents the major carnitine transporter in *Listeria* (7, 28), and carnitine is most likely the predominant osmolyte in animal tissues (4). Further evidence to suggest that OpuC may represent an important virulence factor in *Listeria* is the existence of two PrfA boxes. PrfA, the global regulator of virulence potential in *L. monocytogenes*, may thus control the transcription of *opuC*, or at least a component thereof. Located at positions 1458048 and 1457691 on the genome, the putative PrfA boxes carry only a single and double mismatch, respectively (10). The existence of such highly conserved regulatory domains, further supports our finding that carnitine uptake, at least via OpuC, plays an important role in listerial pathogenesis.

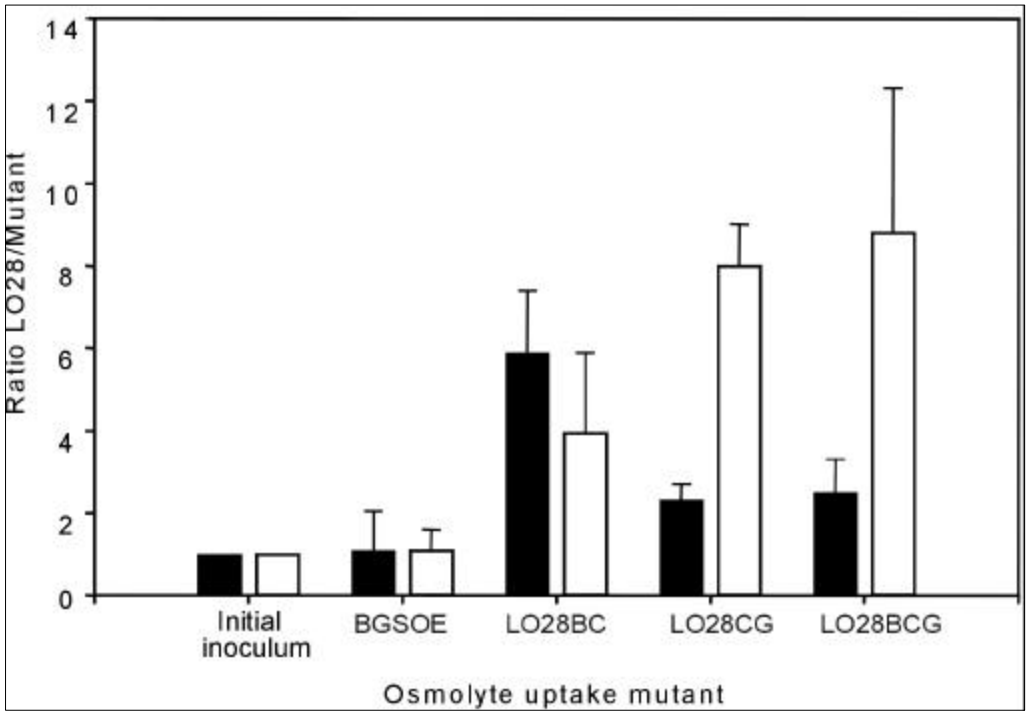

Figure 3. Virulence potential of the double and triple mutants following peroral co-inoculation of BALB/c mice. The ratio of the strains was determined both for the inoculum and the livers (black bars) and spleens (white bars) of infected animals three days post infection (*n* = 4).

Effect of osmolyte transporter deletions on betaine uptake. Radiolabelled osmolyte uptake studies were performed in order to assess the ability of the various osmolyte uptake mutants to transport betaine (Fig. 4). Betaine uptake was 43 nmol mg cell protein^{-1} min^{-1} in wild type LO28. Glycine betaine transport was reduced only slightly in LO28C (Δ*opuC*) (37 nmol mg cell protein^{-1} min^{-1}), indicating that transport of betaine is mainly via Gbu and BetL. This is supported by the finding that in strain BGSOE (Δ*betL*Δ*gbu*) no residual betaine transport could be detected. In strain LO28CG (Δ*opuC*Δ*gbu*) betaine transport was severely reduced (12 nmol mg cell protein^{-1} min^{-1}), however, its growth in DMS with added betaine is comparable to the growth of wild type LO28. Consistent with the results obtained for Δ*betL*, *gbu*, we were unable to detect any betaine uptake in the triple mutant. However, growth of these strains was stimulated slightly upon addition of betaine to DMS. We were only able to detect very low levels of carnitine uptake for the wild type LO28 and the mutants, as was also described by Sleator *et al.* (28).

Accumulation of betaine and carnitine. The concentrations of betaine and carnitine in cells growing at elevated osmolarity in BHI were analysed to determine the effects of mutating the different osmolyte transporters, and to detect differences in preferences of the respective transporters for the two osmolytes. Growth of the wild type and the mutants in BHI

at 30°C is similar (data not shown) and the concentration of accumulated betaine was low, about 70 μmol/g dry weight cells, while carnitine was accumulated to a non-detectable level,

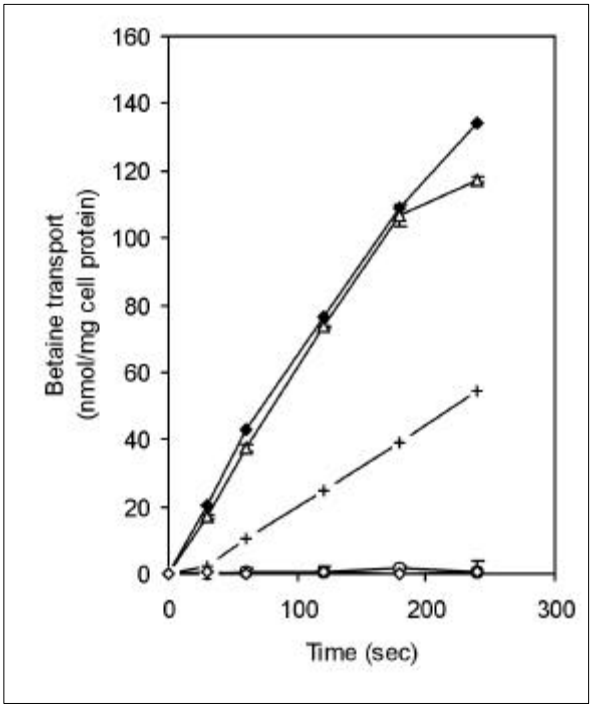

Figure 4. Glycine betaine transport. Wild-type strain LO28 (♦); LO28C (Δ*opuC*) (Δ); LO28CG (Δ*opuC*Δ*gbu*) (+); BGSOE (Δ*betL*Δ*gbu*) (?) and the triple mutant (Δ*betL*Δ*opuC*Δ*gbu*) (?) were assayed for *N,N,N*-[1-^{14}C]trimethylglycine uptake in the presence of 3% NaCl.

i.e. below 40 μmol/g dry weight cells (Fig. 5). The level of betaine accumulation increased with increasing osmolarity, addition of 3 or 6% NaCl resulted in 200 and 300 μmol/g dry weight betaine respectively. For the mutants BSOE (Δ*betL*) and LO28C (Δ*opuC*) the concentrations of accumulated betaine were similar to that of the wild type, and also in these mutants no carnitine accumulation was observed (Fig. 5). In GSOE (Δ*gbu*) less betaine was accumulated, compensated by increased accumulation of carnitine. Growing this strain in BHI with 6% added NaCl resulted in betaine levels of 125 μmol/g dry weight, while carnitine was accumulated to 300 μmol/g dry weight. For BGSOE (Δ*betL*Δ*gbu*), which lacks the two principle betaine transporters, no betaine accumulation from BHI was observed neither at low nor elevated osmolarity. However, carnitine levels reached almost 600 μmol/g dry weight, when this strain was cultured in BHI with 6% added NaCl. For the double mutant LO28CG (Δ*opuC*Δ*gbu*), betaine accumulation is possible via BetL, however, as this strain was found to be severely reduced in its ability to transport betaine (Fig. 4), also betaine accumulation levels were found to be threefold lower than in the wild-type strain. Moreover, carnitine accumulation was observed in this mutant, even though uptake of betaine by BetL is known not to be inhibited by carnitine (9). Also the triple mutant was able to accumulate carnitine to a concentration of about 600 μmol/g dry weight, although all the osmolyte transporters characterized to date are deleted in this strain. Furthermore, its growth was promoted by the

addition of carnitine to DMS. In combination, these results seem to indicate the presence of a fourth functional uptake system transporting carnitine from BHI at elevated osmolarity. Although the concentration of accumulated carnitine was very high in the triple mutant, its growth is severely impaired, indicating that carnitine might be a less effective osmoprotectant than betaine.

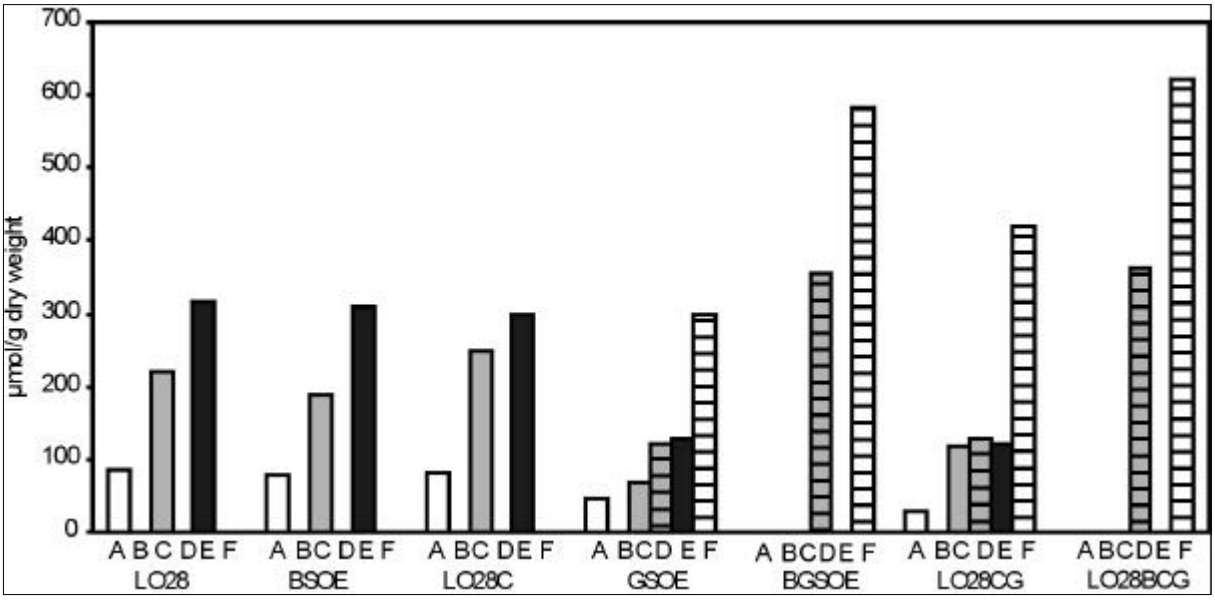

Figure 5. Accumulated betaine and carnitine from BHI by *L. monocytogenes* LO28 and osmolyte transporter mutants in µmol/g dry weight. A; betaine accumulated from BHI with 0% NaCl. B; carnitine accumulated from BHI with 0% NaCl. C; betaine accumulated from BHI with 3% NaCl. D; carnitine accumulated from BHI with 3% NaCl. E; betaine accumulated from BHI with 6% NaCl. F; carnitine accumulated from BHI with 6% NaCl.

Discussion

L. monocytogenes is a food-borne pathogen capable of growing at high osmolarity (NaCl concentrations of up to 10%). This characteristic growth and survival at elevated osmolarities results mainly from the accumulation of betaine and carnitine, the most effective osmoprotectants in *L. monocytogenes*. Molecular characterization of the salt tolerance of *L. monocytogenes* has been the focus of much attention in recent years (e.g. 27, 7, 15, 24, 26, 28). Combined with previous physiological investigations, genetic analysis has provided new insights into the mechanisms of listerial osmotolerance. With the use of multiple deletion mutants in the known osmolyte transporters we were able to describe the role of betaine and carnitine during growth at high osmolarity and in virulence.

In wild type LO28 betaine is the preferred osmolyte when it is grown in BHI at elevated osmolarity, whereas carnitine was only accumulated by the mutant strains impaired in their betaine uptake. The ability of glycine betaine to suppress the accumulation of other

osmolytes, both exogenously and endogenously produced, is typical in those bacterial species that can accumulate glycine betaine along with other osmolytes (13, 20, 22). For the single mutants only deletion of Gbu reduced betaine accumulation, indicating that Gbu is the main betaine transporter for *L. monocytogenes* LO28. The double mutant BGSOE (Δ*betL*Δ*gbu*) is dependent on OpuC for its osmolyte uptake. No accumulated betaine could be detected and its growth at high osmolarity was severely impaired. However, a high concentration of carnitine was present in the cells. These data indicate that carnitine is less effective than betaine in promoting listerial osmotolerance, a result which is in accordance with the lower growth rate observed for wild type cells grown in DMS supplemented with carnitine as opposed to the same concentration of betaine (29). In *Escherichia coli* it was also found that betaine is a better osmoprotectant than carnitine, and it was suggested that the longer carbon chain length of carnitine decreases its osmoprotective function (19).

In virulence assays, a significant reduction in virulence potential was observed following peroral co-inoculation of the wild type and the triple mutant. In a previous study, LO28C (Δ*opuC*) exhibited similar effects (28), leading to the assumption that OpuC, and herewith carnitine uptake, is an important virulence factor in *L. monocytogenes*. The relative abundance of carnitine in mammalian tissues (4) makes it the most readily available and thus possibly the most important osmolyte contributing to the survival of *L. monocytogenes* during infection (28). Growth experiments in DMS with carnitine as the only available osmolyte, showed an increase in growth rate for wild type LO28 and BGSOE (Δ*betL*Δ*gbu*) mutant. Carnitine only slightly promotes growth for LO28CG (Δ*opuC*Δ*gbu*) and the triple mutant, indicating that these strains are not as well protected as the wild type in high osmolarity environments, a factor which may play a role during virulence, particularly given the elevated osmolarity of the blood stream (equivalent to 0.15 M NaCl) and gastrointestinal tract (equivalent to 0.3 M NaCl). Analysis of the genome of *L. monocytogenes* EGD-e (at http://genolist.pasteur.fr/listilist) revealed a putative fourth osmolyte transporter, with homology to *opuB* of *B. subtilis* (12) and exhibiting a functional organization similar to that of BusA (OpuA) in *Lactococcus lactis* (17). The operon consists of two genes, *opuBA* and *opuBB*, located in the vicinity of and oppositely orientated with respect to the *opuC* operon on the *L. monocytogenes* EGD-e genome. In *B. subtilis* OpuB transports choline, which is converted into betaine by two intracellular enzymes (GbsA and GbsB) (6). The transport of choline and its subsequent conversion to betaine affords considerable osmoprotection to the cells (5). We propose that the listerial OpuB transporter is also capable of transporting carnitine. Transport studies using the double mutant BGSOE (Δ*betL*Δ*gbu*) and the triple mutant fail to show any betaine transport, indicating that the remaining transporter OpuB may not have a high affinity for betaine.

The fact that at least three, and probably four, osmolyte transporters are present in *L. monocytogenes*, emphasizes the physiological significance of osmoregulation for this strain, similar to that for *B. subtilis* and *C. glutamicum*. The need for several osmoregulatory systems

may lay in the need to mediate the uptake of structurally diverse compatible solutes available in diverse ecological niches, the involvement of different transporters in response to environments with different osmolalities and the associated need for differential regulation of transporter expression. Betaine, for example, is abundant in plant tissue (23), while carnitine is a major component of muscle tissue (29). BetL is relatively effective only when the osmotic stress is provided by or accompanied by sodium ions, since transport of betaine *via* this transporter is known to be coupled to the influx of Na^+ ions (9). Hence, this transport system cannot explain the osmotically activated transport observed at low Na^+ concentrations. In such an environment *L. monocytogenes* must possess an active ATP-dependent osmolyte transporter. Another benefit of the ATP-dependent transporter Gbu is that it is able to concentrate osmolytes to a much higher level than ion-metabolite symporters. From RNA slot blots and transport studies it is clear that, as is also observed for OpuD in *B. subtilis* (11), both the expression of *gbu* and *betL* as well as the activity of the encoded enzymes is subject to regulation by the external osmolarity (16, 26). The stress-inducible sigma factor, σ^B, may play a role in regulating the expression of both betaine and carnitine transporters. A *L. monocytogenes* mutant lacking σ^B is defective for growth in a defined medium with elevated osmolarity in the presence of either betaine or carnitine, whereas in defined medium at normal osmolarity no significant difference in growth is observed between the mutant and wild type (2).

Currently, the OpuB transporter is being analyzed and a mutant for this transporter is being generated in our laboratory. The generation of a quadruple mutant would make it possible to investigate the role of osmolytes in several stress conditions in more detail, since it is predicted that this strain will be totally impaired in the uptake of betaine and carnitine.

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Chapter 5

Molecular and physiological analysis of the role of the osmolyte transporters BetL, Gbu and OpuC in the growth of *Listeria monocytogenes* at low temperatures

Summary

Listeria monocytogenes is a ubiquitous food-borne pathogen, found widely distributed in nature as well as an undesirable contaminant in a variety of fresh and processed foods. This ubiquity can be at least partly explained by the ability of the organism to grow at high osmolarity and reduced temperatures, a consequence of its ability to accumulate osmo- and cryoprotective compounds termed osmolytes. Single and multiple deletions of the known osmolyte transporters BetL, Gbu and OpuC significantly reduce growth at low temperature. During growth in BHI at 7°C, Gbu and OpuC had a more pronounced role in cryoprotection than did BetL. However, upon addition of betaine to defined media the hierarchy of transporter importance shifted: Gbu>BetL>OpuC. Upon addition of carnitine, only OpuC appeared to play a role. Measurements of the accumulated osmolytes showed that betaine is preferred over carnitine, while in the absence of a functional Gbu, carnitine was accumulated to higher levels than betaine at 7°C. Transcriptional analysis of the genes encoding BetL, Gbu and OpuC revealed that each is induced to varying degrees upon cold shock of *L. monocytogenes* LO28. Additionally, despite being transcriptionally upregulated upon cold shock, a putative fourth osmolyte transporter OpuB (identified by bioinformatic analysis and encoded by *lmo1421* and *lmo1422*) showed no significant contribution to listerial chill tolerance. Growth of the quadruple mutant LO28ΔBCGB (Δ*betL,* Δ*opuC,* Δ*gbu,* Δ*opuB)* was comparable to the triple mutant LO28ΔBCGsoe (Δ*betL,* Δ*opuC,* Δ*gbu)* at low temperatures. Here, we can conclude that betaine and carnitine transport upon low temperature exposure is mediated *via* three osmolyte transporters, BetL, Gbu and OpuC.

H. H. Wemekamp-Kamphuis, R. D. Sleator, J. A. Wouters, C. Hill and T. Abee. Molecular and physiological analysis of the role of the osmolyte transporters BetL, Gbu and OpuC in the growth of *Listeria monocytogenes* at low temperatures. Submitted for publication.

Introduction

The gram-positive food-borne pathogen *Listeria monocytogenes* accounts for almost 35% of all deaths in the USA due to known food-borne bacterial pathogens (27). A number of recent outbreaks have been associated with ready-to-eat foods that have been minimally processed (13). As the demand for fresh food products is increasing, cold storage of these products is becoming more widespread. This continuing trend toward minimal food processing and reliance on refrigeration as a preservation technique has in turn been accompanied by a steady increase in the incidence of food poisoning, particularly by psychrotrophic pathogens such as *L. monocytogenes*, which can grow at temperatures as low as –0.1°C (43).

Mechanisms that allow low-temperature growth of microorganisms involve the maintenance of the cellular membrane fluidity, structure stabilization of macromolecules such as ribosomes, necessary for continued protein synthesis, and the uptake or synthesis of compatible solutes (21). For *L. monocytogenes*, the adaptation of membranes to low temperature is accomplished by altering branching in the methyl end of the fatty acid from iso to anteiso, and by shortening of the fatty acid chain length, resulting mainly in an increase of anteiso C15:0 fatty acids (6). In *L. monocytogenes* instability in the 70S ribosomal particle structure is found upon cold shock (8), which must be overcome to allow normal protein synthesis. In *Escherichia coli*, protein synthesis after cold shock is related to the synthesis of so-called cold-shock proteins (CSPs) (30). In *L. monocytogenes* LO28 four cold-shock proteins have been identified, two of which are produced in increased amounts following a cold shock from 30 to 10°C (44).

With the exception of proline (35), *L. monocytogenes* appears unable to synthesize osmolytes (25), either *de novo*, or from precursor compounds (36). However, transport of the principal osmolytes glycine betaine (*N,N,N*-trimethylglycine) and carnitine (β-hydroxy-γ-N-trimethyl aminobutyrate) upon low temperature stress was found to be increased 15- and 4.5-fold, respectively (25, 4).

