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Molecular Characterisation of the Mechanisms of Compatible Solute Accumulation in *Listeria monocytogenes*

A Thesis Presented to the National University of Ireland
for the Degree of
Doctor of Philosophy

by

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February 2001

Supervisor: Dr. Colin Hill

For my parents

CONTENTS

Abstract

The ability of the Gram-positive foodborne pathogen *Listeria monocytogenes* to survive and grow in environments of elevated osmolarity can be attributed, at least in part, to the accumulation of a restricted range of low molecular mass solutes compatible with cellular function. Accumulated to high internal concentrations in hyper-saline environments, compatible solutes, either transported into the cell or synthesised *de novo*, play a dual role: helping to stabilise protein structure and function while also counterbalancing external osmotic strength, thus preventing water loss from the cell and plasmolysis. While previous physiological investigations identified glycine betaine, carnitine, and proline as the principal compatible solutes in the listerial osmostress response, genetic analysis of the uptake/synthesis systems governing the accumulation of these compounds has, until now, remained largely unexplored.

Representing the first genetic analysis of compatible solute accumulation in *L. monocytogenes*, this thesis describes the molecular characterisation of BetL; a highly specific secondary glycine betaine transport system, OpuC; a multicomponent carnitine/glycine betaine transporter, and finally *proBA*; a two-gene operon encoding the first two enzymes of the listerial proline biosynthesis pathway.

In addition to their role in osmotolerance, the potential of each system in contributing to listerial pathogenesis was investigated. While mutations in each gene cluster exhibited dramatic reductions in listerial osmotolerance, OpuC$^-$ mutants were additionally shown to exhibit reduced virulence when administered *via* the oral route. This represents the first direct link between the salt stress response and virulence in *L. monocytogenes*.

Chapter I

Bacterial Osmoadaptation: A Review

CONTENTS

1. INTRODUCTION

Bacterial species are perhaps the most versatile of all living organisms, inhabiting almost every environmental niche known to, and including, man. This successful occupancy of what are often hostile environments, uncongenial to other life forms, can be attributed at least in part to the development of complex stress management strategies, which have evolved to allow the bacterial cell to sense and respond to changes in its external environment. One such environmental parameter is the osmolarity of the extracellular medium. Bacterial cells are, in principle, required to maintain an intracellular osmotic pressure greater than that of the growth medium in order to generate cell turgor; generally considered to be the driving force for cell extension, growth and division (Csonka, 1989; Taiz, 1984). The ability to adapt to changes in the osmolarity of the external environment is therefore of fundamental importance for growth and survival, and as such, bacterial cells have evolved a number of osmoadaptive strategies to cope with fluctuations in this important environmental parameter.

This review begins with an outline of the principal strategies used by bacteria to overcome salt stress, and continues with an in-depth analysis of the molecular mechanisms governing such responses. The second part of the review deals with the possible signals regulating these responses, and outlines the current knowledge on bacterial osmotic signal transduction pathways. The final section includes an analysis of the possible roles of some of these osmo-stress responsive mechanisms in contributing to the virulence potential of a number of pathogenic bacteria.

2. OSMOADAPTATION

The term osmoadaptation describes both the physiological and genetic manifestations of adaptation to a low water environment (Galinski, 1995). In principle, two strategies of osmoadaptation have evolved to cope with elevated osmolarity: (*i*) the salt in cytoplasm type and (*ii*) the organic osmolyte type (Galinski and Trüper, 1994).

2.1 Salt in cytoplasm; the halobacterial solution

This mechanism, which was discovered in and is typical of members of the Halobacteriacea (Galinski and Trüper, 1994; Martin *et al.*, 1999), achieves osmotic equilibrium by maintaining a cytoplasmic salt concentration (KCl) similar to that of the bathing solution. As a consequence, the entire cytoplasm is exposed to high ionic strength (up to 7 molal KCl has been recorded in species of *Halobacterium* (Lanyi, 1974)) and as such requires extensive structural adaptations.

To achieve salt tolerance, halobacterial proteins have undergone extensive amino acid substitutions, involving enrichment in aspartyl, glutamyl and weakly hydrophobic residues (Lanyi, 1974). The halophilic malate dehydrogenase (hMDH) from *Halobacterium marismortui*, for example, has an excess of 20 mol% acidic over basic residues as compared with only 6 mol% in the non-halophilic enzyme (Mevarech *et al.*, 1977). These modifications can be explained by the need to attract a hydration shell in a surrounding environment of low water activity. For example while native hMDH binds 0.8-1.0 g water and approximately 0.3 g salt/g protein the binding capacity of non-halophilic globular protein is much less (0.2-0.3 g water and approximately 0.01 g salt/g protein) (Zaccai *et al.*, 1986; 1989). Since the unusual hydration properties of the enzyme are absolutely dependent on its native structure, Zaccai *et al.* (1989) proposed a model for the stabilisation of halophilic proteins in which the enzyme's tertiary or quaternary structure is essential to coordinate hydrated salt at a local concentration higher than that in the solvent. The model proposed for hMDH (based on X-ray and neutron scattering studies) sees the protein with a core similar to that of its

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Organisms exhibiting the salt in cytoplasm mechanism of osmoadaptation are thus strictly confined to environments of elevated osmolarity. In contrast, all other organisms possess an adaptation strategy (involving organic osmolyte accumulation) that has as its hallmarks a minimal requirement for genetic change (so called 'genetic simplicity', (Yancey *et al*., 1982)) and a high degree of flexibility in allowing organisms to adapt to significant fluctuations in external osmolarity.

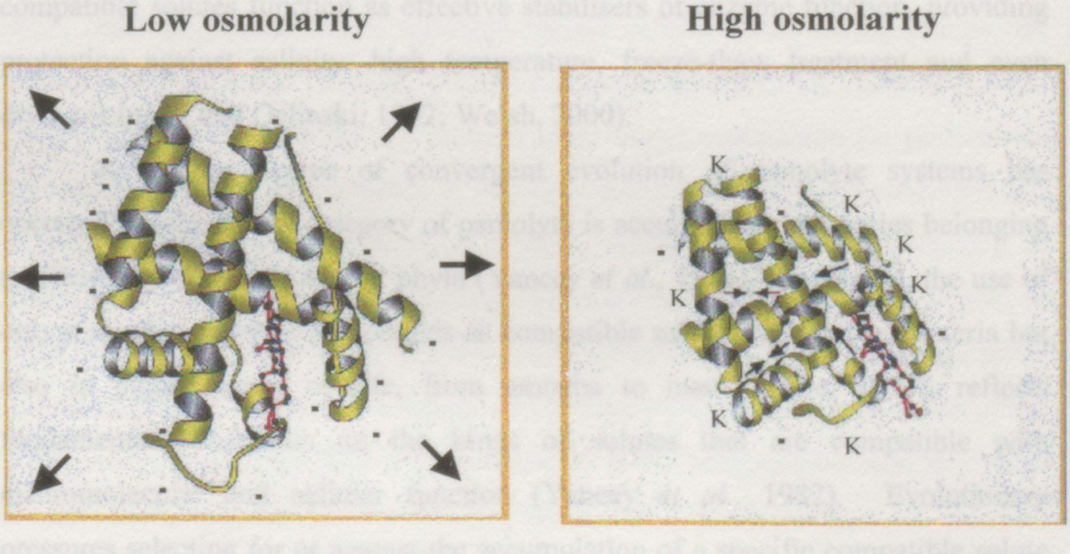


Fig. 1. Effects of low and high osmolarity on the structure and function of halophilic enzymes. Repulsive forces due to the net negative charge on the enzyme surface results in denaturation of the native protein structure at low osmolarity. At high osmolarity shielding cations (K^+) neutralise the negative charge, thus reducing the repulsive forces on the enzyme surface. ( ) Enzyme active site.

2.2 Compatible solutes

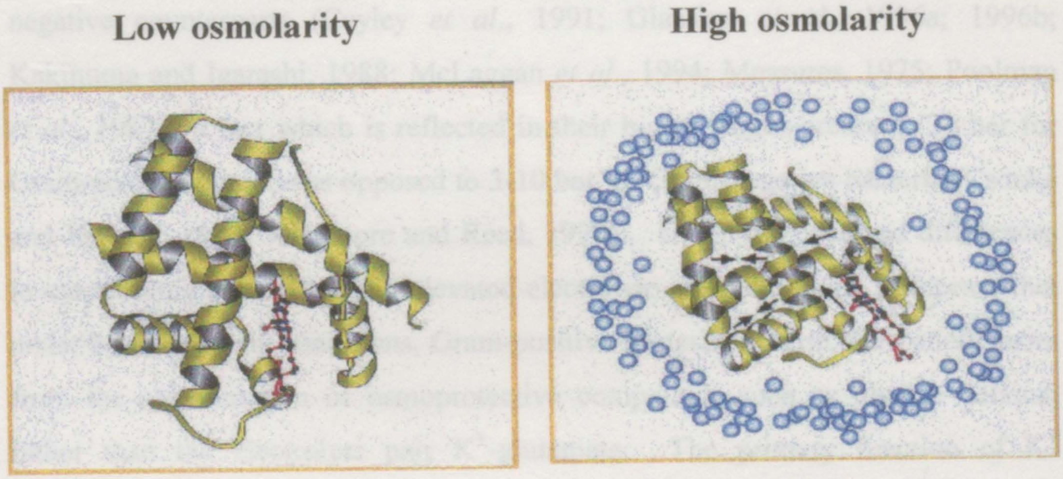
The compatible solute answer to elevated osmolarity involves a bi-phasic response in which increased levels of K^+ (and its counter ion glutamate) have been observed as a primary response phenomenon (Epstein, 1986), followed by a dramatic increase in the cytoplasmic concentration (either by synthesis and/or uptake) of osmoprotective compounds, representing the secondary response. Such compounds, owing to their compatibility with cellular functions at high internal concentrations, are often referred to as compatible solutes (Brown, 1976). In general, compatible solutes are highly soluble molecules which carry no net charge at physiological pH (Galinski, 1995) and do not interact with proteins; factors facilitating their accumulation to high intracellular concentrations (> 1 mol/Kg water (Galinski and Trüper, 1994)) without disrupting vital cellular processes such as DNA repair, DNA-protein interactions and the cellular metabolic machinery (Record *et al.*, 1998a; 1998b; Strøm and Kaasen, 1993; Yancey, 1994). In addition to their role as osmotic balancers (Brown, 1976), compatible solutes function as effective stabilisers of enzyme function, providing protection against salinity, high temperature, freeze-thaw treatment and even drying (Lippert and Galinski, 1992; Welsh, 2000).