To date, three osmolyte transporters: Gbu, OpuC, and BetL (4, 14, 18, 19, 24, 28, 34, 36, and a review in 39), dedicated to betaine and carnitine uptake in *L. monocytogenes* have been identified and characterized experimentally. However recent *in silico* analysis of the *L. monocytogenes* EGD-e genome sequence (20) revealed a fourth putative osmolyte transporter with significant homology to the high-affinity choline uptake system OpuB of *B. subtilis*. Consisting of two genes, *lmo1421* and *lmo1422*, this operon is located approximately 2.4 kb downstream of *opuC* on the listerial chromosome (36). While a possible role for OpuB as a carnitine uptake system in *Listeria* has previously been suggested (15, 45), Angelidis and Smith (3) recently demonstrated that, at least in *L. monocytogenes* 10403S, carnitine uptake is mediated exclusively by OpuC and Gbu. However, the existence, or potential impact, of OpuB was not examined in this strain.

The principal betaine uptake system Gbu is a binding-protein-dependent ATP-binding cassette (ABC) transporter homologous to OpuA in *B. subtilis* (24). The growth rate of *L. monocytogenes* 10403S in the absence of a functional Gbu transporter was significantly lower than that of the wild type at 7°C, with uptake rates for [^{14}C]glycine betaine being reduced ~8-fold in this mutant (24). Moreover, *in vitro* activation of the Gbu transport activity was demonstrated to occur in membrane vesicles at reduced temperatures (18). OpuC, the principal carnitine transporter, encoded by the *opuCABCD* operon (14, 39), is homologous to *opuC* and *opuB* in *B. subtilis* and is also an ABC transporter, coupling ATP hydrolysis to osmolyte transport across the membrane. An interesting feature of *opuC* is that it is preceded by a consensus σ^B-dependent promoter binding site (14, 16), which may also imply chill-stimulated osmolyte uptake, since transcription of σ^B has itself been shown to be upregulated in response to temperature downshift (9). Indeed, a σ^B deletion mutant of *L. monocytogenes* 10403S exhibited reduced growth rates at 8°C in DM supplemented with either betaine or carnitine (0.011 and 0.010 h^{-1}, respectively) compared with wild-type 10403S (0.018 and 0.017 h^{-1}, respectively) (10). Finally, BetL, a secondary betaine uptake system that couples a Na^+-motive force to solute transport across the membrane, is homologous to OpuD of *Bacillus subtilis* and BetP of *Corynebacterium glutamicum* (34). The reduced growth rate for the 10403S σ^B deletion mutant at 8°C upon addition of betaine (10) might reflect the presence of a putative σ^B dependent promoter binding site upstream of *betL* (37). However, the growth rate of a strain in which *betL* is functionally inactivated was not affected at 4°C (37), in addition, *in vitro* betaine transport *via* BetL in proteoliposomes does not appear to be activated by cold (19).

Previously we constructed a set of mutants carrying deletions in the known osmolyte uptake systems Gbu, BetL and OpuC, and determined the role of these systems in listerial growth and survival at elevated osmolarity (37, 39, 45). Here we employ a similar strategy to analyze the role of osmolyte uptake in growth at refrigeration temperature (7°C). Recently, Angelidis *et al*. (5) characterized a Gbu mutant at low temperature and predicted that additionally removing BetL may further impair growth, while deleting *opuC* from this background would possibly eliminate growth altogether under cold stress. Herein, we demonstrate that while the proposed triple mutant LO28ΔBCGsoe (Δ*betL,* Δ*opuC,* Δ*gbu)* is indeed severely affected at low temperature (illustrating the importance of these systems to listerial chill tolerance), growth is not completely abolished, thus suggesting the existence of at least one additional cryoprotective system. However, removing OpuB, a putative fourth osmolyte uptake system, from the existing triple mutant background showed no further reduction in growth at low temperature, thus ruling out a potential cryoprotective role for OpuB in *Listeria*.

Materials and methods

Strains, chemicals and growth conditions. Bacterial strains used in this study are listed in Table 1. Strains were grown either in brain heart infusion (BHI) broth (Oxoid, Basingstoke, United Kingdom), or in defined medium (DM) (31). Where indicated, carnitine and glycine betaine (Sigma Chemical Co., St. Louis, Mo.) were added to DM as filter sterilized solutions to a final concentration of 1 mM. For growth of LO28ΔC (Δ*opuC*), LO28ΔCG (Δ*opuC,* Δ*gbu*), and LO28ΔBCG (Δ*betL,* Δ*opuC,* Δ*gbu*) erythromycin (10 μg/ml) was used as a selection marker. Cell growth was monitored spectrophotometrically by measuring the optical density at 620 nm (OD_{620}) and growth rates were calculated at exponential phase (standard deviations varied within 10% of the values).

Table 1. Bacterial strains.

Strain	Relevant genotype or characteristic	Source or reference
L. monocytogenes		
LO28 (Serotype 1/2c)	*betL*$^+$, *gbu*$^+$, *opuC*$^+$, *opuB*$^+$	P. Cossart, Institut Pasteur
LO28ΔB formerly BSOE	LO28 Δ*betL*	(34)
LO28ΔC formerly LO28C	LO28 Δ*opuC*, *opuC*::pCPL5	(39)
LO28ΔG formerly GSOE	LO28 Δ*gbu*	(45)
LO28ΔBG formerly BGSOE	LO28 Δ*betL*, Δ*gbu*	(45)
LO28ΔCG formerly LO28CG	LO28 Δ*opuC*, Δ*gbu*, *opuC*::pCPL5	(45)
LO28ΔBCG formerly LO28BCG	LO28 Δ*betL*, Δ*opuC*, Δ*gbu*, *opuC*::pCPL5	(45)
LO28ΔBCGsoe	LO28 Δ*betL*, Δ*opuC*, Δ*gbu*	This study
LO28ΔBCGB	LO28 Δ*betL*, Δ*opuC*, Δ*gbu*, Δ*opuB*	Chapter 6

Creation of the triple SOEing mutant LO28BCGsoe. For creation of the triple SOEing mutant LO28BCGsoe, the SOE (oSOE-A, oSOE-B, oSOE-C, and oSOE-D) and forward and reverse (oSOE-F and oSOE-R) primers used were of the same sequence as those used by Angelidis and Smith (3). The hybrid 882-bp *opuC* construct (comprised of two regions in the 5′ and 3′ end of *opuC*) was digested with *Hind*III and *Bam* HI, cloned into pKSV7, and transformed into *E. coli* DH5α to yield pCPL18. This construct was then electroporated into *L. monocytogenes* LO28ΔBG, and successful transformants were selected on BHI plates containing 10 μg/ml chloramphenicol (Cm). Selection at 42°C of cells with chromosomal integration of pCPL18, followed by sequential passaging in BHI broth at 30°C in the absence of Cm, facilitated recovery of cells in which allelic exchange between the intact *opuC* operon and the 882 bp insert on pCPL18 had occurred, thus giving rise to LO28BCGsoe. Confirmation of the deletion event was obtained by PCR using the forward and reverse primers oSOE-F and oSOE-R.

Transcriptional analysis of the osmolyte transporters. RT-PCR experiments were performed essentially as described by Sleator *et al*. (37). *L. monocytogenes* cells were grown to mid-exponential phase at 37°C. Cells were then centrifuged and resuspended in 1 ml of

BHI at 10°C or at 37°C (control temperature). After 15 and 30 minutes incubation, cells were harvested and flash-frozen at -80°C. RNA was extracted using a hot acid phenol procedure described by Ripio *et al*. (33), and cDNA was synthesized by adding 1 µl of total RNA to 4 µl of 5x RT buffer (Roche), 2 µl of 100 mM dithiothreitol, 0.5 µl of a deoxynucleoside triphosphate mix (dATP, dCTP, dGTP, and dTTP; each 10 mM), 0.25 µl of RNasin, 100 ng of the random primer $p(dN)_6$, and 1 µl of Expand reverse transcriptase (Roche). The reaction mixture was incubated at 42°C for 9 h. PCR on cDNA was carried out using the following primers: for *gbu*, *gbu* SOEA and *gbu* SOEB (45), targeting *gbuB*; for *betL*, *betL* SOEC and *betL* SOED (37); for *opuC*, *opuC*-F (5'-CACCAAAAGTAGCGAAC-3') and *opuC*-R (5'-GAATTAAGTCTGGACGGTATAAG-3'), targeting *opuCB*; for opuB, *opuB*-F (5'-ATCTAGAAAATGACTTCGTTAAA-3') and *opuB*-R (5'-ACAAGCTCACCTGAACTTTCC-3'), targeting *lmo1421*; and for 16S RNA, 16SRNA-E (5'-TTAGCTAGTTGGTAGGGT-3') and 16SRNA-B (5'-AATCCGGACAACGCTTGC-3'). PCRs were carried out for 16, 22 and 30 cycles to allow optimal quantification of PCR products.

Uptake studies. *L. monocytogenes* LO28 wild type was grown in DM at 30°C until early exponential phase (OD_{620} ~ 0.25). Where indicated, a cold shock to 10°C was performed, as previously described (44). Osmolyte uptake studies were carried out essentially as described by Verheul *et al*. (40). Cells were concentrated to an OD_{620} of 20 in 50 mM potassium phosphate buffer (pH 6.8) containing 5 mM $MgSO_4$, and chloramphenicol (100 µg/ml) to inhibit protein synthesis. Cells (OD_{620} of 1) were pre-energized at 37°C with 1% glucose for 5 min prior to the addition of radiolabelled osmolyte *N,N,N*-[1-^{14}C]trimethylglycine (final concentration of 19 µM), purchased from Campo Scientific (Veenendaal, The Netherlands). Samples were withdrawn after 0.5, 1, 2, 3 and 4 min and uptake was stopped by addition of 2 ml of cold 50 mM potassium phosphate buffer (pH 6.8) containing 5 mM $MgSO_4$. The cells were immediately after the reaction was stopped collected on 0.2-µm-pore-size cellulose nitrate filters (Schleicher and Schuell GmbH, Dassell, Germany) under vacuum; the filters were washed with another 2 ml buffer and the radioactivity trapped in the cells was measured using a scintillation counter (model 1600TR; Packard Instruments Co., Downers Grove). Uptake of osmolytes by the cells was normalized to total cellular protein. The experiment was performed in duplicate, and the results of a typical experiment are displayed in Fig. 4.

Accumulation studies. Cells were grown in BHI at 30°C or at 7°C until mid-exponential growth-phase (OD_{620} ~ 0.4). 100 ml of culture was pelleted and washed twice in potassium phosphate buffer (50 mM with 5 mM $MgSO_4$, pH 6.8, 7°C) before being resuspended in 750 µl water and freeze-dried for determination of dry weight. The cells were extracted by the procedure of Galinski and Herzog (17), using methanol and chloroform. Betaine and carnitine concentrations were determined as described by Verheul *et al*. (40). Betaine and carnitine were measured by refractive index after HPLC using a LiChrosphere 100-NH_2, 5 µm column (Merck, darmstadt, Germany) at a flow rate of 1 ml/min at 45°C with

a mobile phase of 80:20 (vol/vol) acetonitril-20 mM potassium phosphate (pH 7.0). The concentrations of betaine and carnitine were calculated from the area under the respective peaks using calibration curves. The experiment was performed in duplicate, and the results of a typical experiment are displayed in Fig. 5.

Results

Deletion of osmolyte transporters reduces growth at low temperature. To identify the role of each osmolyte transporters in growth at low temperature, we monitored the growth of various osmolyte uptake mutants in BHI at 7°C (Fig. 1). While growth of the wild type and mutant strains in BHI at 30°C was similar (data not shown), at 7°C the effect of deleting the various osmolyte transporters was clearly observed.

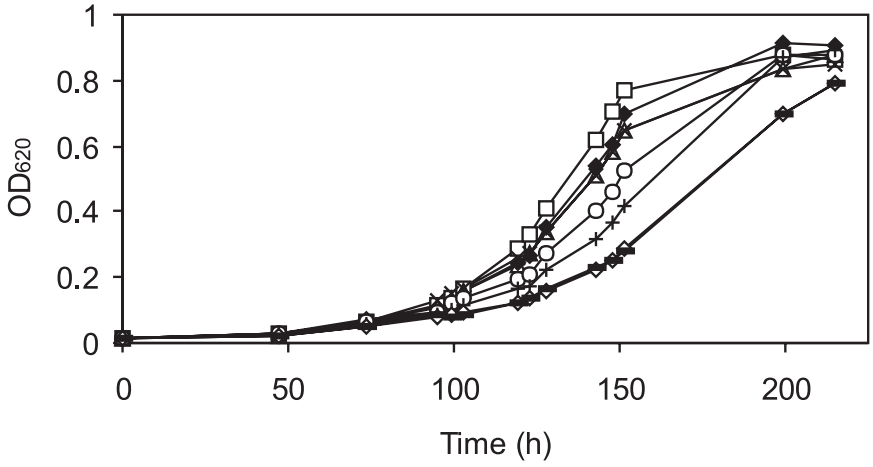

Figure 1. Growth of LO28 and osmolyte uptake mutants in BHI at 7°C. LO28 (♦); LO28ΔB (Δ*betL*) (□); LO28ΔC (Δ*opuC*) (Δ); LO28ΔG (Δ*gbu*) (x); LO28ΔBG (Δ*betL*,Δ*gbu*) (○); LO28ΔCG (Δ*opuC*,Δ*gbu*) (+); LO28ΔBCGsoe (Δ*betL*,Δ*opuC*,Δ*gbu*) (◊); LO28ΔBCGB (Δ*betL*,Δ*opuC*,Δ*gbu*,Δ*opuB*) (-).

The growth rate of the wild type in BHI at 7°C was 0.019 OD_{620} h^{-1} (Table 2), deleting *gbu* or *opuC* decreased growth rates to 0.016 OD_{620} h^{-1}, whereas removing *betL* had no significant effect. Deleting all three transporters in combination had the most dramatic effect resulting in an almost 3-fold reduction in the growth rate (0.019 to 0.007 OD_{620} h^{-1}). Notably, growth of the quadruple mutant LO28ΔBCGB was similar to that of the triple mutant suggesting that, at least under the conditions tested, OpuB is unlikely to contribute to listerial chill tolerance. To study the individual contribution of betaine and carnitine to low temperature adaptation, defined medium (DM) was used. In DM the growth rate for all strains was ~0.0016 (± 0.0001) OD_{620} h^{-1} at 7°C (Table 2).

Table 2. Growth rate (*10^{-3} OD_{620} h^{-1}) of *L. monocytogenes* and mutants at 7°C.

Strain	BHI	DM	DM betaine	DM carnitine	DM betaine carnitine
LO28	19	1.7	3.3	2.7	3.0
LO28ΔB	18	1.7	2.9	2.5	2.6
LO28ΔC	16	1.6	2.8	2.2	2.5
LO28ΔG	16	1.7	2.6	2.7	2.8
LO28ΔBG	15	1.6	2.4	2.9	2.8
LO28ΔCG	11	1.6	2.3	2.3	2.8
LO28ΔBCG	8	1.6	2.1	2.3	2.3
LO28ΔBCGsoe	7	1.6	1.7	1.7	1.6
LO28ΔBCGB	7	1.7	1.7	1.6	1.6

While addition of 1 mM betaine resulted in a doubling of the growth rate of wild type, growth of the mutants was less well induced (Fig 2A). Given that growth rates were most affected following deletion of *gbu* (Table 2), it appears that the Gbu system is the most effective transporter during low temperature growth. Interestingly, it has previously been demonstrated that for growth at elevated osmolarities (3% NaCl), Gbu also plays the dominant role (46). Upon addition of carnitine to DM, the role of OpuC in carnitine transport at low temperature is apparent (Table 2). Only those mutants deficient in their OpuC transporter show reduced growth rates compared to the wild type, indicating the importance of OpuC in carnitine uptake at low temperatures. In addition, deletion of the two principal betaine uptake systems Gbu and BetL (strain LO28ΔBG) does not influence the growth rate or characteristics at 7°C compared with the wild type upon addition of carnitine to DM (Fig. 2B and Table 2). Furthermore, growth of the triple mutant LO28ΔBCGsoe and the quadruple mutant LO28ΔBCGB were not induced upon addition of betaine or carnitine to the medium, supporting our proposal that OpuB is unlikely to play a role in betaine or carnitine uptake and subsequent cryoprotection.

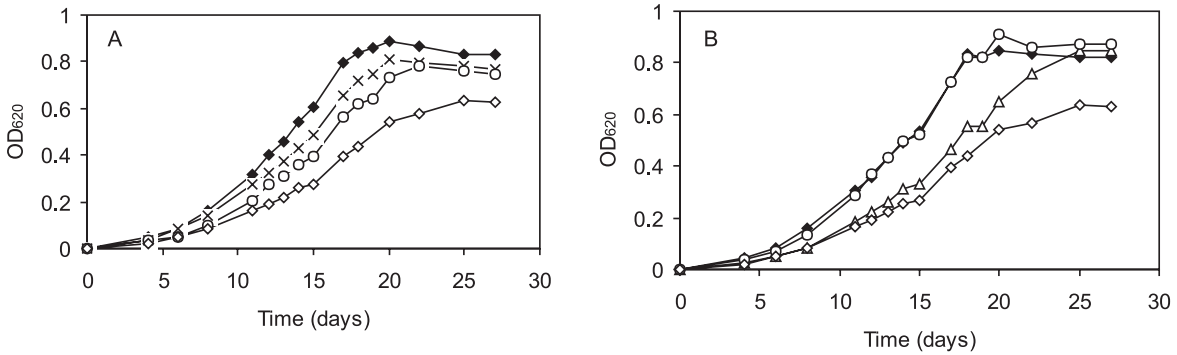

Figure 2. Growth of *L. monocytogenes* LO28 and osmolyte uptake mutants in DM at 7°C with betaine (A) or carnitine (B). (A) LO28 (♦); LO28ΔG (Δ*gbu*) (x); LO28ΔBG (Δ*betL,*Δ*gbu*) (○); LO28ΔBCGsoe (Δ*betL,*Δ*opuC,*Δ*gbu*) (◊). (B) LO28 (♦); LO28ΔC (Δ*opuC*) (Δ); LO28ΔBG (Δ*betL,*Δ*gbu*) (o); LO28ΔBCGsoe (Δ*betL,*Δ*opuC,*Δ*gbu*) (◊).

Induced transcription of osmolyte transporter genes at low temperature. RT-PCR was performed to analyze the transcription of the different transporter genes following cold shock. Though expression levels of the genes at 37°C were just on the detection limit, both *gbu* and *betL*, encoding the principal betaine uptake systems were significantly up-regulated following exposure to 10°C for 30 min (Fig. 3). However, the level of *gbu* up-regulation appears greater than that of *betL* following cold shock. This result is consistent with the observation that Gbu provides significantly more cryoprotection than does BetL. The carnitine transporter-encoding genes were also clearly induced following cold shock. In addition, transcription of the operon encoding OpuB, a putative fourth osmolyte uptake system, was also significantly induced upon low temperature exposure of *L. monocytogenes* cells.

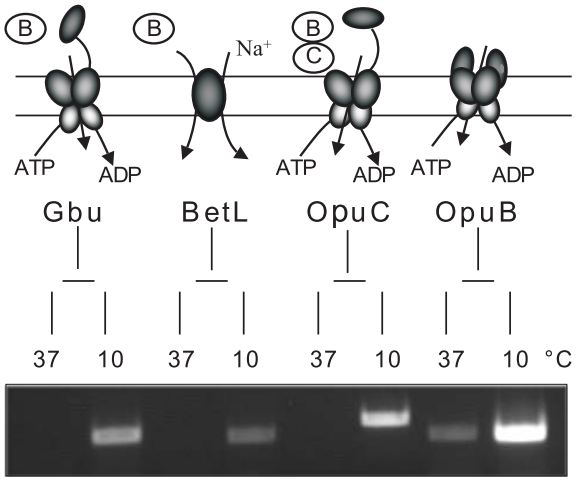

Figure 3. Analysis of the transcription of the four osmolyte transporters in *L. monocytogenes* after cold shock. RNA was isolated from mid-exponential phase cells prior to cold shock and from cells exposed to 10°C for 30 min. The results after 30 cycles are shown.

Betaine and carnitine accumulation at low temperature. Increased transcription of the transporters upon cold shock (Fig. 3) suggests an increased uptake of osmolytes at low temperature. However when *L. monocytogenes* LO28 wild-type cells, cold shocked to 10°C, were tested for betaine uptake after 4 h at 10°C, no increase in betaine uptake at low temperature was observed within the time frame measured (Fig. 4). Furthermore, the uptake rate at 37°C for cold-shocked cells was lower than that for exponential phase cells grown at 37°C. In contrast, Ko *et al*. (25) and Mendum and Tombras Smith (28) showed that *L. monocytogenes* cells (Scott A and 10403S, respectively) grown at low temperature have an increased betaine uptake at this temperature compared to cells grown and tested at 30°C. In addition, cold-shocked 10403S cells tested by Becker *et al*. (10), were found to have comparable betaine uptake levels at 8°C and 25°C.

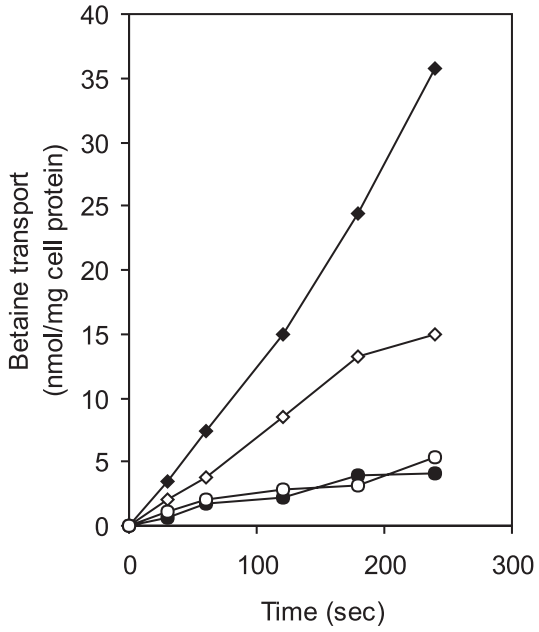

Figure 4. Glycine betaine transport in *L. monocytogenes* LO28 at 37°C: Exponential phase cells (♦); 4h cold-shocked cells (◊). Glycine betaine transport at 10°C: Exponential phase cells (•); 4h cold-shocked cells (o).

Notably, the internal concentration of betaine and carnitine during growth of wild-type LO28 in BHI at 7°C was 300 and 120 μmol/g dry weight cells, respectively (Fig. 5), whereas during growth at 30°C the betaine concentration was 70 μmol/g dry weight cells, while carnitine was not accumulated to detectable levels (45). This indicates that *L. monocytogenes* LO28 cells do accumulate betaine and carnitine at low temperature. For the single mutants LO28ΔB and LO28ΔC similar accumulation levels were found as in the wild type (Fig. 5).

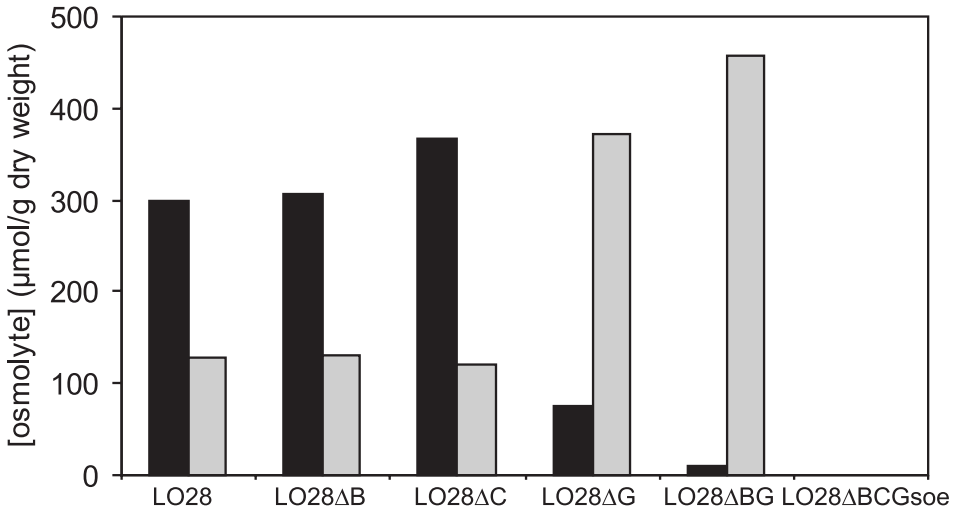

Figure 5. Accumulated betaine (black) and carnitine (shaded) by *L. monocytogenes* LO28 and osmolyte transporter mutants grown in BHI at 7°C.

However, in LO28ΔG the betaine concentration is much lower (~70 μmol/g dry weight cells), which is apparently compensated for by the accumulation of carnitine. In strain LO28ΔBG (Δ*betL,* Δ*gbu*) OpuC is the remaining transporter, responsible for the accumulated carnitine (about 450 μmol/g dry weight cells). Analysis of the triple mutant LO28ΔBCGsoe, following growth at 7°C in BHI revealed no accumulated betaine or carnitine (with a detection limit of about 20 μmol/g dry weight cells). Thus, at least under the conditions tested, BetL, Gbu and OpuC appear to be the only effective betaine and carnitine uptake systems effective during low temperature growth in *L. monocytogenes*.

Discussion

L. monocytogenes is a food-borne pathogen capable of growth at low temperatures. Given the increasing reliance on refrigeration as a method of food preservation (1), the psychrotropic nature of the pathogen is an especially important consideration from a food safety perspective. A major factor contributing to low-temperature growth of *L. monocytogenes* is the ability of the organism to accumulate compatible solutes such as betaine and carnitine. Using a set of mutants with deletions in the known osmolyte transporters BetL, Gbu and OpuC, we were able to determine the individual contribution of betaine and carnitine and their transporters to low-temperature growth of *L. monocytogenes*.

In wild-type LO28, growth at low temperature is clearly stimulated by the addition of betaine and/or carnitine to defined medium. However, the growth rate is higher upon addition of betaine than of carnitine. This suggests that betaine functions as a more effective cryoprotectant than carnitine, an observation which appears also to extend to their respective osmoprotective effects (45), a phenomenon which Peddie *et al*. (29) attributed to differences in hydrocarbon chain length. Upon addition of betaine to DM at 7°C the hierarchy of transporter importance is Gbu>BetL>OpuC. However, when betaine is replaced by carnitine, the role of OpuC at low temperature becomes apparent, in that only those mutants deficient in their OpuC transporter show reduced growth rates compared to the wild type. This is consistent with the findings of Becker *et al*. (10) who showed that growth of *L. monocytogenes* after a cold shock from 37 to 8°C is stimulated upon addition of carnitine to DM, thus proving a role for carnitine in low temperature growth. Moreover, it was found that uptake of carnitine *via* OpuC seems to be regulated by the alternative sigma factor σ^B, a stress-related sigma factor, which is itself induced at low temperature (9, 16). As with σ^B, OpuC also appears to play a role in the salt tolerance and virulence potential of *L. monocytogenes*, since mutants with a disrupted *opuC* gene (LO28ΔC, LO28ΔCG and LO28ΔBCG) showed a significant reduction in their ability to cause systemic infection following peroral co-inoculation with the wild-type parent (39, 45). The relative abundance of carnitine in mammalian tissues (11) makes it the most readily available and thus possibly the

most important osmolyte contributing to the growth and survival of *L. monocytogenes* during infection.

In the wild-type strain it is mainly betaine that is accumulated during growth at low temperature. However, in strain LO28ΔG, more carnitine than betaine is accumulated. This increase in internal carnitine concentration upon deletion of Gbu was also observed in *L. monocytogenes* 10403S at low temperature (5) and for LO28 grown at high osmolarity (45). Thus, carnitine was only accumulated to high levels in those strains impaired in their ability to transport betaine *via* Gbu. The ability of betaine to suppress the accumulation of other osmolytes is typical of bacterial species that can accumulate betaine together with other osmolytes (23, 32). Angelidis and Smith (3) have recently proposed that betaine and carnitine uptake in *Listeria* is mediated exclusively by BetL, Gbu and OpuC. An earlier triple mutant created in our laboratory, LO28ΔBCG (45), displayed low levels of carnitine and betaine uptake, which we attributed to the existence of a possible fourth transporter, which we and others (15) suggested to be OpuB, a potential osmolyte uptake system identified by *in silico* analysis of the completed genome sequence (36). Angelidis and Smith (3) suggested that the residual uptake observed could also be explained by the nature of the OpuC mutation, which was created by plasmid integration rather than SOEing mutagenesis. To resolve this issue, in this study we re-created the triple mutant using SOEing to eliminate the OpuC gene. The resulting mutant designated LO28ΔBCGsoe was found to exhibit no detectable betaine or carnitine accumulation, either at reduced temperatures or elevated osmolarities. Thus, it appears that betaine and carnitine uptake in *L. monocytogenes* is indeed mediated by Gbu, BetL and OpuC alone, both at elevated osmolarities as shown by Angelidis and Smith (3), and also at refrigeration temperatures.

RT-PCR analysis revealed that the genes encoding all three transporters, Gbu, BetL, and OpuC, are significantly up-regulated following a rapid decrease in temperature from 37°C to 10°C. Interestingly, the induced transcription of *opuB* after cold shock also suggests a role for this transporter at low temperature. However, no difference in betaine or carnitine accumulation was observed between the triple and quadruple mutants LO28ΔBCGsoe and LO28ΔBCGB, confirming that OpuB is not involved in betaine or carnitine uptake. Furthermore, given that the growth rates and characteristics of both mutants at low temperatures were similar, OpuB is unlikely to play a significant role in listerial cryotolerance.

At low temperature, the growth rate of *L. monocytogenes* in BHI is ~10 times higher than in DM. Given that betaine and carnitine together only result in a 2-fold increase in growth rate in DM, this deficit may at least partly be explained by the presence of additional amino acids (e.g. proline and glutamic acid) and peptides in BHI. *L. monocytogenes* can accumulate amino acids *via* multiple amino acid transporters and peptides *via* a di- and tripeptide transporter, DtpT (41, 46) and an oligopeptide transporter, OppA (42). In addition to a role in osmoregulation (2), and possible nutritive benefits, peptides have also been shown

to stimulate low temperature growth, as mutating *oppA* significantly affected growth at 5°C (12).

In high salt environments, compatible solutes serve to balance the osmotic disturbance. However, their function at low temperature is largely unknown. Moreover, betaine and carnitine might have different roles in cryoprotection. In eukaryotic cells, carnitine plays a role in fatty acid transport across the inner mitochondrial membrane (22). Becker *et al*. (10) suggested that carnitine may also function as a carrier in the process of fatty acid alteration that occurs during low-temperature adaptation of *L. monocytogenes*. If carnitine were to play a role as a cofactor, then it might be needed only at modest concentrations. Moreover, compatible solutes can also provide protection against high temperature, freeze-thaw treatment and even drying (38). The basis for the compatibility of osmolytes is their preferential exclusion from the immediate surface of proteins and other cytoplasmic macromolecules, ensuring solvation in water (7). Some functions of compatible solutes at low temperature have been proposed: they can serve as stabilizers of enzyme function, and as such may function in stabilization of membrane bilayers (26). Interesting in this perspective is the possible overlapping function with cold-shock proteins (CSPs). These proteins have been suggested to play a role in the stabilization of macromolecules at low temperature (and during other stresses). Previously we described the induction of two of the four CSPs in LO28 after cold shock (44). We have also analyzed CSP production 20h after cold shock to 10°C, in wild-type LO28 as well as in the triple mutant LO28ΔBCG. However, no changes in CSP production between the two strains could be detected (results not shown), indicating that increasing the CSP levels does not compensate for osmolyte depletion.

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Chapter 6

Deletion of the putative choline transporter OpuB in *Listeria monocytogenes* affects virulence, but not growth at low temperatures or high osmolarity

Abstract

Listeria monocytogenes is a food-borne pathogen that is found widely distributed in nature as well as in a variety of fresh and processed foods. This ubiquity can be at least partly explained by the ability of the organism to grow at high osmolarity and reduced temperatures as a consequence of the accumulation of osmo- and cryoprotective compounds called osmolytes. Previously, three osmolyte transporters have been characterized in *L. monocytogenes*, BetL, Gbu and OpuC. In this communication we describe a fourth transporter, OpuB, possibly dedicated to the transport of choline. In a number of micro-organisms, e.g. *Bacillus subtilis* and *Escherichia coli*, choline is converted to glycine betaine affording considerable protection to osmotically stressed cells. In *L. monocytogenes* no accumulated betaine could be detected in cells grown in defined medium with 3% NaCl and 1 mM added choline, indicating that choline is not converted into betaine under these conditions. By using an *opuB*-deletion mutant it was found that OpuB appears to play no significant role in low temperature and high osmolarity growth of *L. monocytogenes*. Moreover, growth and survival of the triple mutant LO28ΔBCGsoe (carrying deletions in the osmolyte transporter genes encoding BetL, OpuC and Gbu) did not differ from the quadruple mutant LO28ΔBCGB (Δ*betL*, Δ*opuC*, Δ*gbu*, Δ*opuB*), upon exposure to stress conditions. This indicates that, although the betaine and carnitine transporters are deleted, OpuB has no significant role in growth at low temperature or high osmolarity. Notably, we also analyzed the growth and survival of the *opuB* deletion strain in an animal (murine) model. Interestingly, the deletion mutant showed a dramatic reduction in its ability to cause systemic infection following peroral co-inoculation with the wild type parent, suggesting a significant role for OpuB in virulence of *L. monocytogenes*.

Introduction

Listeria monocytogenes is ubiquitously found in nature and the organism is well known for growth at high osmolarity and low temperature. Exposure to such environments incites integrated physiological adaptation reactions that are aimed at restoring the disturbed water balance, maintaining optimal turgor, and protecting cell components from the detrimental effects of high ionic strength in the case of high osmolarity environments. Upon growth at low temperature, the adaptation processes are aimed at the maintenance of the cellular membrane fluidity, structure stabilization of macromolecules such as ribosomes (necessary for continued protein synthesis), and the uptake or synthesis of compatible solutes.

Previously, three osmolyte transporters have been described and characterized in *L. monocytogenes*: BetL, Gbu, and OpuC (15, 9, 6, 24, 1, 10, 17, 21, and a review in 22). BetL is a secondary transporter, dedicated to the transport of betaine into the cell. Gbu and OpuC belong to the binding-protein-dependent ATP binding cassette superfamily of transporters and are dedicated to the transport of betaine and carnitine, respectively. Previously, analysis of the *L. monocytogenes* EGD-e genome (available at http://genolist.pasteur.fr/listilist) revealed a putative fourth osmolyte transporter (22, 28) with homology to *opuB* of *B. subtilis* (14) and exhibiting a functional organization similar to that of BusA (OpuA) of *Lactococcus lactis* (18). The listerial *opuB* homologue occurs approximately 2.4 kb downstream of the *opuC* operon and consists of two ORFs (*lmo1421* and *lmo1422*) oriented in the same direction and overlapping by 4 nucleotides. Flanked by stem loop structures and preceded by a consensus σ^B-dependent promoter-binding site, this tight genetic organization suggests that both ORFs (designated *opuBA* and *opuBB*, respectively) constitute an operon (22). In *B. subtilis*, OpuB has been demonstrated to be capable of transporting choline. After transport into the cell, choline is converted into glycine betaine by two enzymes, a glycine betaine aldehyde dehydrogenase GbsA and an alcohol dehydrogenase GbsB. The transport of choline and the subsequent conversion to betaine affords considerable osmoprotection to the cells (3). *B. subtilis* shares the ability to oxidize choline to glycine betaine for osmoprotective purposes with a number of gram-negative and gram-positive bacteria (5, 12, 16). In *B. subtilis*, the genes encoding the GbsA and GbsB enzymes are spaced close by the *opuB* operon and are separated by only 44 bases from each other. Analysis of the genome of *L. monocytogenes* reveals several genes exhibiting significant sequence similarities to *gbsA*: *lmo0913*, *lmo0383*, *lmo1179*, and with *gbsB*: *pduQ*, *lmo1634*, *lmo1166*, *lmo0554*. However, none of these genes are as tightly organized as *gbsA* and *gbsB* (22).

In this communication we identify and characterize OpuB in *L. monocytogenes*, a putative binding-protein-dependent ATP-binding cassette (ABC) transporter, by using an LO28 *opuB* deletion mutant strain, LO28BCGsoe (Δ*betL*, Δ*opuC*, Δ*gbu*) and a quadruple mutant, LO28BCGB (Δ*betL*, Δ*opuC*, Δ*gbu*, Δ*opuB*). OpuB proved to play a minor role in growth at high osmolarity and at low temperature, though, interestingly, a major role in virulence.

Materials and methods

Strains, chemicals and growth conditions. *L. monocytogenes* LO28 was used as wild-type strain throughout this study. An *opuB* deletion was created in the wild-type background, giving rise to LO28Δ*opuB*, as well as in LO28ΔBCGsoe, a strain deleted in its three osmolyte transporters, BetL, OpuC and Gbu, giving rise to LO28ΔBCGB. Strains were grown either in brain heart infusion (BHI) broth, or in defined medium (DM) (19). When required the media osmolarity was adjusted by the addition of NaCl (DM + 3% NaCl: DMS). Where indicated, choline, carnitine, or glycine betaine (Sigma Chemical Co., St. Louis, Mo.) were added to DM(S) as filter sterilized stocks to a final concentration of 1 mM. Cell growth was monitored spectrophotometrically by measuring the optical density at 620 nm (OD_{620}).

Generation of the *opuB* deletion mutant and LO28D**BCGB.** A mutant with a deletion in the *opuB* operon was obtained using the SOE-ing (splicing by overlap extension) technique to remove a 945-bp fragment from the center of the operon. Essentially two ~300 bp PCR products (amplified by *opuB* SOEA [5'-ATCTAGAAAATGACTTCGTTAA AGACT-3'] and *opuB* SOEB [5'-ACAAGCTCACCTGAACTTTCC-3'] and *opuB* SOEC [5'-GGAAAGTTCAGGTGAGCTTGTCAAGCCCGTGATGGTTTGGCT-3'] and *opuB* SOED [5'-GATATCTGCAGACTTAATTGCAT-3']) flanking the sequence to be deleted were spliced, giving a ~600 bp hybrid which was subsequently cloned into the temperature-sensitive shuttle vector pKSV-7 and transformed into *Escherichia coli* DH5α. The resulting plasmid, designated pCPL18, was subsequently transformed into wild-type LO28 and the resulting transformants were selected on BHI plates containing 10 μg of chloramphenicol per ml. Selection at 42°C of cells with chromosomal integration of pCPL18, followed by sequential pasaging in BHI broth at 30°C in the absence of chloramphenicol, facilitated the recovery of cells in which allelic exchange between the intact *opuB* operon and the ~650 bp insert on pCPL18 had occurred, thus giving rise to LO28Δ*opuB*.

The quadruple mutant strain LO28ΔBCGB (Δ*betL*, Δ*opuC*, Δ*gbu*, Δ*opuB*) was constructed by electroporating the pKSV-7 plasmid derivative pCPL18, into *L. monocytogenes* LO28ΔBCGsoe (the *betL*, *opuC*, *gbu* deletion mutant), and this was followed by the integration, excision, curing and, screening steps described above.

Transcriptional analysis of *opuB*. RT-PCR analysis was performed as described by Sleator *et al.* (23). Essentially, *L. monocytogenes* cells were grown to mid-exponential phase at 37°C in BHI or DM. A portion of 10 ml was centrifuged and resuspended in respectively 1 ml of BHI with 3% NaCl, in medium of 10°C, or, as a control, of 37°C. After 15 and 30 minutes incubation, cells were harvested and were flash-frozen at -80°C. RNA was extracted using a hot acid phenol procedure (20), and cDNA was synthesized by adding 1 μl of total RNA to 4 μl of 5x RT buffer (Roche), 2 μl of 100 mM dithiothreitol, 0.5 μl of a deoxynucleoside triphosphate mix (dATP, dCTP, dGTP, and dTTP; each 10 mM), 0.25 μl of RNasin, 100 ng of the random primer $p(dN)_6$, and 1 μl of Expand reverse transcriptase (Roche). The reaction mixture was incubated at 42°C for 9 h. PCR was carried out using the

following primers: for opuB, *opuB*-F (5'-ATCTAGAAAATGACTTCGTTAAA-3') and *opuB*-R (5'-ACAAGCTCACCTGAACTTTCC-3'); and for 16S RNA, 16SRNA-E (5'-TTAGCTAGTTGGTAGGGT-3') and 16SRNA-B (5'-AATCCGGACAACGCTTGC-3'). PCRs were carried out for 16, 22 and 30 cycles to allow optimal quantification of PCR products. Template cDNA was used in the reactions at levels that gave similar band intensities for 16S RNA reactions.