A striking degree of convergent evolution of osmolyte systems has occurred in which each category of osmolyte is accumulated by species belonging to often only distinctly related phyla (Yancey *et al.*, 1982). In general, the use of only a small number of compounds as compatible solutes, not just in bacteria but also in higher forms of life, from amoeba to man (Kinne, 1993), reflects fundamental constraints on the kinds of solutes that are compatible with macromolecular and cellular function (Yancey *et al.*, 1982). Evolutionary pressures selecting for or against the accumulation of a specific compatible solute may depend not only on its osmotic function (as influenced by the degree of methylation (Yancey *et al.*, 1982) and hydrocarbon chain length (Peddie *et al.*, 1994)), but also secondary functions such as heat or cold tolerance (Ko *et al.*, 1994).

2.2.1 Molecular principles of compatible solute function

Preferential exclusion from the immediate surface of proteins and other cytoplasmic macromolecules is the basis for the compatibility of nature's osmolytes (Arakawa and Timasheff, 1985) (Fig. 2). There are three possible explanations as to how the exclusion of these solutes from the protein-water interface occurs. A model proposed by Bull and Breese (1974) suggests that compatible solutes may raise the surface tension of water, increasing the cohesive forces within the water structure, thus, making it energetically more difficult to disrupt water-water interactions in favour of protein-water complexes. Because solvation of the protein with the lower surface tension solute water is energetically more favourable, the bulk water will tend to hydrate the protein, expelling the high surface tension solute water from the protein surface. In addition to increased surface tension, steric incompatibility has been proposed to play an important role in osmolyte exclusion from macromolecular surfaces. In contrast to water, which (owing to its small size, polarity and hydrogen-bond potential) is capable of accommodating almost any protein surface geometry, most organic osmolytes are large, rigid molecules, which, although replete in hydrogen-bonding groups, are preferentially excluded from the protein surface in favour of the more accessible water molecules. The third and perhaps most trivial explanation for preferential exclusion centres on the existence of possible repulsive forces between solutes and certain protein surface groups (Low, 1985). Irrespective of the mechanism of solute exclusion, the thermodynamic consequence is the same: a general stabilising effect opposing the unfolding/denaturation of proteins and other labile macromolecular structures (Baskakov and Bolen, 1998; Galinski, 1993; Qu *et al.*, 1998). This stabilising effect extends not only to salt tolerance but also to a range of stress factors such as heating, freezing and drying (Welsh, 2000).

In addition to the solute protection theory, Cayley *et al.* (1992) proposed that it is the free cytoplasmic volume (unbound water) which is the fundamental determinant of growth under hyper-osmotic stress, and that the secondary effect of volume increase by compatible solute accumulation (a consequence of preferential exclusion from cytoplasmic macromolecules and membrane components) is the key to their osmoprotective function. Thus, compatible solutes may serve a dual role in osmoregulating cells; restoring cell volume and stabilising protein structure.


Low osmolarity

High osmolarity

Fig. 2. Structure stabilisation of halotolerant enzymes at elevated osmolarity. Preferential exclusion of compatible solutes (●) from the protein surface helps to maintain enzyme structure at elevated osmolarity, while also helping to increase cell volume.

3. THE HALOTOLERANT RESPONSE

3.1 Hyper-osmotic shock; solute accumulation

3.1.1 Initial phase of osmoadaptation; the primary response

The most rapid response to osmotic up-shock, both in Gram-positive and Gram-negative bacteria, is a stimulation of potassium (K^+) uptake (Epstein, 1986; Whatmore *et al.*, 1990). However not all of the accumulated K^+ is osmotically active, a certain proportion being required to balance the net negative charge of the cytoplasmic macromolecules (Cayley *et al.*, 1991). Only that fraction of the total K^+ concentration that is balanced by other small counter-ions contributes significantly to osmotic activity. The primary charge counterbalance for K^+ influx in *Escherichia coli* is endogenously synthesised glutamate (McLaggan *et al.*, 1994). On the other hand, the nature of the counter-ion in *Bacillus subtilis* is unclear since, in contrast to *E. coli*, glutamate levels increase only slightly after osmotic up-shock (Kempf and Bremer, 1998). Under non-stressed conditions, Gram-positive bacteria already possess a large amino acid pool, of which a significant proportion is glutamate; similarly, the cellular concentrations of K^+ in non-stressed Gram-positive bacteria are usually much higher than their Gram-negative counterparts (Cayley *et al.*, 1991; Glaasker *et al.*, 1996a; 1996b; Kakinuma and Igarashi, 1988; McLaggan *et al.*, 1994; Measures, 1975; Poolman *et al.*, 1987a) a fact which is reflected in their higher turgor pressure (20 bar for Gram-positive bacteria as opposed to 3-10 bar for Gram-negative bacteria (Csonka and Epstein, 1996; Whatmore and Reed, 1990)). Given the observed differences in cytoplasmic osmolality and elevated electrolyte concentrations, it appears that under hyper-osmotic conditions, Gram-positive bacteria in particular benefit more from the accumulation of osmoprotective compounds such as glycine betaine, rather than the electrolyte pair K^+-glutamate. The primary function of K^+ accumulation in Gram-positive bacteria thus may be to signal induction of the secondary response (Booth and Higgins, 1990). This role of K^+ as a second messenger is inferred from the observed dependence of a number of osmotic responses on K^+ uptake (*Section 5.2*).

3.1.1.1 K^+ uptake

As with the majority of systems, molecular characterisation of K^+ uptake is most advanced for Gram-negative bacteria. *E. coli* possesses four constitutive low affinity K^+ transport systems: TrkG, TrkH, Kup (formerly TrkD) and TrkF, as well as an inducible high affinity system, Kdp.

Kdp. The Kdp system is highly specific for K^+, exhibiting a K_m of 2 μM and a V_{max} of 150 μmol/min/g cells (Rhoads *et al.*, 1976; Epstein *et al.*, 1978). A member of the P-type ATPases, the driving force for K^+ uptake *via* Kdp comes from ATP hydrolysis (Epstein *et al.*, 1978). The membrane associated Kdp-ATPase (KdpFABC) is encoded by the *kdpFABCDE* operon, which also encodes the two-component regulatory system KdpDE. Located at the promoter-distal end of the operon the *kdpDE* genes (encoding the sensor kinase KdpD, and soluble transcriptional activator KdpE) are expressed as an operon from a promoter located within *kdpC*, however read-through from the upstream *kdp* promoter has also been observed (Polarek *et al.*, 1992; Voelkner *et al.*, 1993). Kdp thus serves as an osmotically inducible system scavenging K^+ when the ion is present at low concentrations.

Trk. In media containing >1 mM K^+, the predominant uptake system is Trk. Encoded by constitutively expressed genes dispersed on the chromosome (Bakker, 1993), K^+ uptake, previously attributed to TrkA, is now known to be mediated by two integral membrane bound proteins; TrkG and TrkH (Dosch *et al.*, 1991). While both membrane potential and ATP are required for K^+ uptake, ATP is thought to regulate, rather than drive K^+ uptake *via* the Trk system. Existing as both cytoplasmic and membrane-associated forms (Bossemeyer *et al.*, 1989a), TrkA is believed to regulate TrkG/H, mediating activation by ATP, or acting as a protein kinase (Bakker, 1993; Bossemeyer *et al.*, 1989a). In addition to TrkA, *trkE* represents a further regulatory domain which, when disrupted, eliminates and impairs K^+ transport *via* TrkH and TrkG respectively. K_m and V_{max} values for K^+ uptake *via* TrkG and TrkH are 0.3-1 mM and 2.2-3.0 mM and >200 nmol/min/mg protein and >300 nmol/min/mg protein, respectively (Bossemeyer *et al.*, 1989a; Dosch *et al.*, 1991).

Kup. The Kup system (formerly known as TrkD) represents a low affinity K^+ uptake system. Distinguished from the other systems by its ability to transport caesium (Bossemeyer *et al.*, 1989b) Kup exhibits a K_m of 0.3-0.4 mM and a V_{max} of 30 nmol/min/mg protein for K^+ uptake (Bossemeyer *et al.*, 1989a; Dosch *et al.*, 1991). The final and perhaps least studied K^+ transport system in *E. coli* is TrkF, as yet no gene has been linked to this system, which exhibits K_m and V_{max} values for K^+ uptake of 20-30 mM and <15 nmol/min/mg protein, respectively. It is unlikely that K^+ uptake *via* either Kup or TrkF plays any significant role in the osmoadaptation of *E. coli* (Epstein and Kim, 1971; Rhoads *et al.*, 1976).

While considerably less information is available concerning the mechanisms governing K^+ accumulation in Gram-positive bacteria, uptake has been studied in the acidophilic, moderate thermophile *Bacillus acidocaldarius* (Bakker *et al.*, 1987; Hafer *et al.*, 1989; Michels and Bakker, 1987). Two transport systems have been identified in this strain; a high affinity system exhibiting immunological cross-reactivity with the KdpB subunit of *E. coli*, and a low affinity system displaying kinetic and substrate specificities similar to the *E. coli* TrkG/H systems (Michels and Bakker, 1987).

In conclusion then, osmotically induced accumulation of K^+, representing the primary or initial phase of osmoadaptation, is mediated by rapid activation of low and high affinity systems in both Gram-positive and Gram-negative bacteria.

3.1.2 Secondary response; osmoptotectant accumulation

Given an upper limit of ~400 mM for K^+ glutamate accumulation (Dinnbier *et al.*, 1988; Tempest *et al.*, 1970), the cut-off point for the primary response, at least in Gram-negative bacteria, appears set at ~0.5 M NaCl (Galinski, 1995). Increases in the salt concentration above this level triggers the secondary response *i.e.* accumulation of neutral osmoprotectants which, in contrast to the ionic osmolytes of the primary response, can be accumulated to high intracellular concentrations without adversely affecting cellular processes (Brown, 1976; Yancey *et al.*, 1982). While the list of compatible solutes available to both prokaryotes and eukaryotes is extensive and varied (Kempf and Bremer, 1998), Beumer *et al.* (1994) identified the three principal compatible solutes in *Listeria* as glycine betaine, carnitine and proline (listed in decreasing order of importance, in terms of osmoprotection). Herein the molecular mechanisms governing the synthesis and transport of these compounds are reviewed, using *E. coli* and *B. subtilis* as models of Gram-negative and Gram-positive bacteria, respectively (Fig. 3).