Virulence assays. Virulence of wild-type LO28 was compared with virulence of the *opuB*-deletion mutant by peroral co-inoculation of 8- to 12-week-old BALB/c mice as described by Sleator *et al.* (24). Essentially, mutant and wild-type cells were suspended in buffered saline with gelatin (0.85% NaCl, 0.01% gelatin, 2.2 mM K_2HPO_4, and 4.2 mM Na_2HPO_4). Each animal was infected with approximately 5 x 10^9 cells using a micropipette tip placed immediately behind the incisors. Three days postinfection mice were euthanized and the listerial numbers in the spleens of infected animals were determined by spread plating homogenized samples onto BHI agar plates.

Uptake studies. *L. monocytogenes* LO28 was grown to exponential phase. Uptake studies were carried out essentially as described by Verheul *et al.* (26). Cells were harvested by centrifugation (3000 x g, 15 min., 10°C), washed twice and resuspended in 50 mM potassium phosphate buffer (pH 6.8) containing 5 mM $MgSO_4$ (and 3% NaCl when cells were grown in the presence of additional 3% NaCl). Cells were concentrated to an OD_{620} of 20 in this buffer, and stored on ice until use. Cells (OD_{620} of 1) were preenergized at 37°C with 1% glucose for 5 min prior to the addition of radiolabelled choline (final concentration of 100 μM), purchased from Campo Scientific (Veenendaal, The Netherlands). Samples were withdrawn after 0.5, 1, 2, 3 and 4 min and the cells were collected on 0.2-μm-pore-size cellulose nitrate filters (Schleicher and Schuell GmbH, Dassell, Germany) under vacuum after addition of 2 ml of 50 mM potassium phosphate buffer (pH 6.8) containing 5 mM $MgSO_4$ (and 3% NaCl). The filters were washed with another 2 ml buffer and the radioactivity trapped in the cells was measured using a scintillation counter (model 1600TR; Packard Instruments Co., Downers Grove, Ill, USA). Uptake of choline was normalized to total quantity of cellular protein.

Results

Identification of the *opuB* operon encoding a putative choline transporter. The *opuB* operon in *L. monocytogenes* exhibits a functional organization similar to that of OpuA (BusA) of *Lactococcus lactis*, and the deduced amino acid sequences of OpuBA (329 amino acids) and OpuBB (505 amino acids) reveal that the domain organization is also similar to that of OpuA of *L. lactis* (18). The predicted ligand-binding protein is fused to the C-terminal end of the translocator protein (Fig. 1). The translocator segment of OpuB in *L. monocytogenes* is predicted to have 6 transmembrane segments. By analogy with other ABC

transporters, functional OpuB will most likely be composed of two integral membrane subunits and two ATP-binding subunits (Fig. 1). Van der Heide *et al.* (13) proposed for OpuA in *L. lactis* that the oligomeric structure of a translocator subunit fused to a substrate binding protein implies that two receptor domains are present per functional complex.

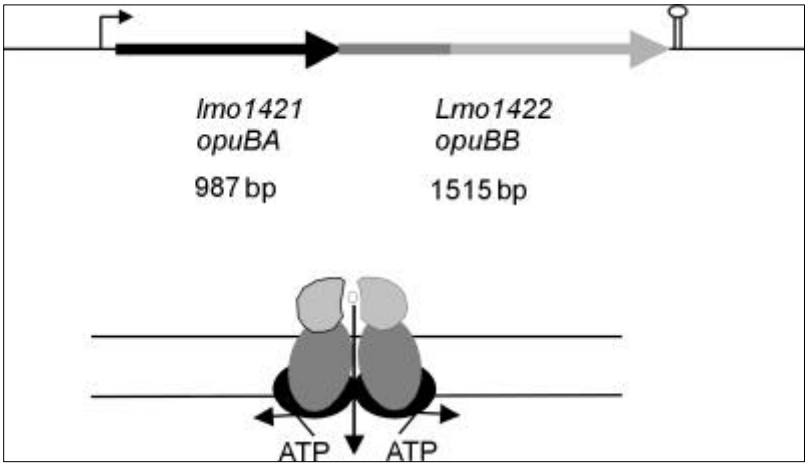

Figure 1. (A) Organization of the *opuB* operon in *L. monocytogenes*. The arrow indicates a putative σ^B–dependent promoter site, the hairpin structure indicates a putative terminator. The numbering of the ORFs follows the numbering of the *L. monocytogenes* EGD-e genome sequence (11). (B) Architecture of the binding-protein-dependent ATP binding cassette transporter OpuB. The transporter is composed of ATPase subunits (black), translocator subunits (dark grey) and substrate binding domains (light grey) (based on OpuA in *L. lactis* (13)).

Induced transcription of *opuB* at high osmolarity and low temperature. RT-PCR was performed to analyze the transcription of *opuB* following cold shock and salt shock (Fig. 2). Already *opuB* transcripts were present at 37°C prior to stress exposure. Following either salt shock or cold shock, transcription of *opuB* was significantly induced, both against the wild-type LO28 and triple mutant LO28ΔBCG backgrounds.

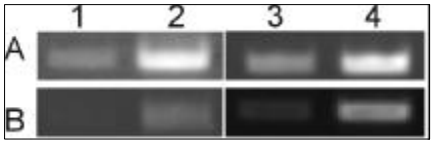

Figure 2. Transcript levels of *opuB* in *L. monocytogenes* LO28 (A) and triple mutant LO28ΔBCG (B) exponential growing cells at 37°C (1 and 3), cells exposed to 3% NaCl for 15 min (2), and cells exposed to 10°C for 30 min (3).

Effect of deleting *opuB* on the growth of *L. monocytogenes* LO28. Growth of *L. monocytogenes* LO28 and the *opuB* deletion mutant was measured in BHI with high osmolarity and at low temperature. No obvious growth effect was observed following *opuB* deletion, either in BHI with 3 or 6% added NaCl, or at 10°C (Fig. 3). To overcome the possibility that any effects of mutating OpuB were being masked by the remaining osmolyte transporters; BetL, OpuC, and Gbu, growth of the triple mutant LO28BCGsoe (in which

OpuB is the only remaining transporter) was directly compared to the quadruple mutant LO28BCGB (from which all the transporters have been removed). However, growth, following exposure to osmotic stress and low temperature, did not differ for these two strains (Fig. 3).

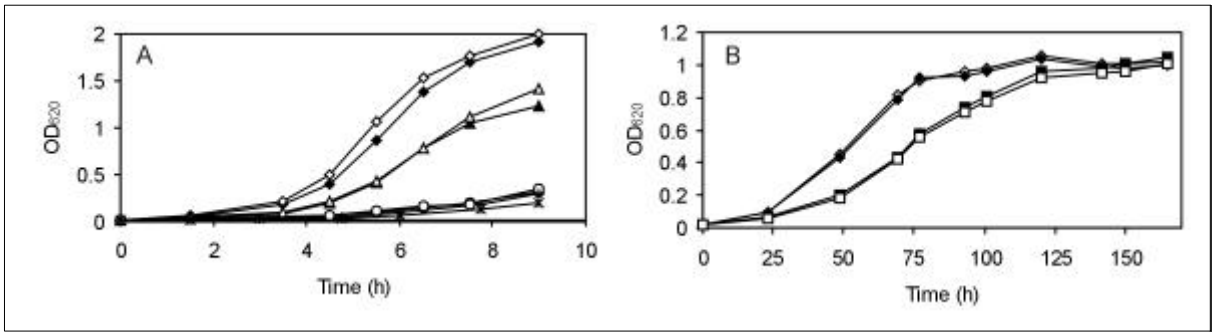

Figure 3. (A) Growth of *L. monocytogenes* LO28 (open symbols) and its *opuB* null mutant (closed symbols) in BHI (?, ♦), in BHI with 3% added NaCl (Δ, ?), and with 6% added NaCl (?, ?). Additionally, growth of the triple mutant LO28ΔBCGsoe (+) and the quadruple mutant LO28ΔBCGB (*) in BHI with 3% NaCl is depicted. Growth of these two mutants does not occur within 10h in BHI with 6% NaCl. (B) Growth of *L. monocytogenes* LO28 (?), its *opuB* null mutant (♦), the triple mutant LO28ΔBCGsoe (?) and the quadruple mutant LO28ΔBCGB (¦) in BHI at 10°C.

To study the individual contribution of betaine and carnitine to the growth of the *opuB* deletion mutant in more detail, defined medium with 3% added NaCl (DMS) was used. Upon addition of betaine or carnitine the *opuB* deletion mutant grew similar as wild-type LO28 (Fig. 4). Additionally, the quadruple mutant LO28ΔBCGB grew similar to the triple mutant LO28ΔBCGsoe. Growth of LO28ΔBCGsoe and LO28ΔBCGB in the presence of either betaine or carnitine was similar to that of growth in the absence of osmolytes, again indicating that the osmoprotective effect of betaine and carnitine is mediated exclusively via the transporters BetL, OpuC, and Gbu, and not OpuB. Thus, OpuB appears to play little if any role in the growth of *L. monocytogenes* at elevated osmolarity and reduced temperatures. Moreover, analysis of the accumulated levels of betaine and carnitine upon growth in BHI with 6% added NaCl showed similar levels for the wild-type strain and the *opuB* deletion mutant, whereas no betaine or carnitine could be detected in LO28ΔBCGsoe and in LO28ΔBCGB (results not shown).

***L. monocytogenes* does not convert choline into betaine.** In many organisms, e.g. *B. subtilis* and *E. coli*, choline is transported into the cell and subsequently converted into glycine betaine by two enzymes, affording the cells significant osmoprotection (3). To analyze the role of choline transport and subsequent conversion in glycine betaine in *L. monocytogenes* LO28, this strain was grown in DMS and in DMS with 1 mM choline. Growth of LO28 was not stimulated upon addition of choline to DMS. Also, mid-exponential phase cells were tested for their internal concentration of betaine, but no betaine could be

detected (results not shown). In uptake studies with radiolabelled [methyl-^{14}C]choline, no uptake of choline was observed in *L. monocytogenes* LO28. Previously, we also found very low uptake rates of radiolabeled carnitine in this strain (24). Despite this observation, accumulated carnitine was found in *L. monocytogenes* LO28 cells grown in DMS with added carnitine (28), indicating that *L. monocytogenes* is able to transport carnitine.

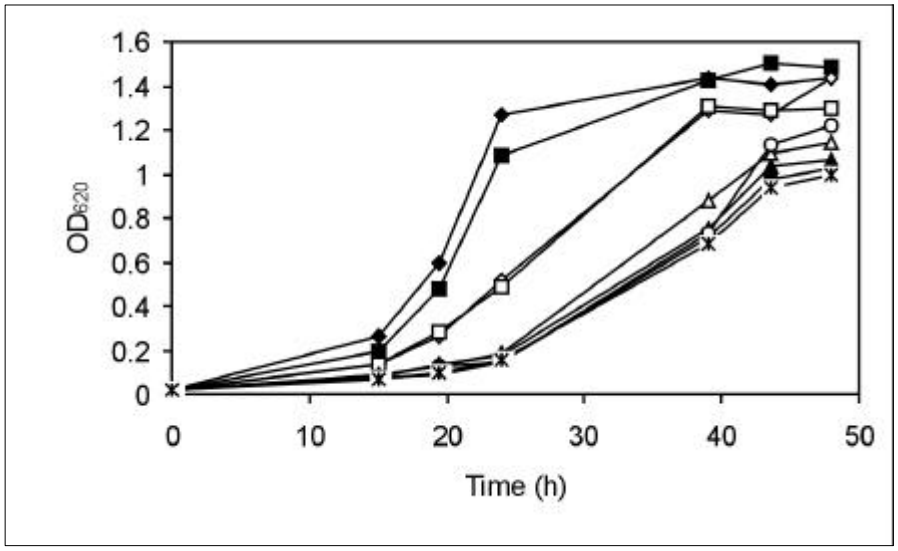

Figure 4. Growth of *L. monocytogenes* LO28 (diamonds), the *opuB* deletion mutant (squares), the triple mutant LO28ΔBCGsoe (triangles) and the quadruple mutant LO28ΔBCGB (circles) in DMS with 1 mM betaine (closed symbols) or with 1 mM carnitine (open symbols). For clarity growth in DMS without any added osmolytes is only depicted for *L. monocytogenes* LO28 wild type (asterisks).

Virulence studies. Given that *Listeria* is a food-borne pathogen, the most common way of infection is *via* the oral route. As for the betaine and carnitine transporter mutants (23, 24, 28) we investigated the ability of the *opuB* deletion mutant to reach and proliferate within a target organ (spleen) following peroral co-inoculation with the wild-type parent. Surprisingly, an almost complete shift to the wild type was observed. From the five animals tested, the spleens of two animals contained only wild-type *L. monocytogenes* cells and three had a highly increased percentage wild type, indicating that the *opuB* deletion mutant is highly impaired in its virulence (Fig. 5). Total cells recovered from the spleens were $5*10^4$ for animals 1 and 2, $5*10^3$ for animals 3 and 5, and $1*10^4$ for animal 4.

Discussion

In this report, we describe the role of the putative choline transporter OpuB in growth during stress conditions and in virulence. Previously, a possible role for OpuB in transport of carnitine was suggested (7, 28). Using an *opuB* deletion mutant strain, we analyzed the role of OpuB in growth at high osmolarity and at low temperature, as these conditions are known to

induce accumulation of osmolytes in *L. monocytogenes*. Under the conditions tested, the *opuB* deletion mutant did not show reduced growth compared with the wild type. If the OpuB transporter was to play a role in osmolyte uptake, it is possible that the other osmolyte transporters, BetL, Gbu and OpuC, are efficient enough to provide the cells with sufficient accumulated osmolytes. Previously, it was found that Gbu is the most important osmolyte uptake system both at high salt concentrations and at low temperature and that carnitine was only accumulated in cells deleted in this transporter (27, 28). Additionally, comparison of LO28ΔBCGsoe and LO28ΔBCGB showed that the OpuB transporter has no significant role in growth at high osmolarity and low temperature, despite the inability of the strains to transport betaine or carnitine *via* the three osmolyte transporters. Moreover, unlike other organisms, such as *B. subtilis* and *E. coli*, *L. monocytogenes* LO28 does not seem to be capable of converting choline into glycine betaine. As choline transport in *L. monocytogenes* LO28 could not be shown, we cannot rule out the possibility that choline is not taken up in this strain, and hence, cannot be converted. However, we did observe choline transport in *L. monocytogenes* EGD-e, and growth of this strain was also not stimulated by the addition of choline to DMS (results not shown).

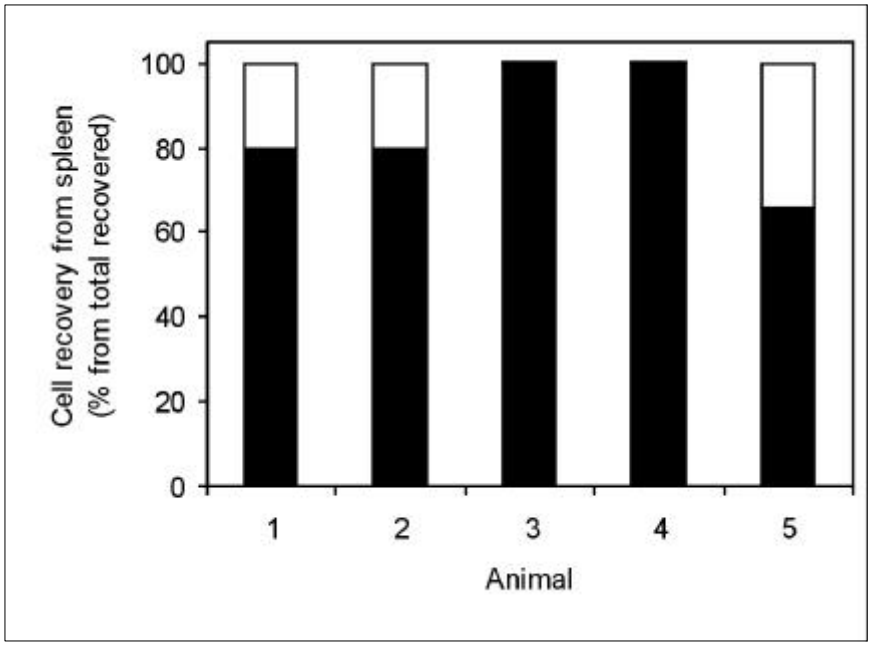

Figure 5. Virulence potential of the *opuB* deletion mutant following peroral co-inoculation of BALB/c mice. The amount of cells of the strains in the spleens was determined for the wild type (black) and *opuB* deletion mutant (white) of 5 infected animals three days post infection.

OpuB in *L. monocytogenes* shows a functional organization similar to that of OpuA in *L. lactis*. In *L. lactis* the mechanism of activation of the OpuA transporter upon osmotic upshock is studied extensively. It was found that the transporter is specifically activated as a consequence of charge concentration in the cell (13). This is achieved not only by a decrease in cell volume but also by the accumulation of potassium ions in the initial response to osmotic upshift. OpuA may not directly sense this signal, rather, the membrane in which the

protein is embedded may serve as mediator. Specific interactions between the membrane lipids and the transporter proteins may alter upon osmotic changes (13).

Although OpuB does not seem to play a major role in growth at high osmolarity or low temperature, transcription of *opuB* was shown to be upregulated upon exposure of cells to 3% NaCl or low temperature. Recently, Fraser *et al*. (8) showed that regulation of *opuB* on the transcriptional level is, at least in part, mediated by the alternative sigma factor σ^B. This sigma factor is shown to be upregulated in *L. monocytogenes* upon exposure to various environmental stresses, e.g. to low temperature, high osmolarity, heat and low pH (2). In *B. subtilis*, σ^B regulates a large general stress response regulon, contributing to the transcription of more than 100 genes that are induced by exposure to environmental stresses. Also in *L. monocytogenes* the consensus promoter sequence specific for the recognition of σ^B can be found in front of many genes (about 50 genes with zero or one mismatch (Chapter 3)), indicating that σ^B regulates a large general stress response regulon in this organism.

Surprisingly, the virulence of the *opuB* deletion mutant is greatly impaired compared with wild-type *L. monocytogenes* LO28. As the *opuB* deletion strain is not impaired in its ability to use the osmolytes betaine and carnitine, it is unlikely that this diminished virulence is due to osmotic stress encountered during pathogenesis, as is the most probable cause for the reduced ability of *opuC* deleted strains to reach, and multiply within the liver and spleens of infected animals (28). It can be speculated that, in case OpuB is a choline transporter, virulence of the deletion mutant is lower since it is impaired in its transport of choline into the listerial cell. Use of choline as the sole carbon and energy source for growth may be important since choline is the phospholipid component of an abundant class of lipids (phosphatidylcholine) present in the host's gut. This phosphatidylcholine is hydrolyzed during the escape of the *L. monocytogenes* cell from the secondary double-membrane vacuole formed during the cell-to-cell spread of *L. monocytogenes* (25). This hydrolyzation of phosphatidylcholine is mediated by PC-PLC (phosphatidylcholine-preferring phospholipase; PC-PLC, encoded by *plcB*), and renders free choline. Choline (*N*,*N*,*N*-trimethyl-ethanolamine), may be converted into ethanolamine upon demethylation of the amino group. In *L. monocytogenes* EGD-e the *eut*-genes encoding the proteins necessary for anaerobic degradation of the carbon source ethanolamine in a coenzyme B12-dependent manner, are present (4). Therefore, diminished choline uptake and consequent depletion of a carbon source, may offer an explanation for the significant reduction in virulence of an *opuB* deleted *L. monocytogenes* strain. However, growth of *L. monocytogenes* in aerobic and anaerobic conditions was not observed when choline or ethanolamine was used in DM (with addition of vitamin B12) as a carbon source (results not shown). Additional experiments with LO28Δ*opuB* showed that this strain has an increased susceptibility to bile (personal communication, M. Begley, UCC, Cork, Ireland). Upon exposure to 30% bovine bile an approximate 2-log reduction in survivors was observed compared to that of the wild type. This may affect the ability of the mutant to reach and subsequently multiply in target organs.

The virulence data presented in this study supply us with interesting new findings and, upon subsequent research, will possibly contribute significantly to our understanding of the intracellular life cycle of *L. monocytogenes*. Additionally, the results from this study contribute to the understanding of listerial stress response, i.e. OpuB does not play a large role in growth and survival during stress conditions and choline is not converted to betaine, under the conditions tested.