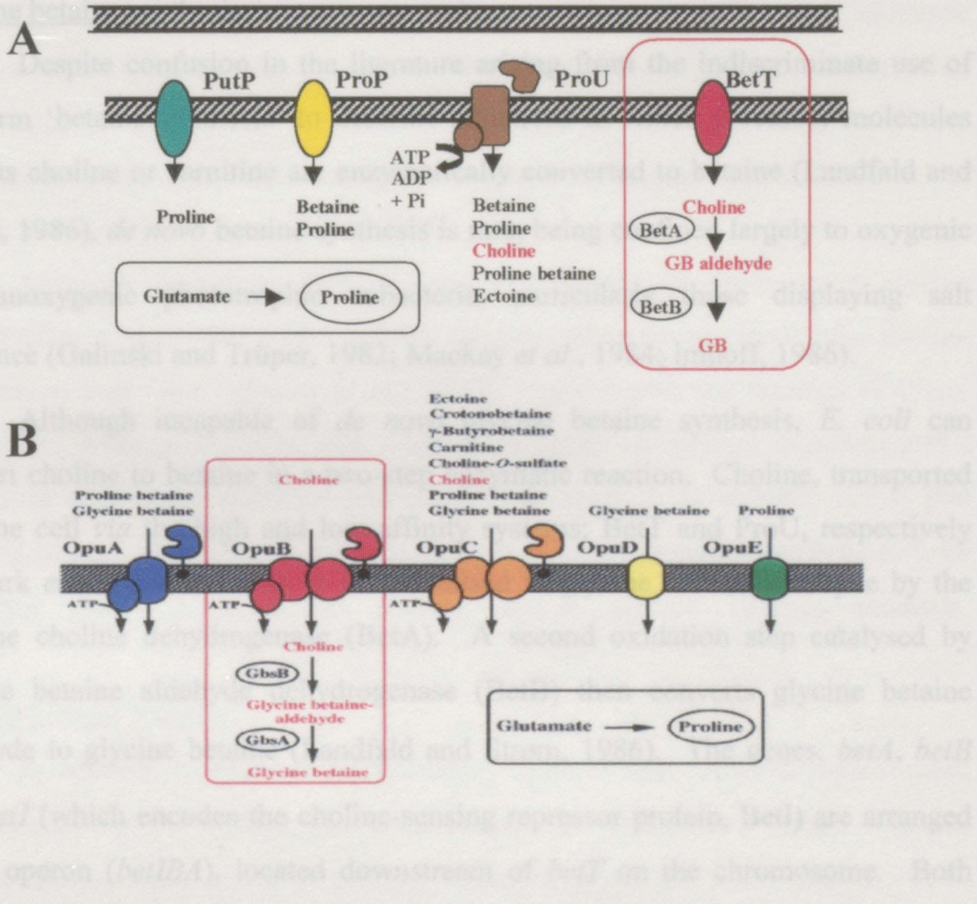
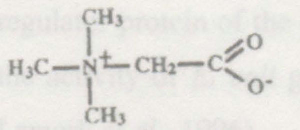


Fig. 3. Compatible solute transport/synthesis systems in (A) *E. coli* and (B) *B. subtilis*; models for Gram-negative and Gram-positive bacteria respectively. Adapted from Bremer and Krämer, 2000.

3.1.2.1 Glycine betaine



$$H_3C-\overset{CH_3}{\underset{CH_3}{N^{+}}}-CH_2-C\begin{smallmatrix}\nearrow O\\ \searrow O^-\end{smallmatrix}$$

The preferred compatible solute for the majority of bacteria and perhaps the most widely utilised osmolyte, spanning both the plant and animal kingdoms, is the trimethylammonium compound glycine betaine (*N,N,N*-trimethyl glycine). The earliest reports of bacterial osmoprotection by glycine betaine were for *Pedicoccus soyae* by Sakaguchi in 1960, however it wasn't until the early 1980s that the full potential of betaine as an effective and versatile compatible solute, both in prokaryotic and eukaryotic systems, was realised (Bagnasco *et al*., 1986; Bellinger and Larher, 1988; Galinski and Trüper, 1994).

Glycine betaine synthesis

Despite confusion in the literature arising from the indiscriminate use of the term 'betaine synthesis' to describe situations in which precursor molecules such as choline or carnitine are enzymatically converted to betaine (Landfald and Strøm, 1986), *de novo* betaine synthesis is rare, being confined largely to oxygenic and anoxygenic phototrophic eubacteria, particularly those displaying salt tolerance (Galinski and Trüper, 1982; Mackay *et al.*, 1984; Imhoff, 1986).

Although incapable of *de novo* glycine betaine synthesis, *E. coli* can convert choline to betaine in a two-step enzymatic reaction. Choline, transported into the cell *via* the high and low affinity systems; BetT and ProU, respectively (Lamark *et al.*, 1991; 1992), is first oxidised to glycine betaine aldehyde by the enzyme choline dehydrogenase (BetA). A second oxidation step catalysed by glycine betaine aldehyde dehydrogenase (BetB) then converts glycine betaine aldehyde to glycine betaine (Landfald and Strøm, 1986). The genes, ***betA***, ***betB*** and ***betI*** (which encodes the choline-sensing repressor protein, BetI) are arranged in an operon (***betIBA***), located downstream of ***betT*** on the chromosome. Both gene systems are transcribed divergently under the control of separate though partially overlapping promoters (Lamark *et al.*, 1991). Expression of ***betA***, ***betB*** and ***betT*** is subject to osmotic induction. Addition of choline (in the absence of betaine) during osmotic stress results in a further induction of ***betT*** and ***betA*** by reducing BetI mediated repression at the promoter region (Røkenes *et al.*, 1996). Under anaerobic conditions expression of both promoters is reduced by ArcA; the regulator protein of the ArcA-ArcB two-component regulatory system, controlling the activity of *E. coli* genes repressed under anaerobic conditions (Eshoo, 1988; Lamark *et al.*, 1996).

Genetic and physiological analysis of the osmoregulatory choline-glycine betaine pathway in *B. subtilis* reveals that, as with *E. coli*, glycine betaine production involves a two-step oxidation process with glycine betaine aldehyde as the intermediate (Boch *et al.*, 1994; 1997). Two enzymes act in concert for glycine betaine synthesis: a type III alcohol dehydrogenase (GbsB) that oxidises choline (transported into the cell by the OpuB and OpuC transporters; Kappes *et* *al.*, 1999) to glycine betaine aldehyde, and a glycine betaine aldehyde dehydrogenase (GbsA), which converts this intermediate to glycine betaine. The structural genes (*gbsAB*) for these enzymes are genetically organised in an operon, expression of which is enhanced by the presence of choline (but not salt) in the growth medium (Boch *et al.*, 1994; 1996).

Although previously believed to be incapable of synthesising glycine betaine (Ko *et al.*, 1994) recent studies demonstrating the existence of a choline transport system OpuC (Fraser *et al.*, 2000), coupled with the findings of Phan-Thanh and Mahouin (1999) that *Listeria* harbours an alcohol dehydrogenase, exhibiting significant sequence homologies to GbsB in *B. subtilis*, prove that *Listeria*, at least in theory, has the necessary machinery to synthesise betaine from precursor molecules such as choline and/or glycine betaine aldehyde.

Glycine betaine transport

In addition to endogenous synthesis, bacteria have evolved sophisticated mechanisms for the uptake and accumulation of osmolytes released into the external environment either by primary microbial producers upon dilution stress, by decaying plant and animals, or by mammals in the form of excretion fluids (e.g. urine) (Galinski and Trüper, 1994; Ventosa *et al.*, 1998). Given that osmolyte uptake is often energetically more favourable than synthesis, accumulation of compatible solutes from exogenous sources generally inhibits endogenous synthesis, at least over a certain range of osmolarities (Dinnbier *et al.*, 1988; Whatmore *et al.*, 1990). In the presence of external glycine betaine, for example, both the *E. coli* Bet and *B. subtilis* Gbs systems are inhibited (Boch *et al.*, 1997; Eshoo, 1988), thus promoting glycine betaine uptake in favour of synthesis. Two osmoregulated permeases, ProP and ProU, mediate uptake of most osmoprotectants in *E. coli* and *Salmonella typhimurium*. First recognised as proline transporters (Anderson *et al.*, 1980; Csonka, 1981; Dunlap and Csonka, 1985; Menzel and Roth, 1980; Wood, 1988), the ProP and ProU systems were subsequently found to transport betaine and other osmoprotectants (Barron *et al.*, 1987; Cairney *et al.*, 1985a; 1985b; Gowrishankar, 1985; Jebbar *et al.*, 1992; Perroud and Le Rudulier, 1985).

ProP. The ProP system transports betaine, proline and ectoine with similar affinities (Jebbar *et al.*, 1992; Wood, 1988). Possessing twelve transmembrane domains, a structural feature common in secondary transport systems (Saier, 1994), it is characterised additionally by the presence of an extended central hydrophilic loop and a carboxy-terminal extension predicted to form an alpha-helical coiled coil (Culham *et al.*, 1993) (Fig.4). Recently Culham *et al.* (2000) demonstrated that this C-terminal extension plays an important role in the osmotic activation of ProP. A similar domain in the betaine transporter BetP of *Corynebacterium glutamicum* has also been linked to the osmosensing and osmoregulatory mechanisms of betaine uptake in this organism (Peter *et al.*, 1998; Rübenhagen *et al.*, 2000) (*Section 5.2.3*). Transcription of *proP* is directed from two promoters, P1 and P2, both of which are activated by osmotic up-shifts. While the cAMP-CRP complex normally represses *proP*-P1, the activity of *proP*-P2 appears dependent on both RpoS and the nucleoid-associated protein FIS (Mellies *et al.*, 1995; Xu and Johnson, 1997). Transport *via* ProP (which exhibits K_m and V_{max} values for betaine uptake of 44 μM and 37 nmol/min/mg protein, respectively) is enhanced by a combination of transcriptional induction (two- to five-fold) and a five-fold stimulation of the activity of the ProP protein (Cairney *et al.*, 1985a; Dunlap and Csonka, 1985; Gowrishankar, 1986) in response to osmotic up-shock.

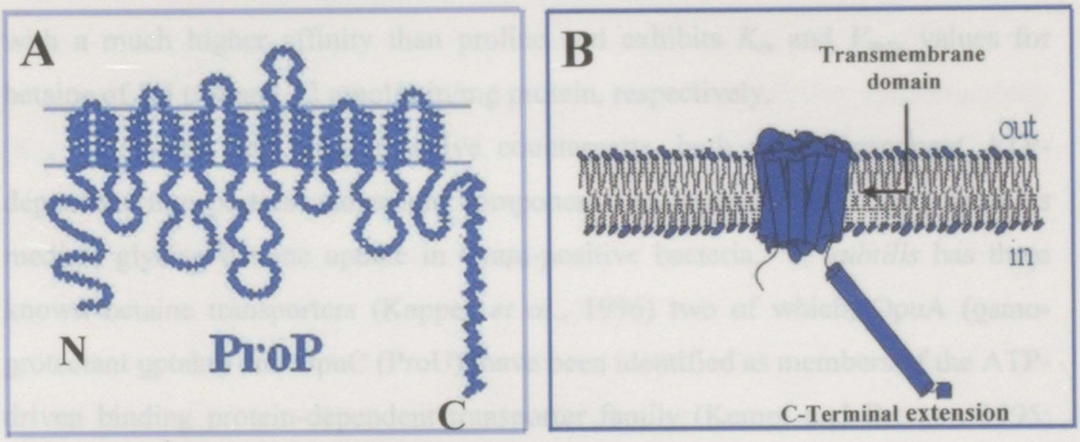


Fig. 4. Structural features of the secondary transporter ProP. (A) secondary structure (B) tertiary structure.

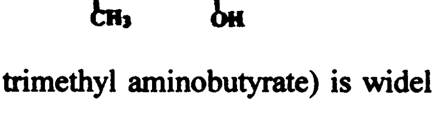
ProU. The multi-component binding-protein dependent transport system ProU belongs to a superfamily of prokaryotic and eukaryotic ATP-binding cassette transporters or traffic ATPases (Dogie and Ames, 1993; Higgins, 1992). The components of the ProU system are encoded by an operon containing three cistrons: *proV*, *proW* and *proX*, encoding two membrane-bound proteins, ProV and ProW; and the periplasmic binding protein ProX (Barron *et al*., 1987; Dattanada and Gowrishankar, 1989; Gowrishankar, 1989; Higgins *et al*., 1987; Stirling *et al*., 1989). Two promoters upstream of *proU* have been identified in *E. coli*; an osmoregulated promoter recognised by the RpoD-RNA polymerase holoenzyme, situated downstream of a weak RpoS-dependent promoter (Dattanada *et al*., 1991; Manna and Gowrishankar, 1994). In addition, evidence exists for the presence of a transcriptional activator site ~200 bp upstream of the RpoD-dependent promoter (Lucht and Bremer, 1991; 1994) together with a negative regulatory sequence within *proV* (Dattanada *et al*., 1991). Both of these AT-rich regulatory regions are easily distorted, thus facilitating attachment of H-NS, a DNA binding protein exhibiting a relatively high affinity for bent DNA (Tanaka *et al*., 1991). As with ProP, maximal betaine uptake at elevated osmolarities is achieved by a combination of transcriptional induction (> 100 fold) and stimulation of enzyme activity (Cairney *et al*., 1885a; 1985b, Gowrishankar and Manna, 1996). However, unlike the ProP system, ProU transports glycine betaine with a much higher affinity than proline and exhibits K_m and V_{max} values for betaine of 1.3 μM and 12 nmol/min/mg protein, respectively.

As with their Gram-negative counterparts, both multi-component ATP-dependent transporters and single component ion-dependent secondary systems mediate glycine betaine uptake in Gram-positive bacteria. *B. subtilis* has three known betaine transporters (Kappes *et al.*, 1996) two of which, OpuA (osmo-protectant uptake) and OpuC (ProU), have been identified as members of the ATP-driven binding protein-dependent transporter family (Kempf and Bremer, 1995; Lin and Hansen, 1995). OpuA, closely related to BusA (the betaine uptake system of *Lactococcus lactis* (Obis *et al.*, 1999; van der Heide and Poolman, 2000a; 2000b)) comprises three compartments: OpuAA, an ATPase; OpuAB, an integral cytoplasmic membrane protein; and OpuAC, an extracellular substrate-binding protein (Kempf and Bremer, 1995). Induced by high osmolarity growth conditions, transcription of *opuA*, like that of *proU*, is controlled by two separately regulated promoters, the osmoregulated *opuA* P-1, and *opuA* P-2, which does not respond to the osmotic stimulus. Both promoters show homology to the consensus sequence of σ^A-dependent promoters (Moran *et al.*, 1982), and are thus likely transcribed by an RNA polymerase complex containing the main vegetative sigma factor (σ^A). With a K_m and V_{max} for betaine uptake of 2.4 μM and 282 nmol/min/mg protein, respectively, OpuA, like ProU in *E. coli*, represents the glycine betaine transporter of highest affinity in *B. subtilis* (Kempf and Bremer, 1995). The OpuC system (exhibiting a K_m of 6 μM and a V_{max} of 65 nmol/min/mg protein for betaine) is related to OpuA but contains an additional integral membrane component (OpuCD). The broad substrate specificity of OpuC (ectoine, crotonobetaine, γ-butyrobetaine, carnitine, choline-*O*-sulphate, choline, proline betaine and glycine betaine (Jebbar *et al.*, 1997; Kappes and Bremer, 1998)) resembles that of EctP, the 'emergency system' accepting all known compatible solutes in *C. glutamicum* (Peter *et al.*, 1998). OpuD, the third betaine uptake system, is a single component transporter exhibiting significant homologies to the betaine transporters BetP of *C. glutamicum* and BetL of *Listeria monocytogenes*, as well as the *E. coli* choline and carnitine transport systems, BetT and CaiT, respectively (Eichler *et al.*, 1994; Lamark *et al.*, 1991; Peter *et al.*, 1996;

Sleator *et al.*, 1999a). High osmolarity stimulates *de novo* synthesis of OpuC and activates pre-existing OpuD proteins to achieve maximal betaine uptake activity (Kappes *et al.*, 1996). The K_m and V_{max} values for betaine uptake *via* OpuD were calculated as 13 μM and 61 nmol/min/mg protein, respectively.

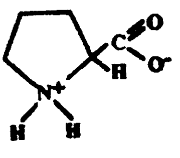
While physiological investigations of osmolyte uptake in *Listeria* identified a single highly specific, constitutive, energy-dependent, secondary transport system (Patchett *et al.*, 1994; Verheul *et al.*, 1997), genetic analysis led to the identification of three independent betaine uptake systems. The first of these, BetL, homologous to OpuD in *B. subtilis*, is a highly specific secondary transporter with a K_m and V_{max} for glycine betaine uptake of 7.9 μM and 134 nmol/min/mg protein, respectively. As with OpuD, BetL is osmotically induced both at the level of transcription (Sleator *et al.*, 2000) and enzyme activity (Verheul *et al.*, 1997). The remaining systems, OpuC (which also transports carnitine and choline (Fraser *et al.*, 2000)) and GbuABC are members of the traffic ATPases and as such resemble the multi-component transporters OpuA and OpuC in *B. subtilis*. An interesting feature of both *betL* and *opuC* is the presence of a consensus σ^B-dependent promoter-binding site upstream of the structural genes (Fraser *et al.*, 2000; Sleator *et al.*, 1999a; 2000). Given that σ^B-minus mutants of *Listeria* (in contrast to *Bacillus*) are significantly affected in their ability to accumulate glycine betaine and carnitine, both at elevated osmolarity and reduced temperatures (Becker *et al.*, 1998; 2000), it is tempting to speculate that the observed phenotype is the consequence of reduced uptake *via* the σ^B-regulated BetL and OpuC transporters.

3.1.2.2 Carnitine



Playing a role in long chain fatty acid transport across the inner mitochondrial membrane of animal cells (Bieber, 1988), the trimethyl amino acid carnitine (β-hydroxy-γ-*N*-trimethyl aminobutyrate) is widely distributed in nature, occurring predominantly in foods of animal origin (present in muscle tissue at concentrations of 0.05 to 0.2% on a fresh weight basis; Beumer *et al.*, 1994). For the majority of bacteria carnitine is transported from the external environment rather than being synthesised endogenously. The first reports of osmoprotection by carnitine were by Kets *et al.* (1994), following NMR spectroscopy of cell extracts from *Lactobacillus plantarum* grown in medium containing added NaCl, and by Beumer *et al.* (1994), who reported stimulation of *L. monocytogenes* by carnitine at elevated osmolarities. Later Verheul *et al.* (1998) demonstrated that carnitine uptake *via* ProP (K_m of 200-250 μM, V_{max} of 1.2 nmol/min/mg protein) and ProU (K_m of 200-250 μM and V_{max} of 1.9 nmol/min/mg protein) is osmotically significant while the CaiT system, implicated in anaerobic catabolism, has no known relationship to osmoadaptation (Eichler *et al.*, 1994; Jung *et al.*, 1990). While the OpuC system appears to function as the sole carnitine transporter in *B. subtilis* (K_m of 5.1 μM, V_{max} of 41 nmol/min/mg protein) (Kappes and Bremer, 1998) OpuC$^-$ mutants of *Listeria* are still capable of accumulating carnitine, albeit at a reduced rate. Thus it would appear that, unlike the situation in *B. subtilis*, carnitine uptake in *Listeria* might well be mediated by more than one system (Chapter IV, this thesis).

3.1.2.3 Proline



First reported as an osmoprotectant in *Salmonella oranienburg* by Christian in 1955 (Christian, 1955a; 1955b), proline has since been shown to accumulate to high intracellular concentrations in a variety of bacteria, following exposure to osmotic stress (Measures, 1975). While many species of Gram-positive bacteria have been shown to increase their internal proline pool size by increased synthesis (Tempest *et al.*, 1970; Whatmore *et al.*, 1990; Whatmore and Reed, 1990), Gram-negative bacteria, in general, achieve high intracellular concentrations of proline during osmotic stress as a consequence of enhanced transport (Brady and Csonka, 1988; Csonka, 1981; Csonka, 1988; Le Rudulier and Bouillard, 1983).

Proline synthesis

For the majority of bacteria, proline is synthesised from glutamate *via* three enzymatic reactions catalysed by γ-glutamyl kinase (GK; *proB* product), γ-glutamyl phosphate reductase (GPR; *proA* product) and Δ^1-pyrroline-5-carboxylate reductase (*proC* product). In general, the *proB* and *proA* genes constitute an operon, which is distant from *proC* on the chromosome. Regulated primarily through feedback inhibition of GK by proline (Leisinger, 1996), mutations in the *proB* gene have previously been linked to proline hyper-production (a consequence of reduced proline mediated feedback inhibition of GK), leading to enhanced osmotic stress tolerance in *E. coli* and other bacteria (Dandekar and Uratsu 1988; Kosuge and Hoshino, 1998; Massarelli *et al.*, 2000; Omari *et al.*, 1992; Rushlow *et al.*, 1984). In addition to *proBA*, sequence analysis of the *B. subtilis* chromosome (Kunst *et al.*, 1997) has recently uncovered an additional proline biosynthesis pathway: *proHJ*, which is apparently responsible for the high-level accumulation of proline under hyperosmotic growth conditions (Bremer and Krämer, 2000).

Work presented in this thesis has led to the identification and disruption of the listerial *proBA* homologue, which has been linked to the salt tolerance of *L. monocytogenes* (Chapter V, this thesis). Interestingly, while mutations in the listerial *proB* gene leading to proline overproduction had no obvious effects on listerial osmotolerance, heterologous expression of the mutated operon in an *E. coli proBA*$^-$ background resulted in a significant increase in the growth rate at elevated osmolarity (Chapter VI, this thesis). In addition, the observed lack of growth of a listerial *proBA*$^-$ mutant in proline deficient minimal medium (either at normal or elevated osmolarity) indicates that unlike *B. subtilis*, *Listeria* possesses only a single proline biosynthesis pathway.

Proline transport

The Gram-negative bacteria *E. coli* and *S. typhimurium* possess three proline transport systems: PutP, ProP and ProU (Wood, 1988). The PutP system serves to transport proline solely for use as a carbon or nitrogen source (Maloy, 1987), and as such plays little if any role in osmoadaptation (Gowrishankar, 1985;

Milner *et al.*, 1987). The osmotically induced systems: ProP and ProU (described earlier in relation to glycine betaine uptake) are, on the other hand, highly responsive to osmotic up-shock. Measurement of growth in high osmolarity medium by mutants deficient in either ProP or ProU revealed that ProP is the major contributor to osmoprotection by proline (Csonka, 1982).