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Chapter 7

Identification and characterization of the di- and tripeptide transporter DtpT of *Listeria monocytogenes*

Summary

Listeria monocytogenes is a gram-positive, intracellular pathogen responsible for oppurtunistic infection in humans and other animals. In the current paper, we describe and characterize the di- and tripeptide transporter DtpT and assess its role in growth in several environments and environmental conditions as well as its role in the virulence of *L. monocytogenes*. Based on homology a *dtpT* homolog is identified in the *L. monocytogenes* EGD-e genome sequence. A *dtp*T chromosomal in frame deletion mutant was constructed by using the temperature-sensitive suicide vector pAUL-A. Here, we demonstrate that the transport of Pro-[^{14}C]-Ala can be contributed to the proton-motive force dependent transporter DtpT. The transporter DtpT was shown to be required for growth when the essential amino acids leucine and valine were supplied as peptides. The protective effect of glycine and proline-containing peptides during growth in defined medium containing 3% NaCl was only noted in *L. monocytogenes* WT and not in the *dtpT* deleted strain indicating that the DtpT transporter is involved in salt stress protection. The role of DtpT in virulence was studied in a mouse model. It was found that the virulence of the EGD-e strain lacking DtpT was higher than that of wild-type *L. monocytogenes* EGD-e. A possible explanation for this observation is the upregulation of complementary peptide transporters upon deletion of DtpT by which the survival of the *dtpT* mutant increases.

J. A. Wouters, T. Hain, A. Darji, E. Hüfner, H. H. Wemekamp-Kamphuis, T. Chakraborty, and T. Abee. Identification and characterization of the di- and tripeptide transporter DtpT of *Listeria monocytogenes*.

Introduction

Listeriosis is a food-borne disease and the causitive agent is *Listeria monocytogenes*. *L. monocytogenes* is a ubiquitous gram-positive, intracellular pathogen that is responsible for the opportunistic infection in humans and other animals. *L. monocytogenes* is often isolated from foods of plant or animal origin and is one of the food-related pathogens of greatest concern in the last decades. Various foods, such as meat, milk and other dairy products, and vegetables contaminated with *L. monocytogenes* have been associated with human listeriosis (7). Unlike other common food-borne diseases, listeriosis is associated with a mortality rate of over 20%. Listeriosis occurs primarily in high-risk groups, such a neonates, elderly, pregnant women, and immuno-compromised individuals. The infectious dose for humans remains unknown and varies depending on the immune status of the individual. Important aspects of the presence of *L. monocytogenes* in food products are its capabilities to acquire the required nutritive compounds and to survive a variety of environmental stresses.

L. monocytogenes has a limited biosynthetic capacity as judged by its complex nutritional requirements, whereas most bacteria are able to synthesize all 20 amino acids necessary for protein biosynthesis by utilizing inorganic ammonium salts as a nitrogen source. Listeria is apparently unable to hydrolyze proteins, and its growth depends upon other proteolytic systems that allow degradation of food proteins. It is believed that peptides and free amino acids present in food result from the activity of indigenous proteinases and or proteinases from divers populations of microorganisms, such as lactic acid bacteria. Verheul *et al.* (24) demonstrated that the growth of *L. monocytogenes* was enhanced by *Pseudomonas fragi* and *Bacillus cereus* in a medium containing casein as the sole source of nitrogen. Previously, it has been shown by Premaratne *et al.* (16) that *L. monocytogenes* ScottA requires the addition of six amino acids (Leu, Ile, Arg, Met, Val and Cys). In *L. monocytogenes* EGD-e the biosynthesis pathways for all amino acids could be identified after metabolic reconstruction. Glaser *et al.* (8) suggested that the requirement for amino acids may be due to repression of some amino acid biosynthetic pathways in laboratory conditions. In the recently characterized genome sequence of *L. monocytogenes* EGD-e a total of 331 genes encoding different transport proteins are found, which adds up to 11.6% of the predicted genes. This is a relatively large number compared to bacteria for which the genome sequence has been characterized thus far and it reflects the ability of *Listeria sp.* to colonize and grow in a broad range of ecosystems, such as food products and host environments. Verheul *et al*. (23) identified a di- and tripeptide transport system in *L. monocytogenes* ScottA which resembles a proton-motive force dependent carrier protein. This peptide permease has a broad substrate specifity and allows transport of a variety of di- and tripeptides. Upon internalization the peptides can be hydrolyzed, used as substrate and serve as source of amino acids essential for growth. In addition to the di- and tripeptide transport, Verheul *et al*. (24) identified an ATP-dependent oligopeptide transporter capable of transporting peptides up to 8 residues. Recently, Borezee *et al.* (4), identified the genes encoding this transport system. The *opp*

operon consists of five genes and shows typical characteristics of ABC-transporters. Deletion of *oppA* revealed that it is indeed involved in the transport of oligopeptides and that it is required for growth at low temperature. Next, it was observed that OppA is involved in intracellular survival of *L. monocytogenes* in macrophages and in organs of infected mice, however, the virulence in the *oppA* mutant was not altered (4).

L. monocytogenes is able to grow at salt concentrations as high as 10% (14) and at temperatures as low as 0°C (25). For the adaptation to both low temperature as well as high salt conditions the ability to accumulate substances from the external environment is essential. These compounds include compounds such as proline, ectoine, glycine, glutamate, carnitine and glycine betaine. The intracellular accumulation of these solutes allow bacterial survival at high or fluctuating osmolarities, dessication or freezing. This type of compounds can be accumulated up to high concentrations without affecting intracellular processes (reviewed in 20). Effective compatible solutes for *L. monocytogenes* are glycine betaine and carnitine (3) and several transport systems for these substances have been characterized in recent years (19, 11, 21). In addition, it was shown that specific glycine and proline-containing peptides stimulate growth of this bacterium at high osmotic strength. The peptide prolyl-hydroxylproline accumulates in cells to high levels in response to growth at high osmolarity, and the pools of the derived amino acids also show a dependence on the external osmotic pressure (2).

In the current paper, we describe and characterize the di- and tripeptide transporter DtpT of *L. monocytogenes* and assess its role in growth in several environments and environmental conditions as well as its role in virulence in a mouse infection model.

Materials and methods

Bacterial strains, media, growth conditions and chemicals. Bacterial strains and plasmids used in this study are listed in Table 1. *L. monocytogenes* EGD-e was grown in brain heart infusion (BHI) broth (Difco) at 37°C. *Escherichia coli* was grown in Luria Bertani browth (LB) (Difco) at 37°C. Ampicillin (100 μg/ml) and erythromycin (300 μg/ml for *E. coli*, 5 μg/ml for *L. monocytogenes*) were added to broth or agar as needed. *L. monocytogenes* EGD-e and *L. monocytogenes* Δ*dtpT* were also cultured in chemically defined medium (CDM) as described previously (16). To analyze the ability to use di- and tripeptides, the essential amino acids leucine and valine were ommitted from CDM and these amino acids were supplied as di- or tri-peptides (Leu-Ala, Ala-Leu, Leu-Pro, Leu-Gly-Gly, Ala-Leu-Gly, Val-Gly and Ala-Val). Growth was monitored by measuring the optical density at 600 nm (OD_{600}). The di- and tripeptides were obtained from Sigma Chemicals (St. Louis, MO). *L. monocytogenes* EGD-e and *L. monocytogenes* Δ*dtpT* were also grown in sterilized milk and meat bouillion as models for growth of *L. monocyt*ogenes on food products. Growth was measured as colony forming units. To assay the role of the DtpT transporter in salt stress

and/or low-temperature stress, growth of *L. monocytogenes* EGD-e and *L. monocytogenes* Δ*dtpT* was also monitored in BHI containing NaCl at concentrations of 3, 6 and 9% (w/v) at several temperatures (37, 10, 7 and 2.5°C). The possible osmoprotective role of the glycine- or proline-containing peptides Val-Gly and Leu-Pro was analyzed for both *L. monocytogenes* EGD-e and *L. monocytogenes* Δ*dtpT* in CDM containing 3% (w/v) NaCl (CDMS) plus 1 mM of the Leu-Pro or Gly-Val.

Table 1. *L. monocytogenes* strains, plasmids and primers used in this study.

Listeria plasmid or strain	Genotype or description	Resistance marker	Reference
Plasmids			
pCR2.1-TOPO	Shuttle vector	Amp	Invitrogen
pCR-XL-TOPO	Shuttle vector	Kan	Invitrogen
pAUL-A	Shuttle vector, thermosensitive	Em	(5)
pAUL-A+Δ*dtpT*	Shuttle vector with flanked *dtpT* gene regions	Em	This study
pPL2	*L. monocytogenes* site-specific phage integration vector	Cm	(12)
pPL2::*dtpT*	Integration vector harboring the *dtpT* gene	Cm	This study
Strains			
EGD-e	Virulent wild-type, clinical isolate, serovar 1/2a		
Δ*dtpT*	EGD-e deleted in *dtpT*		This study
Δ*dtpT* + pPL2::*dtpT*	Δ*dtpT* complemented with *dtpT*		This study
DH10β	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*Δ*M15* Δ*lacX74 deoR recA1 endAl ara*Δ*139* Δ(*ara leu*)*7697 galU galK* λ⁻ *rpsL nupG*		Invitrogen
Primers			
A Pp994.1 f1	CAAATTCACGCATTTCAGC		
B Pp994.1 r2	GTGGGCGGCCGCTAGGAATTAAGCGCACTTTC		
C Pp994.1 f3	GTGGGCGGCCGCTCCGTAATAGGAGAATCGC		
D Pp994.1 r4	TGGAACCTTATCCAGAGG		
E	GTGGTAGACTTACTCGTT		
F	CCGATCAAGTGTGTTTAA		

Generation of *dtpT* deletion mutant. A *dtp*T chromosomal in frame deletion mutant was constructed by deletion of a 1383 bp internal fragment of the *dtp*T gene. In order to create the *dtp*T mutant the flanking regions of the *dtp*T gene were amplified using PCR. The upstream region was generated using the primers A and B (Table 1), resulting in a 415-bp sized fragment. The downstream region was synthesized with the primer pair C and D to obtain a fragment of 603 bp. Both PCR products harbored *Not*I restriction endonuclease sites,

which were introduced with oligonucleotides B and C. Purified PCR products were digested with *Not*I and ligated. After overnight ligation a part of the ligation mix was added into an amplification using the primers A and D for the flanking fragments to generate a PCR product of 1002 bp harboring the 1383-basepair deletion of the *dtp*T gene. The PCR product was cloned into the shuttle cloning vector pCR2.1-TOPO. The cloned fragment was digested with restriction endonucleases *BamH*I and *Sal*I and ligated directly to the temperature-sensitive suicide vector pAUL-A (5) using the cloning vector restriction endonucleases sites *BamH*I and *Sal*I. Sequence confirmation of the deleted gene was done performing sequence analysis of the cloned insert for the newly constructed vector pAUL-A+Δ*dtpT*. The obtained construct was introduced into wild-type *L. monocytogenes* EGD-e by electroporation (17). The chromosomal deletion mutant was generated by using the procedure described by Schaferkordt and Chakraborty (18). The in-frame chromosomal deletion was confirmed by sequencing the PCR product generated with oligonucleotides A and D from chromosomal DNA of the *dtpT* mutant. The procedures for the isolation of plasmid and chromosomal DNA from *L. monocytogenes* were as previously described (18). Standard protocols were used for recombinant DNA techniques (17).

Complementation of *dtpT* deletion mutant. pPL2; a site-specific phage integration vector (12) was used for complementation of the Δ*dtpT* mutant. The *dtpT* gene and flanking regions were amplified using the oligonucleotides E and F (1876 bp) and introduced into the vector pCR-XL-Topo (Invitrogen). The ligation mixture was transformed by electroporation in *E. coli* DH10β and plated on LB agar plates containing 50 μg/ml kanamycin. Plasmid was isolated from a *dtpT*-harbouring recombinant and digested with the restriction endonucleases *Bam*HI and *Xho*I to release the inserted DNA. Following agarose gel electrophoresis, the *dtpT*-harboring fragment was isolated and ligated to *Bam*HI/*Xho*I-restricted vector pPL2 DNA. Following electroporation of the ligation mixture recombinant *E. coli* DH10β clones were plated on LB agar plates containing 25 μg/ml of chloramphenicol. One representative recombinant pPL2::*dtpT* was sequenced to verify the authenticity of the gene cloned and introduced into the isogenic Δ*dtpT* strain by electroporation. Selection of transformants was performed on BHI plates supplemented with 7.5 μg/ml of chloramphenicol. Insertion of the pPL2::*dtpT* plasmid into the PSA bacteriophage attachment site at $tRNA^{Arg}$- *attBB´* was verified by using the primer pairs NC16 and PL95 (12) to specifically amplify a 499 bp PCR product in the integrant strains. The primer pairs E and F were used to specifically detect the *dtpT* gene region. The complemented strain was examined for further phenotypic analysis as described in the results section.

Transport studies. *L. monocytogenes* EGD-e, *L. monocytogenes* Δ*dtpT* and *L. monocytogenes* Δ*dtpT* complemented with *dtpT* were grown in BHI until mid-exponential phase (OD_{600} = 0.5). Cells were harvested by centrifugation (3000 x g, 15 min., 10°C), washed twice and resuspended in 240 mM NaPipes buffer (pH 6.0) containing 5 mM $MgSO_4$ and 100 μg chloramphenicol per ml. Cells at an OD_{600} of 20 in this buffer were stored on ice until use. Cells (final OD_{600} of 1) were pre-energized at 37°C with 10 mM glucose for 10 min

prior to the addition of radiolabelled Proline-^{14}C-Alanine (final concentration of 6.5 μM). Samples were withdrawn and uptake was stopped by the addition of 2 ml 240 mM NaPipes buffer (pH 6.0) containing 5 mM $MgSO_4$ (containing 3% NaCl when uptake experiments were performed in the presence of NaCl). The cells were collected on 0.2-μm-pore size cellulose nitrate filters (Schleicher and Schuell GmbH, Dassell, Germany) under vacuum. The filters were washed with another 2 ml 50 mM potassium phosphate (pH 6.8) buffer and the radioactivity trapped in the cells was measured with a liquid scintillation counter (model 1600 TR, Packard Instruments Co., Downers Grove, IL, USA). Uptake of osmolytes was normalized to total quantity of cellular proteins, which was determined by using the bicinchoninic acid method as provided by the supplier (Sigma Chemicals, St. Louis, MO) using bovin serum albumin as a standard. Radiolabelled prolyl-alanine dipeptide (Pro-[^{14}C]-Ala) was a generous gift from Gang Fang and Bert Poolman (Univ. of Groningen, Groningen, The Netherlands) and was prepared as described by Hagting *et al.* (10).

Virulence assays. 6-8 weeks old female BALB/c mice (obtained from Harlan Winkelmann, Univ. Giessen, Germany) were used in experiments to study the effect of deletion of *dtpT* on the virulence of *L. monocytogenes*. For the *in vivo* bacterial infection and survival and growth analysis, a group of mice was infected *in vivo* and kinetics of the growth of *L. monocytogenes* in the spleen was monitored. Briefly, fresh bacterial cultures (both wild type *L. monocytogenes* EGD-e and mutant *L. monocytogenes Δdtp*T) were established in BHI medium from o/n cultures and were incubated at 37°C. After 2-3 hours of incubation followed by centrifugation and washing in saline, bacteria were resuspended in saline. Mice were infected *in vivo* in the tail vein with 200 μl saline containing 1000-1500 bacteria.

Results

Identification of the *dtpT* gene encoding a putative di- and tripeptide transporter. Based on homology a *dtpT* homolog can be identified in the *L. monocytogenes* EGD-e genome sequence (8). The 1479-bp gene encodes a single polypeptide of 492 amino acid residues. The listerial DtpT protein is similar to the eukaryotic proton-motive force dependent peptide proteins of the PTR family (22) and several bacterial DtpT systems. The *dtpT* gene shows high identity (53 and 48%) to the *yclF* gene of *B. subtilis* (26) and the *dtpT* gene of *Lactococcus lactis* (9), respectively. DtpT transporter proteins are responsible for the transport of hydrophilic di- and tripeptides and share similarities with eukaryotic peptide transporters, e.g. from yeasts, plants and kidney and small intestine of rabbit, man and rat. Based on homology and the hydropathy profile it can be predicted that the DtpT protein consists of a bundle of 12 transmembrane α helices with the N and C termini located internally.

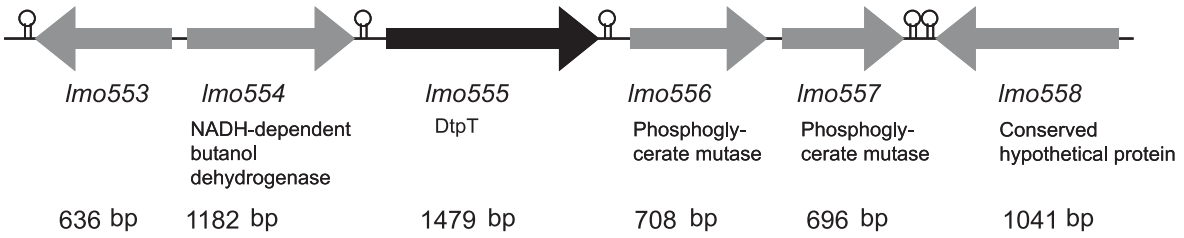

Figure 1. Organization of *dtpT* (black arrow) and surrounding genes (shaded arrows) in *L. monocytogenes* EGD-e. The hairpin structures indicate putative terminators. The numbering of the ORFs follows the numbering of the *L. monocytogenes* EGD-e genome sequence (8).

The *dtpT* gene of *L. monocytogenes* EGD-e is preceded by a putative SigA regulated promoter, suggesting that this gene is expressed under normal growth conditions. 13 bp downstream the open reading frame (ORF) encoding DtpT a putative terminator could be identified. These data would suggest a monocistronic transcript for the *dtpT* gene. The genes surrounding *dtpT* do not seem to be involved in processes related to the transport and/or utilization of di- and tripeptides. Upstream of *dtp*T a gene encoding a NADH-dependent butanol dehydrogenease and an unknown function ORF are found, both containing a separate downstream-located terminator. Downstream of *dtpT* two genes encoding phosphoglycerate mutase and another gene of unknown function are located. Also these genes have separate terminators and do not seem to share genetic elements for their regulation with *dtpT* (Fig. 1).

Transport of Pro-^{14}C-Ala. The transport of dipeptide Pro-[^{14}C]-Ala was analyzed in wild-type *L. monocytogenes*, *L. monocytogenes* Δ*dtpT* and *L. monocytogenes* Δ*dtpT* complemented with a plasmid-encoded *dtpT* gene. In *L. monocytogenes* EGD-e significant Pro-[^{14}C]-Ala transport is observed for mid-exponential cells grown in BHI (Fig. 2). These levels are comparable to the observed uptake rates for *L. monocytogenes* ScottA described by Verheul *et al.* (23). Next, the transport of Pro-[^{14}C]-Ala was analyzed in *L. monocytogenes* Δ*dtpT* and for this strain no transport activity could be measured (Fig. 2). Complementation of the *dtpT* gene in *L. monocytogenes* Δ*dtpT* revealed that di-peptide transport was restored to similar levels as found for wild-type *L. monocytogenes*. These data indicate a role of DtpT in the transport of the Pro-[^{14}C]-Ala di-peptides. Previously, it has been shown that the transport of dipeptides is dependent on the proton motive force and by addition of the potassium ionophores valinomycin and the potassium proton exchanger nigericin this transport could be significantly inhibited (23). Here, we demonstrate that the transport of Pro-[^{14}C]-Ala can be contributed to the proton-motive force dependent transporter DtpT.

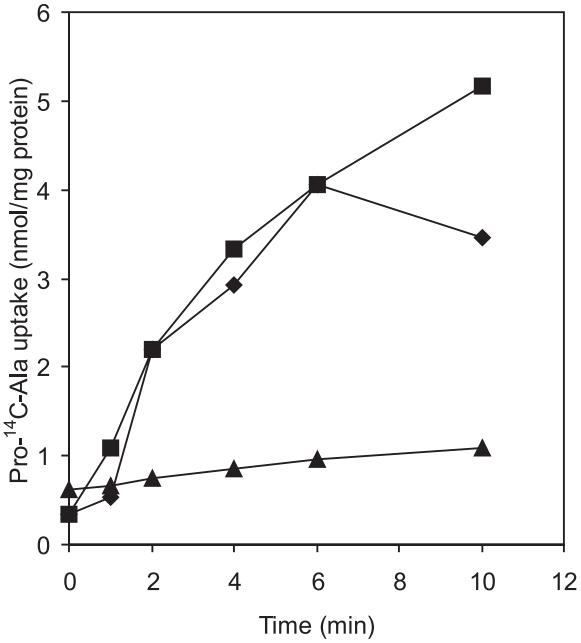

Figure 2. Uptake of Pro-[^{14}C]-Ala peptide in *L. monocytogenes* EGD-e and *L. monocytogenes* Δ*dtpT* transport. Cells were grown in BHI until mid-exponential phase (OD_{600} = 0.5) and washed twice in 240 mM NaPipes buffer (pH 6.0) containing 5 mM $MgSO_4$. Uptake assays of Pro-[^{14}C]-Ala were performed in 240 mM NaPipes buffer (pH 6.0) containing 5 mM $MgSO_4$ with glucose-energized cells of *L. monocytogenes* EGD-e (■), *L. monocytogenes* Δ*dtpT* (▲) or *L. monocytogenes* Δ*dtpT* containing pPL2::*dtpT* (♦).

***L. monocytogenes* Δ*dtpT* is affected in its ability to grow on di- and tripeptides.** The growth of *L. monocytogenes* EGD-e and *L. monocytogenes* Δ*dtpT* on defined medium containing different peptides was monitored. To do so, di- and tripeptides containing the essential amino acids valine or leucine were added to CDM in which either valine or leucine were deleted, respectively. In the absence of either valine or leucine in CDM, no growth of *L. monocytogenes* EGD-e at 37°C was observed within 60 h (Fig. 3), indicating the requirement of both these amino acids for *L. monocytogenes* as was previously reported by Premaratne *et al.* (16). Recently, using the data of the genome sequence it is observed that *L. monocytogenes* contains the genetic information for the biosynthesis of all amino acids (8). Apparently, the observed requirement of *L. monocytogenes* EGD-e for the amino acids valine and leucine might be due to repression of the amino acid biosynthetic pathways at these conditions. Upon addition of leucine containing peptides growth was restored completely (Fig. 3A), indicating the efficient uptake and subsequent hydrolysis of these peptides in *L. monocytogenes* EGD-e. The addition of Val-Gly restored growth of *L. monocytogenes*, whereas the addition of Ala-Val did not completely result in restoration of growth; compared to CDM addition of Ala-Val resulted in a delay in growth and a reduction of growth rate (Fig. 3C).

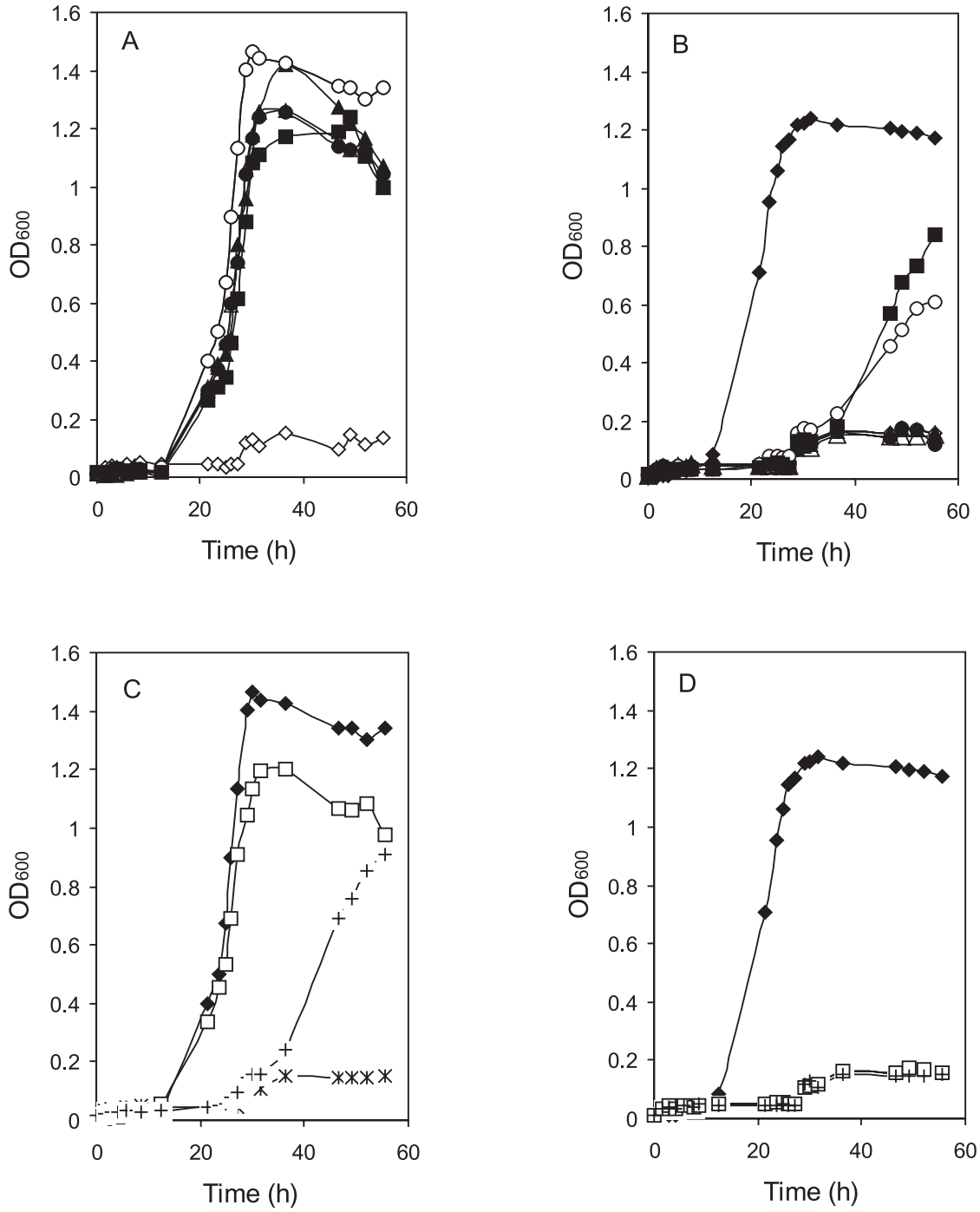

Figure 3. Valine or leucine-containing peptides as sources of essential amino acids for growth of *L. monocytogenes*. (A) Growth of *L. monocytogenes* EGD-e at 37°C in CDM (♦), CDM lacking leucine (◊), CDM lacking leucine supplied with the leucine containing peptides Leu-Ala (▲), Ala-Leu (Δ), Leu-Pro (●), Leu-Gly-Gly (○) and Ala-Leu-Gly (■). (B) Growth of *L. monocytogenes* Δ*dtpT* in CDM (♦), CDM lacking leucine (◊), CDM lacking leucine supplied with the leucine containing peptides Leu-Ala (▲), Ala-Leu (Δ), Leu-Pro (●), Leu-Gly-Gly (○) and Ala-Leu-Gly (■). (C) Growth of *L. monocytogenes* EGD-e in CDM (♦), CDM lacking valine (*), CDM lacking valine supplied with the peptides Val-Gly (□) and Ala-Val (+). (D) Growth of *L. monocytogenes* Δ*dtpT* in CDM (♦), CDM lacking valine (⋆), CDM lacking valine supplied with the peptides Val-Gly (□) and Ala-Val (+). Growth was monitored spectrophotometrically at OD_{600}.

This indicates that Ala-Val can either not be efficiently transported or can not be efficiently hydrolysed upon uptake after which the released amino acid valine can be used as essential amino acid for growth. In the case of *L. monocytogenes* Δ*dtpT* growth on CDM lacking leucine could not be restored by the addition of leucine containing dipeptides (Fig. 3B). These data directly indicate that deletion of DtpT results in the inability to use these dipeptides for growth, and presumably for transport thereof. However, upon longer incubation growth of *L. monocytogenes* Δ*dtpT* was partly restored by the addition of the leucine-containing tripeptides Leu-Gly-Gly and Ala-Leu-Gly (Fig. 3B). This would imply that these tripeptides can be transported via another system than DtpT. It is to be expected that the oligopeptide transporter Opp might be responsible for the transport of tripeptides and thus an overlap in substrate specificity of these two transporters for tripeptides exists. However, the efficacy of DtpT to transport these tripeptides is much higher than the replacement Opp system. Also, for the growth of *L. monocytogenes* Δ*dtpT* in CDM lacking valine supplemented with valine containing dipeptides no outgrowth was observed, indicating the inability to use also these substrates for growth (Fig. 3D).

Growth of *L. monocytogenes* Δ*dtpT* in complex media exposed to stress conditions and in food model systems. The growth of *L. monocytogenes* EGD-e and *L. monocytogenes* Δ*dtpT* was also monitored in rich BHI medium and revealed no differences in growth for these strains at 37°C. Similarly, also in two food products, sterilized milk and beef bouillon, no differences in growth at 37°C, measured by the number of colony forming units, were observed for *L. monocytogenes* EGD-e and *L. monocytogenes* Δ*dtpT*. Deletion of *dtpT* did also not alter the ability of *L. monocytogenes* to grow in BHI at low temperature (10, 7 or 2.5°C). However, addition of different concentrations of salt (up to 10% NaCl) to BHI medium in combination with incubation at low temperatures (10, 7 or 2.5°C) reduced the growth rates for *L. monocytogenes* Δ*dtpT* slightly compared to *L. monocytogenes* EGD-e. Probably, *L. monocytogenes* grown in BHI is not dependent on di- or tripeptides for its supply of amino acids, however, during (multiple) stress conditions certain peptides might provide additional protection, such as for example glycine- or proline-containing peptides as was reported for *L. monocytogenes* ATCC23074 (2). To further investigate this hypothesis we added a glycine- or a proline-containing dipeptide (final concentration 1 mM) to (complete) CDMS. This revealed that for *L. monocytogenes* EGD-e growth at 37°C was stimulated in the presence of the peptides whereas only a minor stimulation was observed for *L. monocytogenes* Δ*dtpT*. This implies that the DtpT transporter is involved in the accumulation of peptides and that these can counter high osmolarity stress, as is observed by the increase in growth rates (Fig. 4).

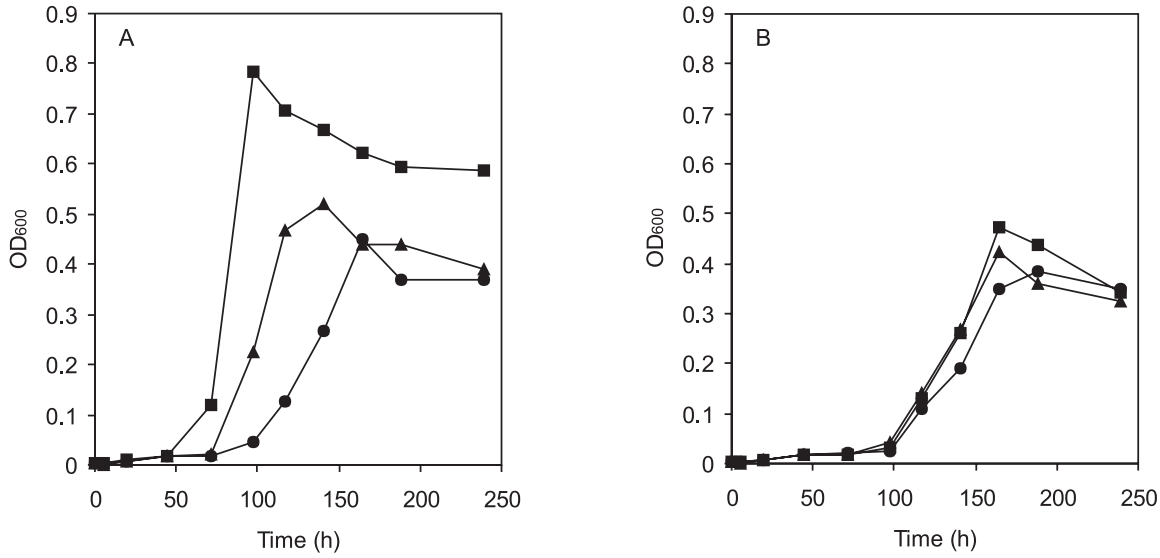

Figure 4. Growth of *L. monocytogenes* EGD-e (A) and *L. monocytogenes* Δ*dtpT* (B) in CDMS containing glycine and proline-containing peptides. Growth was monitored at 37°C by measuring the OD_{600}. Circles indicate growth in CDMS, squares indicate growth in CDMS containing 1 mM Leu-Pro, and triangles indicate growth in CDMS containing 1 mM Gly-Val.

Virulence of *L. monocytogenes* Δ*dtpT*. Using a mouse infection model, we studied the *in vivo* behavior of the mutant *L. monocytogenes* Δ*dtpT* strain. The *in vivo* growth kinetics, as revealed from mouse infection studies, indicated that deletion of *dtpT* did not impair the survival and growth of mutant *L. monocytogenes* Δ*dtpT* strain in the spleen of mice (Fig. 5). On the contrary, in comparison to wild type *L. monocytogenes*, growth of mutant *L. monocytogenes* Δ*dtpT* strain was much faster and persisted longer in mice as even on day 8th post-infection where *L. monocytogenes* wild- type strain was almost eliminated (Fig. 5).

Discussion

In this report, we demonstrate that peptides are nutritionally valuable in providing *L. monocytogenes* with essential amino acids. In addition, by using a strain specifically deleted for the *dtpT* gene we show that the di- and tripeptide transporter DtpT is involved in the translocation of these peptides. The transport of the Pro-[^{14}C]-Ala peptide was abolished in a *dtpT* deletion mutant and transport was restored in a complementation mutant. Growth experiments in CDM demonstrated that DtpT is responsible for the uptake of di- and tripeptides that subsequently can be sources of the essential amino acids leucine and valine. Previously, it has been demonstrated that *L. monocytogenes* has intracellular aminopeptidases responsible for the degradation of the peptides generating an available free amino acid pool

(23). In the presence of the peptides Leu-Gly-Gly and Ala-Leu-Gly growth of the *dtpT* chromosomal deletion mutant is observed after longer incubation periods. This is most likely explained by the presence of another transport system capable of translocating these peptides and the most likely candidate is the oligopeptide transporter Opp previously characterized by Verheul *et al*. (24) and recently genetically characterized (4). Borezee *et al.* (4) demonstrated that Opp is capable of transporting peptides longer than four residues. Apparently Opp is also capable of transporting tripeptides at low rates.

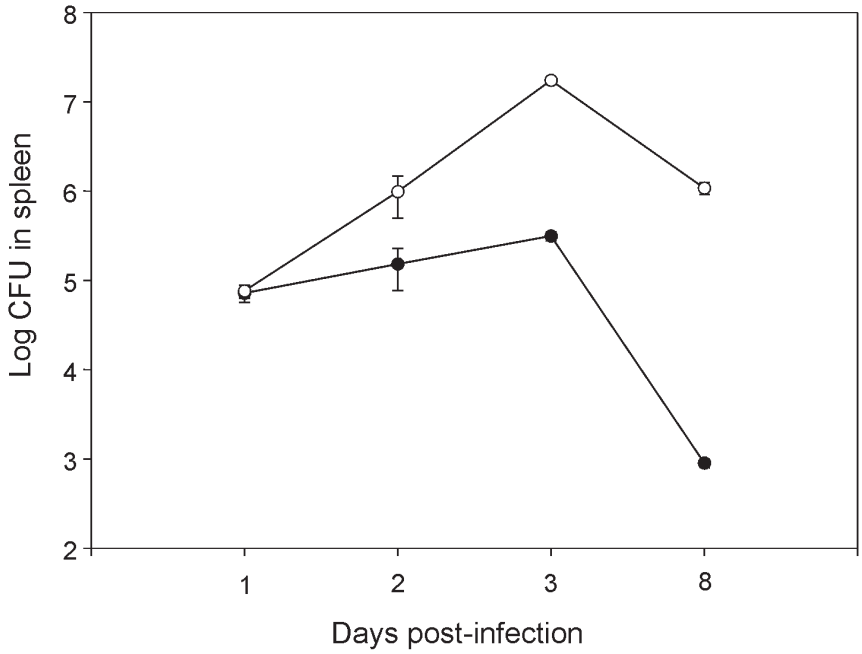

Figure 5. Virulence of *L. monocytogenes* EGD-e (closed symbols) and *L. monocytogenes* Δ*dtpT* (open symbols) in a mouse model. Mice were infected *in vivo* with approximately 1000-1500 bacteria. Numbers of bacteria were enumerated at days 1, 2, 3, and 8 post infection.

L. monocytogenes is a bacterium that is able to adapt to various stressful conditions and a variety of defensive mechanisms have been reported to be involved in these adaptation processes (1). For certain proline and glycine containing peptides a protective effect at high osmolarity conditions has been reported (2). In the current report we demonstrate that in rich BHI medium or in milk or beef bouillon the ability to grow is not dependent on DtpT and this is most likely explained by the availability of free amino acids. Also for growth in BHI at low temperature deletion of *dtpT* does not negatively effect growth. These phenomena are most likely explained by the limited concentration available di- and tripeptides relative to free amino acids and the ability of *L. monocytogenes* to accumulate peptides and amino acids via the Opp transporter and multiple amino acid transporters. Upon combination of various stresses (i.e. high salt and low temperature) a growth defect is noted upon deletion of *dtpT*. In a more controlled setting of CDMS a growth stimulatory effect of peptides containing proline

(Leu-Pro) or glycine (Val-Gly) was noted. It was observed that the DtpT transporter is required for this growth stimulatory effect. In conclusion, these data indicate that DtpT contributes to stress resistance under specific conditions when protective di- or tripeptides can be accumulated from the environment.

In the current paper, an overlap in response mechanisms to various environmental stress conditions, encountered in food environments as well as in host environments was noted. The role of DtpT in the listerial virulence pathway has not been studied before and this study indicated that the cell number of the *dtpT*-deleted *L. monocytogenes* strain in the spleen of mice is higher than for wild-type *L. monocytogenes*. Moreover, the *L. monocytogenes* Δ*dtpT* strain persisted longer in the spleen of mice than the wild type. This phenomenon is most likely explained by regulatory and compensatory mechanisms, e.g. related to the regulator PrfA and /or OppA. Recently a study on PrfA, the major regulator of listerial virulence gene expression (5), indicated that this protein can act as an activator and as a repressor of different sets of genes, also depending on growth conditions (15). One of the genes activated by PrfA is *lmo555*, the *dtpT* gene. Though this gene does not have a putative PrfA box, transcription of this gene in BHI at 37°C was lower in a strain missing PrfA (15). This activation of DtpT by PrfA is an indication that DtpT might play a role in *L. monocytogenes* virulence. Notably, the *L. monocytogenes* Δ*dtpT* strain showed an increased virulence in the mouse model, suggesting that loss of di- and tripeptide accumulation enhances growth capacity *in vivo*. Another explanation for the enhanced virulence of *L. monocytogenes* Δ*dtpT* could be related to the overlap in activity and regulatory mechanism with oligopeptide transporter Opp. Upon deletion of the gene encoding the DtpT transporter, the genes encoding the *opp* transporter might be activated to compensate for the loss of peptide transport. Borezee *et al*. (4) observed that OppA plays a major role in the intracellular survival of *L. monocytogenes*. In the absence of OppA, bacterial growth was delayed in the liver and spleen of mice during the early stages of infection. Also, it was found that OppA favors early escape from phagosomes and intracytoplasmic multiplication in macrophages. This may mean that the peptide uptake plays a role in the intracellular survival of *L. monocytogenes*. The delayed growth of the *oppA* mutant in the macrophage might be due to a limitation of nutrients (4). Indeed, it has been demonstrated that *L. monocytogenes* utilizes intracellular peptides as a source of amino acids during its intracellular replication (13). It might be speculated that the deletion of *dtpT* induces an enhanced quantity of OppA and thus enhances growth in the spleen of mice and macrophages.

The results from this study contribute to our understanding of the utilization of peptides by *L. monocytogenes*. We have shown that DtpT (i) is responsible for the translocation of di- and tri-peptides, (ii) contributes to stress resistance under specific conditions and (iii) has a role in the virulence of *L. monocytogenes*. The complex role of the DtpT transporter makes this protein a interesting and possibly valuable target for the design of specific anti-pathogenic treatments.

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Chapter 8

Summary and concluding remarks

Introduction

Ready-to-eat, fresh food products with extended shelf life are increasingly popular. However, many of these foods contain raw or minimally processed components and contain therefore a risk of surviving pathogenic bacteria. The food-borne human pathogen *Listeria monocytogenes* is of particular concern in those food products due to its wide distribution in the environment and consequently its presence on raw food ingredients. Moreover, it is known for its ability to cope with a variety of environmental stresses, such as high osmotic pressures and low temperatures. These characteristics, and the serious consequences of a listeriosis outbreak (listeriosis accounts for almost 35% of all deaths in the USA due to known food-borne bacterial pathogens (31)), make *L. monocytogenes* an important threat to consumers and the food industry. Understanding the growth and survival of *L. monocytogenes* and knowledge of virulence factors will contribute to the design of safe, minimally processed foods. This thesis deals with the survival strategies of *L. monocytogenes* in relation to environmental conditions encountered in minimally processed foods. Research mainly focused on the transporters BetL, Gbu, OpuC, and OpuB and two types of regulators, the alternative sigma factor σ^B, involved in general stress response, and the cold-shock proteins (CSPs).

Growth of *L. monocytogenes* at high osmolarity

L. monocytogenes is able to grow at salt concentrations up to 10% (30). Growth under such adverse environmental conditions is attributed mainly to the accumulation of compatible solutes that maintain turgor. The major cytoplasmic components of salt-stressed *L. monocytogenes* are, next to the primary response components potassium and glutamate, glycine betaine (*N*,*N*,*N*-trimethylglycine), L-carnitine (β-hydroxy-γ-N-trimethyl aminobutyrate), and the amino acid proline (7, 28, 37).