Among the Gram-positive bacteria, osmoprotection by exogenous proline uptake has been most extensively studied in *Staphylococcus aureus* (Bae and Miller, 1992; Graham and Wilkinson, 1992; Pourkomailian and Booth, 1992; 1994; Townsend and Wilkinson, 1992), *L. lactis* (Molenaar *et al.*, 1993; Obis *et al.*, 1999) and *B. subtilis* (von Blohn *et al.*, 1997). Proline uptake in *S. aureus* appears to be mediated by high and low affinity systems. The high affinity system, PutP, is highly specific for proline and, given its significant homologies with PutP in *E. coli*, appears to function independently of osmotic stimulation for the uptake of proline as a carbon, nitrogen or energy source. The low affinity system on the other hand is extremely responsive to osmotic up-shock and is capable of transporting both proline and glycine betaine. Proline uptake in both *L. lactis* and *L. plantarum* resembles that of *S. aureus*, in that the only osmotically significant proline transporter also functions as the major betaine uptake system in these strains (BusA (OpuA) in *L. lactis* (Obis *et al.*, 1999) and QacT in *L. plantarum* (Glaasker *et al.*, 1996a)).

The situation in *B. subtilis* differs markedly from that in other Gram-positive bacteria studied to date, in that osmotically stimulated proline uptake in this strain is mediated by the high-affinity, substrate-specific OpuE. While closely related to the proline-inducible PutP permeases, which have no apparent role in the osmostress response, expression of *opuE* is strongly induced by the osmolarity of the external environment, but not by proline (Spiegelhalter and Bremer, 1998; von Blohn *et al.*, 1997). Transcribed from two closely spaced, osmoregulated promoters: *opuE* P-1, which is recognised by the vegetative σ^A, and *opuE* P-2, which is dependent on the stress-induced σ^B (Spiegelhalter and Bremer, 1998; von Blohn *et al.*, 1997), *opuE* was the first member of the σ^B regulon with a clearly defined physiological function in the *B. subtilis* osmostress response. However, σ^B is dispensable for the induction of the OpuE system under high-osmolarity growth conditions, indicating that the activity of the σ^A-dependent *opuE*-P1 promoter is sufficient for overall osmotic control of *opuE*.

3.2 Osmotic responses not involving compatible solute accumulation

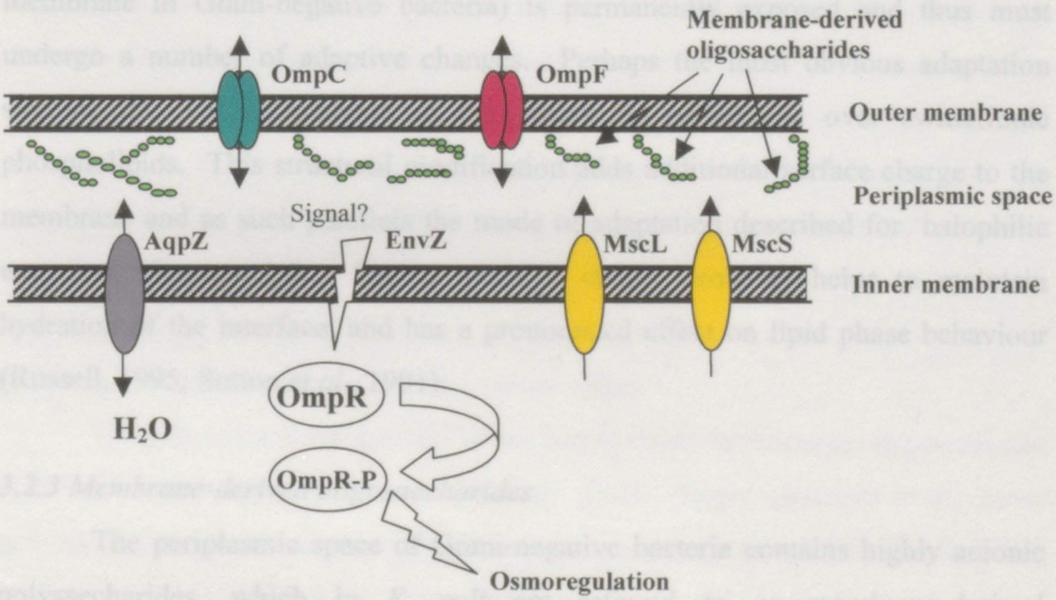


Fig. 5. Osmotic responses not involving compatible solute accumulation.

3.2.1 Outer membrane porins: OmpC and OmpF

The most extensively studied osmoregulated genes which do not directly contribute to compatible solute accumulation are *ompC* and *ompF*; encoding two structurally related Gram-negative outer membrane channel proteins OmpC and OmpF (Fig. 5). Expression of these porins, which facilitate the non-specific diffusion of small (≤ 500 Da) hydrophilic molecules across the outermost permeability barrier of the cell (Nikaido and Vaara, 1987), responds in a reciprocal fashion to the external osmolarity (expression of *ompF* being depressed while that of *ompC* is enhanced at elevated osmolarity) (Csonka, 1989). Not restricted to salt stress, the levels of OmpC and OmpF appear to respond to a variety of environmental parameters including temperature, carbon source and oxygen availability as well as the pH of the medium (Csonka and Hanson, 1991).

3.2.2 Membrane adjustment

While the cytoplasmic interior of a bacterium employing compatible solutes may be protected from the damaging effects of the external salt, the outer surface of the cytoplasmic membrane (as well as the periplasmic space and outer membrane in Gram-negative bacteria) is permanently exposed and thus must undergo a number of adaptive changes. Perhaps the most obvious adaptation strategy involves an increase in the proportion of anionic over zwitterionic phospholipids. This structural modification adds additional surface charge to the membrane and as such parallels the mode of adaptation described for 'halophilic enzymes' (*Section 2.1*). Excess negative charge probably helps to maintain hydration of the interface, and has a pronounced effect on lipid phase behaviour (Russell, 1995; Sutton *et al.*, 1991).

3.2.3 Membrane-derived oligosaccharides

The periplasmic space of Gram-negative bacteria contains highly anionic polysaccharides, which in *E. coli* are referred to as membrane-derived oligosaccharides (MDOs) (Kennedy, 1987). Encoded by constitutively expressed genes; *mdoA* and *mdoB*, these anionic polymers (containing between six and twelve glucose units with an average charge of –5 (Kennedy, 1982)) generate a Donnan potential across the outer membrane, resulting in the accumulation of cations to a higher concentration in the periplasm than in the medium, consequently giving rise to hydrostatic pressure in the periplasmic space (Kennedy, 1982). Unlike intracellular compatible solutes the levels of these oligosaccharides decreases with increasing osmolarity (Miller *et al.*, 1986). Interestingly, while MDOs appear to play an important role in periplasmic osmoregulation of Gram-negative bacteria, blocking MDO synthesis fails to inhibit growth of *E. coli* in media of high or low osmolarity (Fiedler and Rottering 1988; Kennedy, 1982).

3.2.4 Non-accumulated osmoprotectants

Identified by Gouffi and Blanco (2000) as a new class of sinorhizobial osmoprotectants, these non-accumulated disaccharides include: sucrose, trehalose, maltose, cellobiose, gentibiose, turanose and palatinose. Structurally, these disaccharidic osmoprotectants contain either two-glucosyl residues or a glucosyl residue linked to a fructosyl residue (Gouffi *et al.*, 1999). Unlike other bacterial osmoprotectants (*e.g.* betaine, carnitine or proline) these disaccharides do not accumulate as cystolic osmolytes (or immediate osmolyte precursors) in salt-stressed *Sinorhizobium meliloti.* Instead they are catabolised during early exponential growth, contributing indirectly to enhance the levels of two endogenously synthesised osmolytes, glutamate (two-fold increase) and N-acetylglutaminylglutamine amide (six-fold), facilitating growth at elevated osmolarities.

3.3 Hypo-osmotic shock; solute and water efflux

Bacteria in their natural habitat are just as likely to encounter hypo-osmotic or dilution stress, as they are hyper-osmotic shock. Rapid increases in the water activity of the external environment (often a consequence of rainfall, flooding etc) leads to a massive influx of water into the cell, requiring the bacteria to react quickly to avoid cell lysis. As with salt stress, bacteria have evolved a number of mechanisms to counter the potentially detrimental effects of hypo-osmotic shock; essentially rapid increases in water activity are countered by both solute and water efflux.

3.3.1 Solute efflux

Ubiquitous amongst bacterial cells, mechanosensitive or stretch-activated channels are the major routes for the release of cytoplasmic solutes to achieve a rapid reduction of turgor pressure during the transition from media of high to low osmolarity (Berrier *et al.*, 1992; Le Dain *et al.*, 1998; Sukharev *et al.*, 1994; Szabó *et al.*, 1993; Zoratti and Petronilli, 1988). *E. coli* possess between three and five stretch-activated channels, however, with the exception of MscL and MscS (Levina *et al.*, 1999; Sukharev *et al.*, 1997) genetic studies have failed to identify the structural genes for these systems. In addition to stretch-activated channels, specific carrier-like systems (*Section 5.4.1*) appear to contribute to solute discharge, since the initial rapid efflux *via* stretch activated channels is followed in some microbes by a slower process with different kinetic and metabolic parameters (Glaasker *et al.*, 1996a; Strøm and Kaasen, 1993).

3.3.2 Water efflux

Recent evidence suggests that bacteria, like higher plants and animals, possess aquaporins (*e.g.* AqpZ; Calamita *et al.*, 1995; Calamita, 2000); specific water-channels that facilitate the rapid influx/efflux of water thus alleviating water stress without dissipating the transmembrane potential (Engel *et al.*, 2000). Expressed in diverse species (Marples, 2000; Park and Saier, 1996), aquaporins have been shown to play essential roles in maintenance of turgor and transpiration in plants (Maurel *et al.*, 1993) as well as volume regulation and organismal fluid retention in animal cells (Knepper, 1994).

4. OSMOSENSING

While much information is available concerning the genetic and physiological responses of bacteria to environmental osmolarity (as outlined in the previous section) considerably less is known about the signals regulating these responses. While regulation of most biological responses depends on the recognition of signal molecules by specific receptors, osmoregulation differs in that the information from the environment is not a specific molecule but a physiological parameter: the water activity (a_w) of the exterior (Kung *et al.*, 1990). This section reviews the possible parameters, which (being subject to change in osmotically stressed cells) may be used as signals to trigger osmoregulatory responses (Fig. 6).

4.1 Possible Osmosensing Mechanisms

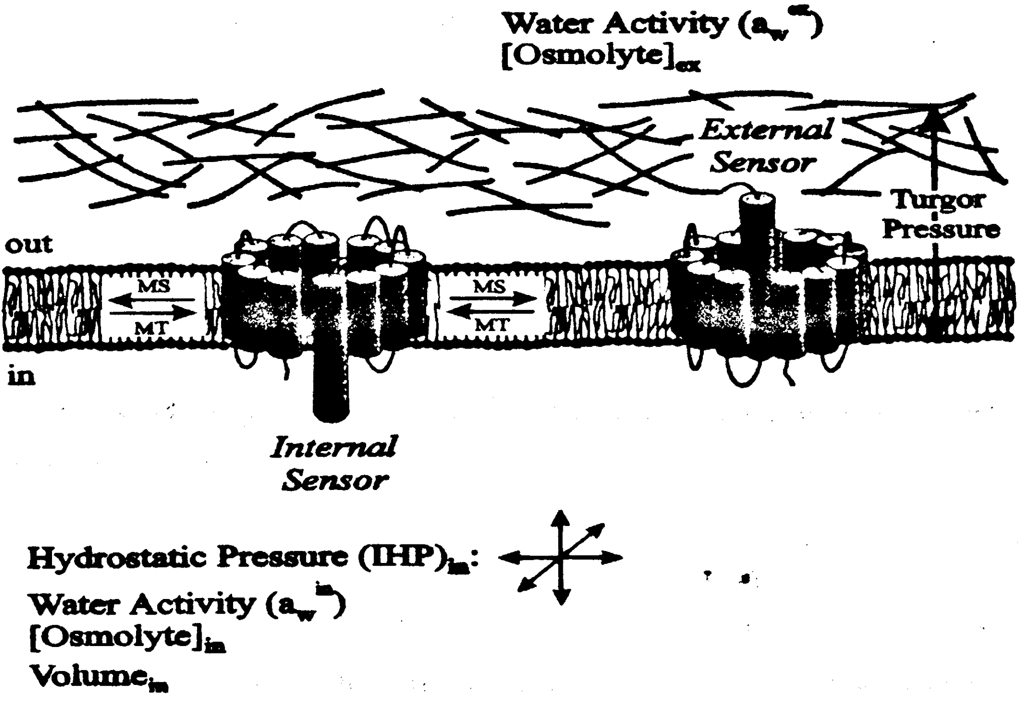


Fig. 6. Physiochemical parameters that may reflect the activity of osmoregulated transport systems. Two transport systems, one with an external and one with an internal osmosensing domain, are depicted schematically. The cell envelope represents that of a Gram-positive bacterium, *i.e.* the cytoplasmic membrane and peptidoglycan layer are shown. MS and MT refer to membrane stretch and tension while a_W (the water activity) refers to the mole fraction of water in solution. From Poolman and Glaasker, 1998.

Internal hydrostatic pressure (IHP)$_{in}$. Within the bulk liquid of the cell interior, changes in hydrostatic pressure are isotropic (uniform in all directions). While the observed pressure changes are low ($\leq$ 0.5 mPa (Csonka and Hanson, 1991)) they may bring about measurable changes in protein-protein and protein-ligand interactions (Heremans, 1982).

Membrane pressure differential. Turgor pressure (the hydrostatic pressure difference which balances the osmotic pressure difference between the cell interior and exterior), acting normal to the wall, and membrane strain (MS/MT which occurs in response to the change in turgor pressure and affects the expansion/compression of the bilayer in the phase of the membrane) may be detected by pressure sensors located in the inner membrane.

Internal osmolarity. Once turgor is lost the cytoplasmic compartment behaves as an osmosensor (Csonka and Hanson, 1991). In principle, either of three parameters: (*i*) cytoplasmic volume, (*ii*) accompanying changes in the concentration of one or more solutes (*e.g.* K^+ (Booth and Higgins 1990; Epstein, 1986)), or (*iii*) the internal a_W, could serve as osmoregulatory signals.

External osmolarity or a_W. Possibly sensed by transmembrane proteins with outward-facing sensing domains.

Cytoplasmic membrane area. As with the cytoplasmic volume, the cytoplasmic membrane area is responsive to changes in medium osmolarity. Such changes (around 7% in an elastic cell for a rise in medium osmolarity of 100 mosmol/kg (Csonka and Hanson, 1991)) may be detected by stretch-activated strain *e.g.* MscL (Wood, 1999).

While not all acting in the same time scale (Poolman and Glaasker, 1998), most of the above mentioned physiochemical parameters are not mutually exclusive but are instead interrelated. The observed flexibility of the cell wall peptidoglycan (Doyle and Marquis, 1994) for example, allows changes in turgor pressure to be accompanied by immediate changes in cytoplasmic volume, concentration of internal solutes and membrane area (Csonka and Hanson, 1991), thus allowing the cell to monitor three or more signals simultaneously.

While these signals are essential to trigger the activation of osmoregulated transport/synthesis systems, they will not solely determine the fluxes of compatible solutes across the membrane. The ultimate activity of an osmoregulated system, after initial activation, will depend on the state of the cell with respect to (*i*) the internal osmotic pressure or related parameter at the time of the shift (as described above); (*ii*) the internal concentration of the compatible solute, which may inhibit through 'feedback' or '*trans*' inhibition; and/or (*iii*) physiological parameters such as the energy status and the internal pH of the cell (Poolman and Glaasker, 1998). Osmoregulatory mechanisms are thus inextricably linked to other cellular processes (Csonka and Hanson, 1991).

5. OSMOREGULATION

To date, extensive analysis of the signal transduction pathways originating from osmotic challenge and leading ultimately to immediate (activity) and long term (expression) modulation of the primary and secondary responses have been restricted to a handful of organisms; namely *E. coli* (Kdp, Trk, ProP, ProU and EnvZ/OmpR), *C. glutamicum* (BetP), *L. lactis* (BusA (OpuA)) and *L. monocytogenes* (BetL).

5.1 The primary response

5.1.1 Kdp

Osmotic regulation of the Kdp system occurs both at the level of transcription and enzyme activity (Epstein, 1992); however, more is known about the transcriptional regulation of the *kdp* genes. Induction of the *kdp* operon (mediated by a sensor kinase (KdpD)/ response regulator (KdpE) system (Polarek *et al.*, 1992; Voelkner *et al.*, 1993)) can be triggered by moderate osmotic pressure increases ($\geq$ 0.2 mPa (Csonka and Hanson, 1991)) elicited only by ionic and non-polar solutes which are excluded from the membrane. While the latter observation rules out sensing by intra- or extracellular a_W, other parameters such as isotropic pressure, intracellular concentration of specific solutes, turgor pressure and membrane stretch remain as possibilities (Csonka and Hanson, 1991). Given that amphipathic compounds (which intercalate into the lipid bilayer altering the curvature stress of the membrane (Epand and Epand, 1994)) elicit a similar effect as osmotic up-shock, membrane stretch has been proposed to be the most likely osmotic signal sensed by the transmembrane domain of the KdpD sensor kinase (Sugiura *et al.*, 1994). However, since K^+ uptake is still observed in the presence of glycine betaine (which restores turgor and consequently membrane stretch, to a normal level), stretch alone appears unlikely to function as the sole regulatory signal for the *kdpABC* operon. Evidence that intracellular K^+ may function as a second signal regulating expression of the operon was originally put forward by Rhoads *et al.* (1976) and later Gowrishankar (1987). Furthermore, Sugiura *et al.* (1994) demonstrated that K^+ sensing can be separated mechanistically from medium osmolarity signals, as mutants which fail to perceive the K^+ signal respond normally to hyper-osmotic stress. Indeed, while the autophosphorylation of wild-type KdpD is negatively regulated by K^+, medium osmolarity has a positive effect.

5.1.2 Trk

In contrast to Kdp, osmotic regulation of Trk is mainly at the level of transport activity. While the activity increases upon osmotic up-shift, the initial rate of influx appears dependent on the intracellular osmolarity as opposed to the external environment (Meury *et al.*, 1985). Since intracellular osmolarity and K^+ concentration are not well separated in the experimental setup, it has been proposed that the actual rate is determined by the intracellular K^+ concentration through feedback regulation (Poolman and Glaasker, 1998).

Alkalisation of the cytoplasm, a consequence of K^+ uptake (Dattanada and Gowrishankar, 1989; Kregenow, 1981), has been suggested as a possible signal for increased glutamate (the K^+ counter ion) synthesis following hyper-osmotic shock.

5.2 The secondary response

5.2.1 ProP

Effectively regulated by the external osmolarity, both at the level of expression and activity (Cairney *et al.*, 1985a; Dunlap and Csonka, 1985; Gowrishankar, 1986), it is the biochemical activation of the ProP protein that contributes most to the osmostress response. A number of possible signals have been proposed to modulate the activity of ProP including turgor pressure (Milner *et al.*, 1988), K^+ concentration (Koo *et al.*, 1991) and intracellular pH (Poolman and Glaasker, 1998). Since 'activated' uptake occurs irrespective of whether turgor has been restored *via* uptake of K^+, it seems unlikely that ProP senses turgor pressure *per se* (Poolman and Glaasker, 1998). The requirement for K^+ to stimulate ProP activity, although well documented (Koo *et al.*, 1991; Marshall, 1996) remains ill defined since K^+ is also required to support respiration (Padan *et al.*, 1976) and energisation of ProP (Racher *et al.*, 1999). While stimulation of uptake *via* ProP can be observed following an increase in the intracellular pH upon K^+ uptake (Poolman *et al.*, 1987a; 1987b), the resulting activation is transient (Koo *et al.*, 1991).

Perhaps the most likely mechanism governing sustained ProP activation at elevated osmolarity involves modulation of the α-helical coiled-coil formation of the ProP carboxy terminus (Culham *et al.*, 2000; Racher *et al.*, 1999). Stability of the coiled-coil structure may be modulated in response to either varying a_W or cytoplasmic solvent composition (Leikin *et al.*, 1993). One cytoplasmic element exhibiting a significant influence on the osmotic activation of ProP is the 232-amino-acid, basic, hydrophilic protein ProQ (Milner and Wood, 1989). Mutating *proQ* reduces both the rate and extent of ProP activation by an osmotic up-shift (Kunte *et al.*, 1999). Since neither transcription nor translation of *proP* appears to be altered by the mutation, it is proposed that ProQ may influence the osmotic activation of ProP at a post-translational level (Culham *et al.*, 2000; Kunte *et al.*, 1999).