In Chapter 4 the role of accumulated betaine and carnitine during high osmolarity growth of *L. monocytogenes* was analyzed using single, double and triple deletions in the osmolyte transport systems BetL, Gbu and OpuC. Analysis of the intracellular levels of osmolytes revealed that betaine is accumulated up to high levels in wild type *L. monocytogenes* LO28 when grown in BHI with 6% added NaCl, whereas no carnitine accumulation could be detected. However, upon deletion of Gbu, also carnitine was accumulated in salt-stressed cells. During growth at high osmolarity, Gbu was found to be the most important osmolyte transporter, as altering Gbu had the most drastic effect on growth rate in BHI with 6% added NaCl. However, additional roles in osmo-adaptation can be ascribed to BetL and OpuC, as the highest reduction in growth rate was found for the triple mutant LO28ΔBCG (Δ*betL*, Δ*opuC*, Δ*gbu*). The growth rate of mutant LO28ΔCG was higher

than that of LO28ΔBG. In conclusion, in BHI with 6% NaCl the hierarchy of transporters is Gbu>BetL>OpuC.

Several bacteria possess the ability to synthesize glycine betaine from its precursor choline (16, 22, 29). Choline itself has no osmoprotective role, it has a net positive charge at physiological pH. In *B. subtilis*, transport of choline and a two-step oxidation via the intermediate glycine betaine aldehyde yields glycine betaine, conferring considerable osmoprotection (9, 10). In *L. monocytogenes* a putative choline transporter, OpuB, encoded by two genes, *lmo1421* and *lmo1422*, was identified (Chapter 6). Additionally, several genes exhibiting significant sequence similarities to *gbsA* (a betaine aldehyde dehydrogenase): *lmo0913, lmo0383, lmo1179*, and to *gbsB* (an alcohol dehydrogenase): *pduQ, lmo1634, lmo1166, lmo0554* were found in the genome of *L. monocytogenes* EGD-e (42). However, growth of *L. monocytogenes* in defined minimal medium with added salt was not stimulated upon addition of choline. Moreover, no accumulated betaine was found in cells grown in the presence of choline, indicating that choline transport is absent or that choline is not converted to betaine in *L. monocytogenes*, and hence, is unable to supply osmoprotection. Additionally, growth at high osmolarity of a quadruple mutant, LO28ΔBCGB (Δ*betL,* Δ*opuC,* Δ*gbu,* Δ*opuB*) was similar of that of the triple mutant LO28ΔBCGsoe, indicating that additional deletion of *opuB* does not significantly alter high-osmolarity growth. Moreover, for both strains no intracellularly accumulated betaine or carnitine was observed.

In addition to betaine and carnitine, certain peptides, specifically the proline-containing di- and tripeptides prolyl-hydroxy-proline (PHP) and prolyl-glycyl-glycine (PGG), containing also glycine, can function as osmoprotectants (1). In Chapter 7 we demonstrated that growth of *L. monocytogenes* EGD-e in DM with elevated osmolarity is enhanced upon addition of the dipeptides Leu-Pro and Gly-Val. This protective effect of the glycine and proline-containing peptides was only noted in *L. monocytogenes* wild type and not in the *dtpT* deleted strain, indicating that the di- and tripeptide transporter DtpT is involved in stress protection.

Growth of *L. monocytogenes* at low temperature

Refrigeration is a common preservation technique for a large variety of foods, including ready-to-eat foods. Insight into the mechanisms allowing low-temperature growth of *L. monocytogenes* is essential to understand and possibly control growth at low temperature. These mechanisms involve the maintenance of the cellular membrane fluidity (2), the uptake or synthesis of compatible solutes (28), and structure stabilization of macromolecules such as ribosomes, necessary for continued protein synthesis (50).

In Chapter 5 the uptake of compatible solutes at low temperature was studied. Single and multiple deletions of the osmolyte transporters BetL, Gbu, and OpuC, were shown to reduce growth at low temperature. During growth in BHI at 7°C, Gbu and OpuC had a more

pronounced role in cryoprotection than BetL. However, during growth in DM at low temperature the addition of betaine shifted the hierarchy of the transporters to Gbu>BetL>OpuC. Upon addition of carnitine, only OpuC plays a major role. The most dramatic effect of low temperature on growth rate was observed for the triple mutant LO28ΔBCGsoe (Δ*betL,* Δ*opuC,* Δ*gbu)*. Measurements of the accumulated osmolytes showed that betaine is preferred over carnitine, while in the absence of a functional Gbu, carnitine was accumulated to higher levels than betaine at 7°C. Transcriptional analysis of the genes encoding BetL, Gbu and OpuC revealed that each is induced to varying degrees upon cold shock of *L. monocytogenes* LO28. Both *gbu* and *betL,* encoding the principal betaine uptake systems in *L. monocytogenes* are significantly up-regulated following exposure to 10°C for 30 min. However, the level of *gbu* up-regulation appears greater than that of *betL* following cold shock, consistent with the observation that Gbu provides significantly more cryoprotection than BetL. Additionally, the carnitine transporter-encoding gene *opuC* is also significantly induced after a cold shock.

Upon exposure of bacteria to low temperature, the synthesis of the majority of proteins is blocked, possibly due to a cold-sensitive block in initiation of translation, resulting in a decrease in polysomes and an increase in 70S monosomes and ribosomal subunits. It is therefore proposed that the ribosome might be the temperature sensor in bacteria (48). Also in *L. monocytogenes* an instable 70S ribosomal particle structure is found upon cold shock (4), representing a problem that must be overcome to allow normal protein synthesis. Cold-shock proteins (CSPs) are among a group of proteins that are induced upon low temperature exposure of cells. These small, mainly acidic proteins are highly induced upon a cold shock. The translation of *csp* mRNA upon a cold shock is possible because of the presence of a DB (downstream box) element on the mRNA ensuring additional binding to the impaired ribosome (34). The induction of cold-shock specific ribosomal factors leads to restoration of the ribosomal structure and the ability to form intact translation initiation complexes for translation of non cold-shock mRNAs (27, 34). In chapter 2 we demonstrate that the adaptation process of *L. monocytogenes* LO28 to low temperature includes elevated levels of CSPs. Four 7 kDa proteins were found, named Csp1, Csp2, Csp3 and Csp4. The genome sequence of *L. monocytogenes* EGD-e shows three genes encoding for CSPs, *cspL*, *cspB,* and *cspD* (21), possibly indicating strain differences between *L. monocytogenes* LO28 and EGD-e. After a cold shock, levels of Csp1 and Csp3 increased 10-fold and 3.5-fold, respectively, the levels of Csp2 and Csp4 were not elevated. Remarkably, upon high hydrostatic pressure (HHP) treatment also enhanced levels of CSPs were observed. Pressurization of *L. monocytogenes* LO28 cells resulted in 3.5 and 2-fold increased levels of Csp1 and Csp2, respectively. It has been suggested that pressurization affects ribosomes in a similar way as low temperature, suggesting a similar role for CSPs after HHP treatment as upon cold-shock. Applying a combination of these preservation techniques to foods, notably low temperature storage and subsequent HHP treatment, might yield more bacterial survivors than HHP treatment of non cold-adapted cells. It can be speculated that this cross-protection is due to the

enhanced production of CSPs during the low temperature exposure. An additional cold-adaptative mechanism that might increase pressure resistance of cells is the adaptation of membranes to maintain cellular membrane fluidity at low temperature. Hydrostatic pressure decreases, as low temperature, the membrane fluidity and thereby increases membrane permeability and leakage of sodium and calcium ions (44, 47). The increased pressure resistance of cold adapted cells might thus be mediated *via* an increased amount of CSPs and a more fluid cellular membrane.

Regulation of compatible solute transport in *L. monocytogenes*

Betaine and carnitine contribute significantly to growth of *L. monocytogenes* at high osmolarity and at low temperature. These compatible solutes are accumulated via three transporters, Gbu, BetL, and OpuC. A predicted fourth osmolyte transporter, OpuB, does not seem to play a role in accumulation of betaine or carnitine (Chapter 6).

Control of osmolyte accumulation via these transporters is achieved at the transcriptional, translational and post-translational levels. Control of the osmolyte transporters at the transcriptional level is achieved via several mechanisms. The alternative transcription factor σ^B, involved in stress response, was found to be responsive to temperature and osmotic stress. A *sigB* deletion mutant strain was impaired in its ability to use betaine and carnitine as osmo- and cryoprotectants (5, 6). Fraser *et al*. (18) were able to assign the impaired carnitine uptake to the transcriptional regulation of *opuC* by σ^B. Also betaine accumulation was shown to be partially σ^B-dependent, and although this could not be unambiguously assigned to one of the transporters, *betL* seems the most likely candidate, as this transporter has an upstream promoter sequence matching the consensus σ^B-dependent sequence (with 4 mismatches). It is unlikely that σ^B represents the sole factor regulating transcription of the osmolyte transporters, as neither betaine nor carnitine transport is completely abolished in a *sigB* deletion mutant strain (6, 18). Analysis of the *betL* promoter region, for example, indicates the presence of a putative σ^A-dependent promoter upstream of the σ^B-dependent promoter, as is the case for e.g. the proline transporter *opuE* in *B. subtilis* (46). Transcription of *opuE* from the σ^B-dependent promoter increased transiently after an osmotic upshock, whereas transcription from the σ^A-dependent promoter rose in proportion to the external osmolarity and was maintained at high levels (46). Sleator *et al*. (41) suggested that this adoption of "redundant" promoters, and, moreover, of whole gene systems, may have led to a dissemination of the role of σ^B in coordinating transcriptional control among other regulatory systems. One such group of alternative regulators is the GntR transcription regulator family. Possessing 19 members, this group represents the largest family of transcriptional regulatory proteins in *L. monocytogenes* (21). In addition, *L. monocytogenes* may possess proteins that merely modulate transcriptional acitivity, as might be the case for FlaR, a functional homologue of H-NS (involved in transcriptional control of *proU* in *E. coli*) and induced at

high osmolarity (40). The genome of *L. monocytogenes* EGD-e revealed the presence of 15 histidine kinases and 16 response regulators (21). It is likely that at least one of the resulting two-component systems may also play a role in the listerial salt and chill stress response (41), analogous to two-component systems in e.g. *Escherichia coli* and *Salmonella*.

Translation efficiency is another important factor in determining the final yield of functional proteins. The strength of the ribosomal binding site, the choice of initiation codon, and spacing differences all determine the efficiency with which the ribosome-binding site (RBS) is recognised by the ribosome. Four of the osmolyte transporter genes in *L. monocytogenes* have an alternative initiation codon, namely TTG (for *betL*, *gbuA*, and *gbuC*) and GTG (for *opuCA*). In *E. coli* it is apparent that the use of non-ATG initiation codons serves to limit expression at the translational level. Thus these genes in *L. monocytogenes* are presumably regulated, at least to some extent, at the level of translation (41).

Finally, the accumulation of osmolytes is also controlled by the activity of the transport proteins. Verheul *et al.* (49) showed that transport of both betaine and carnitine is subject to *trans* inhibition by preaccumulated solute. Internal betaine did not only inhibit the transport of external betaine, but also that of carnitine and vice versa. The *trans* inhibition was alleviated upon osmotic upshock, suggesting that alterations in membrane structure are transmitted to the binding pockets for betaine and carnitine of both transporters at the inner surface of the membrane. Recent research in *L. lactis* showed that its betaine transporter OpuA is specifically activated as a consequence of increased charge concentration in the cell (25). This increase can be caused by a decrease in cell volume but also by the accumulation of potassium ions in the initial response to osmotic upshift. The OpuA protein may not directly sense this increased charge, rather, the membrane in which the protein is embedded may serve as mediator by alterations in specific interactions between the membrane lipids and the transporter (25).

The fact that three betaine and carnitine transporters are present in *L. monocytogenes* emphasizes the physiological significance of osmoregulation for this strain. Most bacteria capable of adaptation to relatively high salinity have redundant osmolyte transporters. In Table 1 the uptake systems for compatible solutes in the gram-negative bacterium *E. coli*, the low G/C containing gram-positives *B. subtilis* and *L. monocytogenes*, and the high G/C containing gram-positive *Corynebacterium glutamicum* are listed. The need for several osmoregulatory systems may lay in the need to mediate the uptake of structurally diverse compatible solutes available in diverse ecological niches, the involvement of different transporters in response to environments with different osmolalities and the associated need for differential regulation of transporter expression. Betaine, for example, is abundant in plant tissue (39), while carnitine is a major component of muscle tissue (45). BetL is relatively effective only when the osmotic stress is provided by or accompanied by sodium ions, since transport of betaine via this transporter is known to be coupled to the influx of Na^+ ions (20). Hence, this transport system cannot explain the osmotically activated transport observed at

low Na^+ concentrations. In such an environment the organism must have (an) other active osmolyte transporter(s).

Table 1. Uptake systems for compatible solutes; adapted from Bremer and Krämer (12); Sleator *et al.* (41); Mendum and Tombras Smith (33); and this thesis.

Organism	System	Type of mechanism	Substrate spectrum	Major substrate(s)[a]	Regulation at the level of:	
					Expression	Activity
E. coli	ProP	H^+ symport	Broad	GB, PB, Pro, Car, Ect, others	+	+
	ProU	ABC transporter[b]	Broad	GB, PB, Pro, others	+	+
	BetT	Secondary transport[c]	Narrow	Cho	+	+
B. subtilis	OpuA	ABC transporter	Medium	GB, PB, others	+	ND[d]
	OpuB	ABC transporter	Narrow	Cho	+	ND
	OpuC	ABC transporter	Broad	GB, PB, Cho, Car, others	+	ND
	OpuD	Na^+ transport	Narrow	GB, others	+	+
	OpuE	Na^+ transport	Narrow	Pro	+	-
L. monocytogenes	Gbu	ABC transporter	Medium	GB, Car, others	+	+
	OpuB	ABC transporter	Narrow	Cho (?)	+	+[e]
	OpuC	ABC transporter	Medium	Car, GB, others	+	+
	BetL	Na^+ transport	Narrow	GB	+	+
C. glutamicum	BetP	Na^+ transport	Narrow	GB	+	+
	EctP	Na^+ transport	Broad	GB, Pro, Ect	-	+
	ProP	H^+ transport	Medium	Pro, Ect	+	+

[a] GB, glycine betaine; PB, proline betaine; Cho, choline; Pro, proline; Car, carnitine; Ect, ectoine.
[b] ABC, ATP-binding cassette
[c] The cotransported ion (Na^+ or H^+) is not known
[d] ND, not determined
[e] Choline uptake is salt-stimulated in *L. monocytogenes* EGD-e (data not shown)

Role of osmolyte transporters in virulence of *L. monocytogenes*

For many food-borne pathogens the ability to sense and respond to the high osmolarity of the gastrointestinal lumen is a key component of virulence. The shift in osmolarity between the external aqueous environment and the small intestine functions to trigger the synthesis of virulence factors essential for subsequent pathogenesis (14). In addition, in order to survive and grow in the lumen of the gastrointestinal tract bacteria must adapt to an environment with an osmolarity equivalent to 0.3 M NaCl. A significant reduction in the ability to colonize the upper small intestine and cause subsequent systemic infection following peroral inoculation in mice was observed in mutants lacking OpuC (Chapter 4, 43, 51), leading to the assumption that OpuC, and herewith carnitine uptake, is an important virulence factor in *L. monocytogenes*. The relative abundance of carnitine in mammalian tissues (8) makes it the

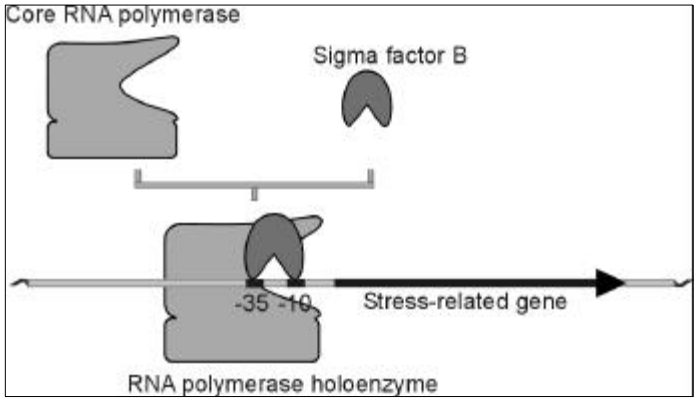
most readily available and thus possibly the most important osmolyte contributing to the survival of *L. monocytogenes* during intracellular growth following infection (43).

Interestingly, OpuB seems to play a major a role in virulence, as cells with an *opuB* deletion were almost not recovered from mice when they were co-inoculated perorally with the LO28 wild type, as described in Chapter 6. Transport of choline and use of it as the sole carbon and energy source for growth may be important since this carbon source is a constituent of an abundant class of lipids (phosphatidylcholine) present in the host's gut. This phosphatidylcholine is hydrolyzed during the escape of the *L. monocytogenes* cell from the secondary double-membrane vacuole formed from the cell-to-cell spread of *L. monocytogenes*. This hydrolyzation of phosphatidylcholine is mediated by PC-PLC (phosphatidylcholine-preferring phospholipase; PC-PLC, encoded by *plcB*), rendering free choline. Choline (*N,N,N*-trimethyl-ethanolamine), may be converted into ethanolamine upon demethylation of the amino group. In *L. monocytogenes* EGD-e the *eut*-genes encoding the proteins necessary for anaerobic degradation of the carbon source ethanolamine in a coenzyme B12-dependent manner, are present (13). Therefore, diminished choline uptake and consequent depletion of a carbon source, may be the explanation for the significant reduction in virulence of an *opuB* deleted *L. monocytogenes* strain. Additionally, an *opuB* deletion mutant showed an increased susceptibility to bile (personal communication, M. Begley, UCC, Cork, Ireland), which may affect the ability of the mutant to reach and subsequently multiply in target organs.

Regulation of gene expression

Bacteria have evolved several strategies to survive or even adapt to adverse environmental conditions such as the various stresses encountered in minimal processing. The survival strategy of most bacteria involves entry into the stationary phase. Stationary phase *E. coli* cells show significant physiological changes to confer protection to several environmental insults (36). Additionally, in some gram-positive micro-organisms such as *B. subtilis*, the survival strategy involves differentiation to spores.

For all bacteria adaptation to sudden adverse conditions in the environment requires the ability to respond rapidly. This involves activation of existing enzymes and transcription of genes resulting in enhanced levels of (stress-related) proteins. Alternative sigma factors recognize different promoter sequences and redirect transcription to, for example in *B. subtilis*, genes involved in heat-shock response, chemotaxis, sporulation, and general stress response (24). In Chapter 3 the role of the alternative sigma factor σ^B, involved in general stress response in *L. monocytogenes* and other gram-positive organisms, was studied in acid stress resistance of *L. monocytogenes*.



	Promoter sequence			
Gene	-35	spacing	-10	# mismatches
sigB	GTTTTA	-N14-	GGGTAA	
opuC	GTTTAA	-N14-	GGGAAA	2
opuB	GGAATA	-N15-	GGGTAA	3
betL	GTTACC	-N14-	GGGAAA	4
cspL	GATTTA	-N10-	GAGAAA	3
cspD	GTGTTA	-N10-	CGGCAA	3
cspB	GATTTA	-N12-	CGTTAA	3
gadD	TTTTTA	-N12-	CGGTAA	2
gadC	GTTTGT	-N14-	GGGTAT	3

Figure 1. Schematic representation of σ^B-related transcription and alignment of putative σ^B-dependent promoters in *L. monocytogenes* (Chapter 3, ref. 5, 18).

Survival at a lethal pH (pH 2.5) and adaptation to a non-lethal pH (pH 4.5) was shown to be σ^B-dependent. An important characteristic of survival in acidic conditions is the maintenance of a relatively high intracellular pH. For this two specific mechanisms have been identified, the glutamate decarboxylase acid resistance system (GAD) and F_0F_1-ATPase (15). Three of the five genes in the glutamate decarboxylase system, *gadD* and the operon *gadCB* were found to be σ^B-dependent (Fig. 1). This can partly explain the loss of acid tolerance of the *L. monocytogenes sigB* mutant strain, as also cells with null mutations in the *gad*-genes (*gadA*, *B*, and *C*) show reduced acid resistance and adaptation (15). In addition, two-dimensional gel-electrophoresis revealed nine proteins that were enhanced in the wild type but not in the *sigB*-deleted strain upon acid exposure. Five of these proteins were analysed with a Maldi-TOF mass spectrometer and were identified as Pfk, GalE, ClpP and Lmo1580. Pfk, 6-phosphofructokinase, and GalE, UDP-glucose-4-epimerase, are enzymes involved in glycolysis and sugar metabolism, respectively. ClpP is the ATP-dependent Clp protease proteolytic subunit, involved in the prevention of accumulation of proteins that are misfolded by heat, osmotic stress, or other stresses, and that might be toxic for the cell. Moreover, it was shown that ClpP plays a major role in intramacrophage survival in *L. monocytogenes* (19). Exposure to pH 4.5, in order to preload cells with σ^B and consequently with σ^B-dependent

general stress proteins, conferred also considerable protection against high hydrostatic pressure treatment and freezing. These combined data suggest that the expression of σ^B-dependent genes provides *L. monocytogenes* with a nonspecific multiple stress resistance that may be relevant for survival in the natural environment as well as during food processing.