5.2.2 ProU

As with ProP, the ProU system is regulated both at the level of transcription and enzyme activity. However, unlike ProP, it is transcriptional activation of *proU* that is most important in terms of the osmostress response (Cairney *et al.*, 1985a; 1985b). As with the *Kdp* operon, transcription of *proU* can be induced only by high concentrations of solutes that do not cross the membrane (Csonka and Hanson, 1991), thus ruling out regulation by intra- or extracellular a_W. Also, since regulation by either turgor pressure or membrane stretch are unlikely given their transient nature, by elimination the most likely signal is the concentration of a specific solute or solutes. Intracellular K^+ concentration was originally proposed as a possible signal for *proU* expression by Sutherland *et al.* (1986) and later by Ramirez *et al.* (1989), who reported that expression of the operon *in vitro* was increased in proportion to the K^+-glutamate concentration in the assay buffer. Other workers have disputed this proposal and eliminated glutamate (but not K^+) as the inducing signal (Csonka *et al.*, 1994). However, since multiple cellular processes are stimulated by K^+ (Leirmo *et al.*, 1987), the dependence of *proU* transcription on K^+ may be a reflection of the general stimulatory effect of the ion on enzymatic reactions in general, rather than evidence for a specific osmoregulatory signal (Csonka and Epstein, 1996).

DNA supercoiling has also been suggested to function as a regulator of *proU* expression (Higgins *et al.*, 1988; Ní Bhrían *et al.*, 1989). Mutations in *topA* (encoding topoisomerase I) were shown to increase *proU* expression (Higgins *et al.*, 1988), while disrupting *gyrA* and *gyrB* (genes specifying the two subunits of DNA gyrase) reduces expression of the operon (in *Salmonella* but not *E. coli*) at low osmolarity (Higgins *et al.*, 1988). However, as with K^+, the effects of DNA supercoiling on the expression of *proU* may be the result of pleotrophic effects of supercoiling on transcription, rather than proof that supercoiling is a specific osmoregulatory signal (Pruss and Drlica, 1989).

In addition to *topA*, *gyrA* and *gyrB*, mutational alterations of a number of other DNA binding proteins (which have no direct role in supercoiling) have been linked to modified *proU* expression. Mutations in *hns* (*osmZ*), encoding the DNA binding protein H-NS, results in a moderately elevated expression of *proU* at all osmolarities. Under normal growth conditions H-NS binds to sites both up- (Ueguchi and Mizuno, 1993) and downstream of the promoter (Lucht and Bremer, 1994) forming an extended nucleo-protein complex, which prevents binding of the RNA polymerase, thus blocking transcription. Dissociation of this complex occurs by an unknown mechanism at high osmolarity. Additionally, deletion of the negative regulatory sequence within *proV*, to which H-NS binds, prevents the formation of the nucleo-protein complex, consequently increasing expression of *proU* by up to 25-fold in low osmolarity medium (Dattanada *et al.*, 1991). Mutations in the gene for IHF (integration host factor), on the other hand, decrease the induced level of *proU* expression two-fold (Lucht and Bremer, 1991), while mutations in the gene for HU-B (histone-like protein) reduces both basal and induced levels of *proU* expression (Manna and Gowrishankar, 1994). Since expression of *proU* remains osmotically controlled in strains mutated in H-NS, IHF or HU-B, it is apparent that these proteins function as modulators rather than regulators of *proU* expression (Kempf and Bremer, 1998).

Regulation of ProU at the level of enzyme activity has been linked to the periplasmic tail of the ProW protein, which is predicted to form an amphiphilic α-helix (Haardt and Bremer, 1996). This protein domain has been implicated in osmosensing by monitoring alterations in membrane tension as well as changes in the intracellular osmolarity (Poolman and Glaasker, 1998).

5.2.3 BetP

Although regulated both at the level of gene expression and enzyme activity, recent studies on betaine uptake in *C. glutamicum* have focused primarily on osmoregulation of the BetP protein (Peter *et al.*, 1998; Rübenhagen *et al.*, 2000). Modulation of the activity of the protein by the amphipathic compound tetracaine indicates that at least part of the primary signal transferred to BetP comes directly from the membrane (Peter *et al.*, 1998; Rübenhagen *et al.*, 2000). Additional evidence that a major factor modulating BetP activity originates *via* the membrane was obtained following heterologous expression against an *E. coli* background. A shift in the optimum of osmotic stimulation from 1.3 osmol/kg (in *C. glutamicum*) down to 0.5 osmol/kg (when expressed in *E. coli*), initially attributed to the difference in turgor pressure between *E. coli* and *C. glutamicum* (Peter *et al.*, 1996), is now known to be linearly related to the increase in the content of phosphatidyl glycerol in the *E. coli* lipids (Rübenhagen *et al.*, 2000). Peter *et al.* (1998) recently demonstrated that both the N- and C-terminal extensions of BetP function as putative osmosensory domains. Deletions in the N-terminus (a 62 amino acid domain with an excess of negatively charged residues) shift the optimum of activation from 1.3 to 2.6 osmol/kg, while similar mutations in the C-terminus (a 55 amino acid extension with a large excess of positive residues) result in a complete loss of regulation.

5.2.4 BusA (OpuA)

As with BetP, a major factor modulating BusA (OpuA) activity originates *via* the surrounding membrane directly, as demonstrated both by the influence of tetracaine and the fatty acid composition of the membrane (Guillot *et al.*, 2000; van der Heide and Poolman, 2000a). In addition to modulation of its translocation activity, *busA* is osmotically regulated at the level of gene expression (van der Heide and Poolman, 2000b).

5.2.5 BetL

While the secondary glycine betaine uptake system, BetL, has been shown to be effectively regulated at the level of gene expression (Sleator *et al.*, 2000), Verheul *et al.* (1997) demonstrated that both betaine and carnitine uptake in *Listeria* is additionally regulated at the level of enzyme activity by a novel osmolyte sensing mechanism, in which regulation of uptake of both betaine and carnitine is subject to inhibition by pre-accumulated solute. Internal betaine inhibits not only transport of external betaine but also that of carnitine and *vice versa.* The observed *trans*-inhibition is alleviated upon osmotic up-shock, which suggests that alterations in membrane structure are transmitted to the allosteric binding sites for betaine and carnitine of both transporters at the inner surface of the membrane. The linkage of the *trans*-inhibitory effect to the osmotic strength of the environment is also observed in *L. plantarum* (Poolman and Glaasker, 1998) and *S. aureus* (Pourkomailian and Booth, 1994) and thus may form a general strategy to tune the intracellular osmolarity and maintain the cell turgor within certain limits.

5.3 Outer membrane porins: OmpC and OmpF

The OmpC and OmpF porin levels are controlled predominantly at the level of gene expression, by the two-component regulatory system EnvZ/OmpR but fine-tuning requires an additional level of control involving the antisense RNA MicF. Maximally expressed at 37°C (Coyer *et al.*, 1990), this 174-nucleotide RNA sequence, transcribed from a promoter upstream of the *ompC* gene, is highly complementary to the 5′ region spanning the translation initiation site of *ompF.*

MicF thus acts as a negative regulator of *ompF* expression at the post-transcriptional stage (Pratt *et al.*, 1996).

While the signal transduction pathway for the transcriptional and post-transcriptional control of *ompC* and *ompF* has been well characterised with respect to the structures and interactions of its components, the signal or signals to which EnvZ responds remains to be determined. Given that activation occurs in response to both permeant and impermeant solutes (Gutierrez *et al.*, 1987) the most likely signals appear to be either the levels of specific solutes or the cytoplasmic, periplasmic or extracellular a_W (Csonka and Hanson, 1991). Since the response to high osmolarity in minimal medium is markedly reduced in the presence of betaine (Barron *et al.*, 1986), extracellular a_W is unlikely to function as the signal. This, together with the localisation and structure of the EnvZ sensor protein, which spans the cytoplasmic membrane, exhibiting both periplasmic and cytoplasmic domains (Igo and Sihavy, 1988; Igo *et al.*, 1989; 1990), points to the sensing of a signal within either the periplasm or cytoplasm as opposed to the cell exterior. In this connection, sensing of the periplasmic derived MDOs has been proposed as a possible signal for EnvZ (Fiedler and Rottering, 1988).

5.4 Solute efflux

5.4.1 Specific efflux systems

Osmoregulated efflux activity with specificity for compatible solutes has been described for a number of microbes (Glaasker *et al.*, 1996a; Ruffert *et al.*, 1997; Schleyer *et al.*, 1993). In general, specific compatible solute efflux upon osmotic down shock is characterised by two kinetic components; one with a t ½ < 1 sec and the other with t ½ of 4-5 min. Although the molecular nature of the rapid (t ½ < 1 sec) efflux activities is unknown, these systems exhibit properties that mimic mechanosensitive channels (*i.e.* rapid stretch-activated efflux (Sukharev *et al.*, 1997)) and are discriminated from the slower mechanisms (most probably mediated by bi-directional secondary transporters *e.g.* BetP and BetL (Poolman and Konings, 1993)) by a number of features including; a function independent of metabolic energy, and an observed insensitivity to substrate on the *trans* site of the membrane (Poolman and Glaasker, 1998).

5.4.2 Mechanosensitive channels

Mechanosensitive or stretch-activated channels (of which MscL and MscS of *E. coli* are the best characterised members) are, as their name suggests, activated by membrane stretch (a fact demonstrated by their observed activation in the presence of amphipathic compounds which intercalate into the lipid bilayer (Martinac *et al.*, 1990)). Blount *et al.* (1996) proposed that the mechanosensing domain of MscL might be confined to the hydrophobic core (composed of two transmembrane segments TMS1 and 2) and the periplasmic loop in between the TMS. This proposal was later confirmed by the isolation of mutations in TMS1, which result in an increased sensitivity of the channel to mechanical stress (Blount *et al.*, 1997). While MscL has been implicated in the release of both K^+ and small proteins such as thioredoxin during osmotic downshock (Ajouz *et al.*, 1998; Blount *et al.*, 1997) it is not known whether it also mediates the efflux of other ions or nonionic cosolvents (Wood, 1999). While patch clamp analysis of *E. coli* revealed the existence of multiple mechanosensitive channel conductances, it seems likely that one or more of these activities correspond(s) to the observed efflux of compatible solutes upon hypo-osmotic shock (as described in *section 5.4.1*).

6. OSMOSTRESS AND VIRULENCE

Bacteria capable of causing foodborne illness must negotiate a long and tortuous passage from the environment to the site of infection of the susceptible host. As well as the stresses encountered during the production, preparation and storage of food, bacterial foodborne pathogens are additionally faced with the formidable defences of the host immune system. Following consumption they are exposed to the low pH of the stomach and subsequently the volatile fatty acids, bile salts, high osmolarity and low oxygen content of the small intestine. Bacteria surviving to this point are forced to compete with the established gut flora for niches and nutrients and encounter, among other insults, anti-microbial peptides produced by their competitors (Dunne *et al.*, 1999). Organisms capable of invasion subsequently penetrate the gut epithelium (possibly *via* M cells located in Peyer's patches) and are rapidly engulfed by macrophages before being internalised by phagosomes; specialised organelles that prevent bacterial multiplication by means of acidic pH and the production of defensins (oxygen-independent mechanisms) as well as peroxide and superoxide radicals (oxygen-dependent mechanisms) (Gahan and Hill, 1999).