Other regulators of gene expression in bacteria are cold-shock proteins. CSPs can activate transcription of certain cold-induced proteins (11, 23, 35) and act as anti-terminators (3). Another function of CSPs is to stabilize RNA at low temperature, thereby minimizing the secondary folding of mRNA (26). As mentioned previously, in Chapter 2 we identified four CSPs in *L. monocytogenes* LO28. Whereas all 4 CSPs were expressed in exponential growth phase, protein levels of two CSPs (Csp1 and Csp2) were shown to be enhanced upon a cold shock. Also, levels of two CSPs (Csp1 and Csp3) were induced after HHP treatment. In *E. coli*, recent research showed that the expression of RpoS, the alternative sigma factor involved in stress response in most gram-negative bacteria, is regulated by CspC and CspE. These two CSPs (of the nine CSPs present in *E. coli*) are constitutively expressed and may thus act as regulatory elements for the expression of the stress proteins in the complex stress-response network of the cell (38).

Perspectives for further research on survival strategies of *L. monocytogenes*

Although to date much is known about the physiology and pathogenesis of *L. monocytogenes*, the recent elucidation of its genome sequence has opened new possibilities for research on safe and minimally processed foods. Molecular mechanisms of stress adaptation and subsequent effects on virulence factors can be revealed using genome wide approaches, e.g. DNA micro-arrays in combination with proteome analysis. Previous work (5, 6, 18) and Chapter 3 of this thesis show that in *L. monocytogenes* general stress regulation through the alternative sigma factor σ^B has an important role in regulation of the production of proteins involved in stress resistance, and thus is a factor playing a major role in survival of *L. monocytogenes* in minimally processed foods. However, thus far the full impact of this sigma factor is not completely understood. Additional research, e.g. using micro-arrays with a *sigB*-deletion mutant or a σ^B-overexpression strain, will render additional and new information on the exact genes through which σ^B exerts its function.

The uptake of compatible solutes upon hyperosmotic shock has been extensively studied in *L. monocytogenes*. In this thesis the transport mechanisms and the accumulation of betaine and carnitine at high osmolarity and low temperature is described. However, the primary response to an osmotic upshock, i.e. the uptake of potassium ions and glutamate, is poorly understood in *L. monocytogenes*. Additionally, the events after a hypoosmotic shock (osmotic downshock) have received little attention. From the genome of *L. monocytogenes* EGD-e, several genes encoding putative transporters involved in osmo-adaptation were identified; *kdpABC* coding for a transporter involved in the uptake of potassium ions (possibly regulated by KdpDE), *lmo1013* and *lmo2064* coding for putative mechanosensitive channels,

releasing solutes upon hypoosmotic shock (41). Moreover, the mechanism of osmotic stress sensing and subsequent induction and activation of osmolyte transporters is largely unknown, not only in *L. monocytogenes*, but also in other bacteria. Induction of transcription of the genes encoding the OpuC and OpuB transporters in *L. monocytogenes* is regulated by the alternative sigma factor σ^B (18), whereas regulation of transcription of Gbu and BetL is largely unknown. Activation of the osmolyte transporters upon osmotic upshock is subject to trans inhibition by preaccumulated solute in *L. monocytogenes* (49). Recent research in *L. lactis* showed that its betaine transporter OpuA is specifically activated as a consequence of charge concentration in the cell (25). However, the activation of the osmolyte transporters in *L. monocytogenes* is largely unexplored.

Application of research on *L. monocytogenes* in the design of safe foods

In this thesis, it is demonstrated that *L. monocytogenes* has a robust physiology and is able to adapt to diverse environments using general and specific mechanisms. One such general mechanism is gene regulation via the alternative sigma factor σ^B. Insight into the mechanism of action of σ^B might clarify possibilities in blocking stress sensing, regulation, and the adaptation pathway. Additionally, it is envisaged that more effective hurdle technology can be designed upon extensive knowledge of the σ^B regulon, i.e. following a first hurdle involving σ^B-related cellular defense (e.g. acid stress), a second stress in which cellular defense is not σ^B-related might be more effective than an additional hurdle in which the response includes the σ^B regulon. However, the efficiency of a preservation technology should not only be tested on the sequenced strain EGD-e, since differences with naturally occurring strains may occur. Moreover, due to population heterogeneity, even within a population differences in stress adaptation and survival can occur.

Though several functions for CSPs have been suggested, cross-protection of low-temperature adapted cells, containing high levels of CSPs, to other, new preservation techniques, has not been examined thoroughly. In Chapter 2 we described the increased survival of cold-shocked cells exposed to high hydrostatic pressure. These data imply cross protection of cold shocked cells to HHP exposure, which may negatively affect the efficiency of combined preservation techniques. From the increased knowledge on CSPs useful information can be gained to understand low-temperature adaptation and the deleterious effects of cold shock on certain bacteria. In this respect the release of genome sequences of more and more bacteria plays an important role, as the presence of cold-shock proteins, and thus the likelihood of being able to recover from a cold shock, is easily checked upon. However, one has to bear in mind that subtle differences within the regulation and/or structure of CSPs might already imply an altered psychrotrophic growth pattern of strains, e.g. discrimination between psychrotrophic and mesophilic strains of *B. cereus* involves three nucleotide changes in the major cold shock protein gene *cspA* (17). The increased knowledge

on low-temperature adaptation may also offer perspectives for methods to control the growth of other microorganisms that limit the shelf life and safety of refrigerated foods.

It has been suggested that a detailed understanding of the listerial osmolyte uptake mechanisms may lead to the control of this pathogen in foods. Suggested possible control measurements include deliberately subverting the essential osmolyte transporters, *i.e.* smugglin technology, or limiting the availability of osmolytes. However, despite the previous described smuggling activity of e.g. triethylglycine, which inhibits betaine uptake via both BetL and Gbu (32), a practical application of this smuggling technology is probably inadequate. Additionally, since both betaine and carnitine occur wide-spread in food products, limiting the availability of osmolytes in foods is probably not feasible. The possibly most effective manner of inhibiting growth of *L. monocytogenes* in minimally processed foods is the use of hurdle technology, though the height and the sequence of the hurdles must be chosen carefully, as was demonstrated in this thesis. A possible sequence of hurdle exposure that might provide additional killing of *L. monocytogenes* is exposure to acidic conditions followed by high osmolarity. The internal pH of cells exposed to acidic conditions decreases, impairing the efficiency of transporters depending on ATP hydrolysis. A subsequent exposure to high salinity, for which adaptation relies for a large part on the ABC-transporters Gbu and OpuC, might further decrease survival or growth. Determination of the optimal sequences and height for hurdle technology in minimal processing will be challenging. Ultimately, understanding the growth and survival of *L. monocytogenes* and knowledge of virulence factors will contribute to the design of adequate food-safety measures.

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Samenvatting

De consument van vandaag wil zijn maaltijden gemakkelijk en in korte tijd bereiden. Daarbij verlangt hij versheid, geen chemische conserveringsmiddelen, en bovenal (microbiologische) veiligheid. Deze combinatie van wensen heeft geleid tot de introductie van diverse kant-en-klaar maaltijden die na productie voornamelijk geconserveerd worden door koeling. Ze bevatten rauwe of minimaal verhitte bestanddelen, hetgeen de aanwezigheid van diverse micro-organismen impliceert. De meeste bacteriën kunnen niet uitgroeien bij lage temperatuur, waardoor het product veilig in de koelkast bewaard kan worden. Echter, een bacterie die algemeen in de natuur voorkomt en ook bij lage temperatuur kan groeien, is de voedselpathogeen *Listeria monocytogenes*. Deze pathogeen kan listeriose veroorzaken, een ziekte die onder andere kan leiden tot hersenvliesontsteking met een dodelijke afloop in circa 25% van de gevallen. De meeste infecties treden op bij personen met een verzwakt afweersysteem. De consequentie van listeriose bij zwangere vrouwen is vaak een spontane abortus. *L. monocytogenes* kan groeien in de koelkast en vormt daardoor een potentieel gevaar voor bovengenoemde kant-en-klaar maaltijden. Andere factoren die bijdragen aan de persistentie van de bacterie in de voedselketen zijn het vermogen om te groeien bij relatief hoge zoutconcentraties, om te overleven bij lage pH, en om biofilms te vormen op diverse oppervlakken. Kennis omtrent de groei en overleving van *L. monocytogenes* zijn essentieel om adequate behandelingen aan voedsel te geven. Deze conserveringmethoden dienen mild te zijn teneinde te voldoen aan de eisen die de consument aan levensmiddelen stelt (natuurlijk en vers). Voorbeelden van milde conserveringstchnieken zijn een hittebehandeling bij relatief lage temperatuur, een ultra-hogedrukbehandeling (UHD), verpakken onder gewijzigde gascondities en natuurlijk bewaren bij lage temperatuur. Dit proefschrift beschrijft de processen van adaptatie van *L. monocytogenes* aan lage temperatuur, hoge zoutconcentraties en andere conserveringsmethoden. De rol van koude-schok eiwitten (CSPs) en de rol van de osmolietopnamesystemen BetL, Gbu, OpuC en OpuB in de groei bij lage temperatuur worden behandeld. Ook wordt de rol van osmolietopname in groei bij hoge zoutconcentraties beschreven, de functie van de alternatieve sigmafactor σ^B in stress-adaptatie, en de opname van di- en tripeptiden door *L. monocytogenes*.

In Hoofdstuk 2 wordt een belangrijk onderdeel van de adaptatie aan lage temperatuur door *L. monocytogenes* beschreven. Met behulp van twee-dimensionale gel-electroforese werd aangetoond dat *L. monocytogenes* LO28 vier CSPs heeft. In andere organismen zoals *Bacillus subtilis* en *Lactococcus lactis* is eerder aangetoond dat deze eiwitten essentieel zijn voor adaptatie van de cellen aan lage temperatuur. Twee van deze eiwitten, Csp1 en Csp2, zijn in verhoogde concentratie aanwezig in *L. monocytogenes* cellen die aan lage temperatuur blootgesteld zijn. Daar deze eiwitten waarschijnlijk een effect hebben op ribosoom- en mRNA-stabilisatie bij de translatie van mRNA in eiwit, en deze stabilisatie ook tijdens andere processen van belang kan zijn, werd onderzocht of deze eiwitten tijdens een UHD behandeling ook verhoogd aanwezig zijn in *L. monocytogenes* cellen. Het bleek dat de

niveaus van twee van de vier CSPs (Csp1 en Csp3) verhoogd zijn na een hogedrukbehandeling. Ook blijken *L. monocytogenes* cellen een UHD-behandeling beter te kunnen overleven wanneer ze zijn aangepast aan lage temperatuur. Het percentage cellen dat een hogedrukbehandeling overleefde, is 100 maal hoger voor cellen die een koudeschok hadden ondergaan dan cellen die groeiden bij 30°C. Dit impliceert dat een koudeschok kruisbescherming geeft voor een UHD behandeling, wat de effectiviteit van gecombineerde conserveringstechnieken kan beïnvloeden.

In Hoofdstuk 3 wordt de rol van de alternatieve sigmafactor σ^B in *L. monocytogenes* beschreven. Een sigmafactor is verantwoordelijk voor het herkennen van een specifieke promotersequentie voor een gen en voor het starten van de transcriptie, zodat transcriptie kan worden gereguleerd door de verschillende sigmafactoren. De alternatieve sigmafactor σ^B wordt actief wanneer *L. monocytogenes* zich in ongunstige omgevingen bevindt en reguleert transcriptie zo dat stress-gerelateerde genen worden overgeschreven. De eiwitten die dan geproduceerd worden, kunnen de cel beschermen. Met behulp van een stam met een *sigB* deletie werd aangetoond dat σ^B een belangrijke rol speelt in overleving in en adaptatie aan lage pH omgevingen. Aangetoond werd dat het glutamaatdecarboxylase-systeem (Gad-systeem), een mechanisme dat in belangrijke mate de overleving bij lage pH bepaalt door intracellulaire verzuring te voorkomen, voor een gedeelte σ^B-afhankelijk is. Dit systeem bestaat uit 5 eiwitten, GadA, GadB, GadC, GadD en GadE, waarvan werd gevonden dat de transcriptie van de genen coderend voor GadB, GadC en GadD (waarbij *gadCB* een operon vormt) σ^B-afhankelijk is. Naast inductie van het Gad-systeem zijn met behulp van twee-dimensionale gel-electroforese negen eiwitten (o.a. metabole enzymen en stress-gerelateerde eiwitten) gevonden die in wild-type cellen verhoogd aanwezig zijn na een blootstelling aan pH 4,5, terwijl ze dat niet zijn in cellen van de stam met de *sigB* deletie, en dus waarschijnlijk σ^B-gereguleerd zijn. Verder blijkt uit Hoofdstuk 3 dat deze sigmafactor ook een rol speelt in de overleving van *L. monocytogenes* tijdens een UHD behandeling en na invriezen.

Groei van *L. monocytogenes* in omgevingen met hoge osmolariteit en/of lage temperatuur wordt gestimuleerd door accumulatie van betaine en carnitine in de cel. Dit zijn osmolieten, kleine moleculen zonder netto lading die tot hoge concentraties in de cel kunnen worden opgenomen zonder dat ze celfuncties storen. Betaine en carnitine zijn met name aanwezig in levensmiddelen van respectievelijk plantaardige en dierlijke oorsprong. Deze osmolieten worden over de celmembraan getransporteerd via drie transport systemen: BetL, OpuC en Gbu. Het eiwit BetL transporteert in co-transport met een natrium ion betaine de cel in (met behulp van de electro-chemische gradiënt). Gbu transporteert ook voornamelijk betaine, maar deze transporter gebruikt ATP voor dit transport. Ook OpuC gebruikt ATP voor transport, en transporteert voornamelijk carnitine. In Hoofdstuk 4 en 5 wordt de rol van deze transporters, tijdens respectievelijk groei bij hoge zoutconcentraties en lage temperatuur uitgebreid besproken. Om de functie van de verschillende transporters en van de geaccumuleerde betaine en carnitine te achterhalen, werden verscheidene mutanten gebruikt, inclusief een drievoudige mutant waarin alle drie transporters uitgeschakeld zijn. Van de drie

systemen is Gbu de belangrijkste tijdens groei bij hoge osmolariteit en lage temperatuur. Deletie van deze transporter leidt tot een verlaging van de groeisnelheid bij beide condities. Wild-type cellen die gekweekt waren in BHI met 6% zout hebben betaine intracellulair geaccumuleerd (300 μmol/gram drooggewicht), maar geen carnitine. In de stammen waarbij BetL of OpuC is uitgeschakeld is de geaccumuleerde hoeveelheid betaine hetzelfde, terwijl ook de groei van deze stammen niet is aangetast. Echter, bij deletie van Gbu is de intracellulaire hoeveelheid betaine verminderd (125 μmol/gram drooggewicht), en werd ook carnitine gevonden (300 μmol/gram drooggewicht). In experimenten met muizen werd gevonden dat OpuC, de carnitine transporter, een rol speelt in de virulentie van *L. monocytogenes*. Dit zou verklaard kunnen worden doordat er tijdens de pathogenese condities voorkomen met hoge osmolariteit, en carnitine veel in zoogdieren voorkomt. Geconcludeerd kan worden dat transport van betaine en carnitine in *L. monocytogenes* verzorgd wordt door BetL, Gbu en OpuC, en dat de eventuele vierde transporter, OpuB geen rol speelt in betaine- of carnitine-accumulatie. Dit werd bevestigd door het gebruik van een viervoudige mutant, waarbij naast BetL, Gbu en OpuC ook OpuB werd uitgeschakeld. Deze stam vertoont dezelfde groei karakteristieken bij lage temperatuur en hoge zoutconcentraties als de drievoudige mutant. In Hoofdstuk 6 wordt deze vierde transporter uitgebreid beschreven. De genen coderend voor OpuB (*lmo1421* en *lmo1422*) vertonen gelijkenis met die voor de cholinetransporter OpuB in *B. subtilis*. Het kon echter niet onomstotelijk worden bewezen dat OpuB in *L. monocytogenes* een cholinetransporter is. Wel kon met behulp van een *opuB* mutant in *L. monocytogenes* LO28 worden vastgesteld dat deze transporter geen rol heeft in de groei bij lage temperatuur of hoge zoutconcentraties. Ook werd ontdekt dat OpuB een duidelijke rol speelt in de virulentie van *L. monocytogenes*.

In Hoofdstuk 7 wordt de di- en tripeptiden transporter DtpT beschreven. Deze transporter maakt het mogelijk voor *L. monocytogenes* om di- en tripeptiden op te nemen zodat deze als bron van aminozuren gebruikt kunnen worden. Deze transporter blijkt onmisbaar te zijn voor groei in gedefinieerd medium waarbij valine en leucine, twee essentiële aminozuren, als dipeptiden werden aangeboden. Bij groei in complexe media en levensmiddelen blijkt deze transporter geen grote rol te spelen. Groei van *L. monocytogenes* in gedefinieerd medium met verhoogde osmolariteit werd gestimuleerd in aanwezigheid van proline-bevattende dipeptiden, echter niet in de stam met een *dtpT* deletie. Dit wijst op een rol van DtpT in bescherming tegen hoge osmolariteit.

Het onderzoek dat beschreven is in dit proefschrift heeft een belangrijke bijdrage geleverd aan het begrip van de fysiologie en virulentie van de voedselpathogeen *L. monocytogenes*. Het is duidelijk dat verschillende mechanismen een rol spelen bij de adaptatie van *L. monocytogenes* aan ongunstige omgevingsfactoren als lage temperatuur en hoge osmolariteit. Voor een uiteindelijk veilig product heeft de toepassing van meerdere, verschillende milde processen waarschijnlijk de beste kans van slagen. De verkregen kennis van de groei en overleving van *L. monocytogenes* en de kennis van virulentie-factoren kunnen bijdragen aan het ontwerp van adequate conserveringsmethoden.

Nawoord

Hier is het dan, het boekje. Na vier jaar van proeven doen, schrijven, plezier hebben en doorbijten, is het af. De afgelopen jaren zijn voor mij een periode geweest waarin ik op meerdere vlakken veel heb geleerd. Diverse personen hebben hierin een bijdrage gehad, en hier wil ik graag een aantal van deze personen in het bijzonder bedanken.

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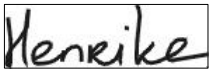

Curriculum vitae

Hendrikje Hermina Kamphuis werd op 22 december 1975 in Den Ham (Ov.) geboren. Ze volgde vanaf 1988 de middelbareschoolopleiding aan het Christelijk Lyceum Almelo waarvan zij het VWO-diploma behaalde in 1994. In datzelfde jaar startte ze met de opleiding Levensmiddelentechnologie aan de Landbouwuniversiteit te Wageningen (thans Wageningen Universiteit). Gedurende de doctoraalfase participeerde zij in onderzoek naar de invloed van verschillende eiwitbronnen in de voeding op het risico van dikke darmkanker (prof. dr. ir. M. A. J. S. van Boekel, dr. G. M. Alink en dr. ir. E. H. Vis) en de karakterisatie van een familie van koudeschokeiwitten in *Lactococcus lactis* (prof. dr. ir. F. M. Rombouts, dr. T. Abee en dr. ir. J. A. Wouters). Stage werd gelopen bij Friesland Coberco Dairy Foods Research Centre te Deventer. In maart 1999 behaalde zij het doctoraaldiploma met lof.

Van juni 1999 tot juli 2003 deed zij een promotie-onderzoek bij de leerstoelgroep Levensmiddelenmicrobiologie van de Wageningen Universiteit. Het onderzoek maakte deel uit van het onderzoeksproject ‘Microbial stress response in minimal processing’ gesponsord door het Wageningen Centre for Food Sciences (WCFS). Tijdens haar promotie-onderzoek heeft zij vier maanden onderzoek gedaan in het Department of Microbiology, University College, Cork, Ireland. Het in dit proefschrift beschreven onderzoek werd uitgevoerd onder begeleiding van prof. dr. ir. F. M. Rombouts, dr. T. Abee en dr. ir. J. A. Wouters.

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