In view of the variety of stresses encountered by pathogenic bacteria during the course of infection, it is becoming increasingly evident that in addition to 'true' virulence factors (those encoding toxins or invasins, for example), there also exists an additional class of proteins or contributory factors, involved in the complex stress management strategies which are essential for the pathogen to mount a successful infection. This section focuses on the link between osmostress and virulence, and reviews the role of various osmoregulatory systems in contributing to the virulence potential of certain pathogenic bacteria.

6.1 Osmoprotectant accumulation

In addition to their role in the salt tolerance response, there is increasing evidence to suggest that osmoprotective compounds, together with their transport/synthesis systems, may function as important virulence factors for certain pathogenic bacteria. Gowrishankar and Manna (1996) first proposed that *proU* may function as a virulence gene in the pathogenic enterobacteria, while in *E. coli*, a strain capable of causing urinary tract infections and pyelonephritis has been shown to exhibit an abnormally high level of ProP activity. In addition, deletion of *proP* dramatically reduces the ability of the strain to colonise mouse bladders (Culham *et al.*, 1998). Similarly, inactivation of the *putP* homologue in *S. aureus* significantly reduces virulence in an experimental endocarditis model (Bayer *et al.*, 1999). Work presented in this thesis has demonstrated that knockout of *opuC* in *L. monocytogenes* LO28 can reduce the virulence potential of this strain following intraperitoneal infection. Interestingly, this effect appears to be strain specific and was not seen in a knockout mutant in *L. monocytogenes* ScottA. However, elimination of OpuC in both strains significantly reduced the ability to colonise the upper small intestine in mice following peroral administration

(Chapter IV, this thesis).

Thus, while a number of osmolyte transport systems have clearly been linked to the virulence potential of certain pathogenic bacteria, the role of osmolyte synthesis in microbial pathogenesis has received considerably less attention. Chapters V and VI of this thesis investigate the effects of osmolyte synthesis, specifically proline synthesis, in contributing to listerial pathogenesis. While knockout of the *proBA* locus reduces salt tolerance in complex broth, it does not appear to affect virulence potential when administered to mice by the intraperitoneal or peroral routes (Chapter V, this thesis). This finding reflects that of an earlier study in which Marquis *et al.* (1993), using an uncharacterised proline auxotroph, showed that proline auxotrophy fails to exhibit reduced virulence, suggesting that the host tissue contains a relatively abundant source of free proline or proline containing peptides. Furthermore, manipulation of the system resulting in proline overproduction also failed to alter the virulence potential in *L. monocytogenes* (Chapter VI, this thesis).

6.2 EnvZ-OmpR

The EnvZ-OmpR two-component regulatory system, originally identified as a regulator of the outer membrane porins OmpC and OmpF (Fig. 5), has emerged as a global regulator of virulence potential. Mutating *ompR* dramatically reduces virulence of both *Shigella flexineri* and *S. typhimurium*, suggesting a major role for this locus in both pathogens (Bernardini *et al.*, 1990; Dorman *et al.*, 1989). In *S. typhimurium*, OmpR mutants fail to lyse infected macrophages and so fail to induce a key step in pathogenesis (Lindgren *et al.*, 1996). While insertion mutations in *ompC* and *ompF* alone failed to affect virulence, strains carrying mutations in both porins are significantly attenuated (though not to the same extent as an OmpR mutant (Chatfield *et al.*, 1991)). A specific role for these porins in intestinal survival is supported by the fact that double mutants in *ompC* and *ompF* are severely attenuated when administered *via* the oral route, but only marginally affected when administered intravenously. Since conditions of high salinity (*e.g.* 0.3 M NaCl in the intestinal lumen (Chowdhury *et al.*, 1996)) and high temperatures (37°C) favour synthesis of OmpC over OmpF, Nikaido and Vaara

(1987) conjectured that OmpC may be synthesised preferentially when the pathogen is present in the intestinal tract of the animal host. In this environment the small pore size of OmpC may help to exclude harmful molecules such as bile salts, while facilitating uptake of nutrients present at high concentrations. OmpF, on the other hand, exhibiting a larger pore size than OmpC (Nikaido and Rosenberg, 1983), is most likely expressed outside of the host where temperature and salinity are lower and nutrients are likely to be more dilute.

Given that *ompR* mutants of both *S. typhimurium* and *S. typhi* are significantly more attenuated than OmpC:OmpF double mutants, the influence of EnvZ-OmpR on virulence potential is expected to extend beyond the regulation of outer membrane porins (Mahan *et al.*, 1996). Other genes regulated by OmpR in *S. typhimurium* include *tppB*, which encodes a tripeptide permease (Gibson *et al.*, 1987) and *aas*: a gene, induced within macrophages, encoding 2-acylglycerolphosphoethanolamine acyltransferase (Valdivia and Falkow, 1997). While mutations in either *tppB* or *aas* had no significant effect on virulence (Lee *et al.*, 2000), deletion of *sifA* (an OmpR regulated gene responsible for the formation of *Salmonella*-induced filaments within HeLa cells (Mills *et al.*, 1998)) results in partial attenuation of virulence, indicating some requirement for filament formation during infection (Stein *et al.*, 1996). Bernardini *et al.* (1990) showed that transcription of the *mxi* operon (membrane expression of invasion plasmid antigens) of *S. typhi* is induced at high osmolarity. Furthermore, this osmoregulation was not seen in an *ompR* deletion background. Indeed, expression of the operon was reduced 10-fold in the *ompR* mutant. In addition, Pickard *et al.* (1994) demonstrated that the Vi capsule in *S. typhi* was also affected by mutations in *ompR*. Strains carrying an *ompR* mutation were no longer agglutinated by Vi antiserum. The authors concluded that the mutation was a consequence of reduced production (as opposed to decreased export) of the polysaccharide, a defect that could be complemented by a plasmid containing the *ompR* gene.

Since mutations of individual components of the OmpR regulon have only a marginal effect on virulence potential, researchers have continued the search for the key component of the regulon. In this regard a most interesting recent discovery is the fact that OmpR regulates the two-component system SsrA-SsrB in

Salmonella pathogenicity island SPI2, which in turn regulates a type III secretion system required for both murine infection and replication within macrophages (Fig. 7) (Lee *et al*., 2000). Evidence suggests that EnvZ, sensing both the low pH and osmolarity of the phagosome, activates OmpR, which in turn stimulates rapid expression of *ssrA* and *ssrB*. The SsrA-SsrB two-component system then detects another signal (possibly mediated by PhoP-PhoQ) and in turn activates expression of the SPI2 typeIII secretion system (Deiwick *et al*., 1999; Lee *et al*., 2000).

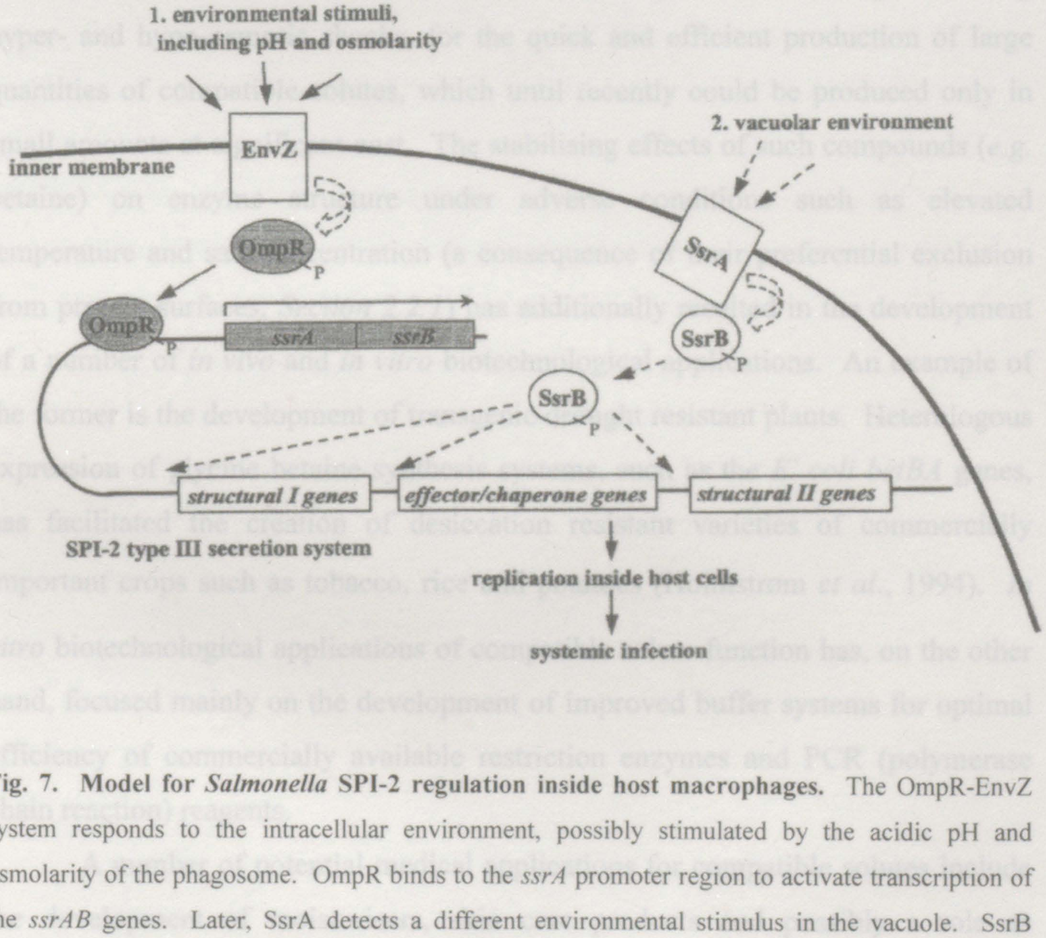


Fig. 7. Model for *Salmonella* SPI-2 regulation inside host macrophages. The OmpR-EnvZ system responds to the intracellular environment, possibly stimulated by the acidic pH and osmolarity of the phagosome. OmpR binds to the *ssrA* promoter region to activate transcription of the *ssrAB* genes. Later, SsrA detects a different environmental stimulus in the vacuole. SsrB activates expression of the type III secretion system encoded within SPI-2, which then allows for replication inside cells and systemic infection in mice. From Lee *et al*., 2000.

7. FUTURE PROSPECTS: Commercial applications

Detailed genetic and physiological analysis of bacterial stress responsive systems, particularly salt stress, as outlined in this review, has provided the basis for a number of recent advances both in the fields of biotechnology and medicine. Taking advantage of the existence of specific solute efflux systems and stretch activated channels (dedicated to the release of compatible solutes following osmotic down shock; *Section 3.3*) Sauer and Galinski (1997) developed a technique known as bacterial milking: a novel bioprocess involving alternating hyper- and hypo-osmotic shocks, for the quick and efficient production of large quantities of compatible solutes, which until recently could be produced only in small amounts at significant cost. The stabilising effects of such compounds (*e.g.* betaine) on enzyme structure under adverse conditions such as elevated temperature and salt concentration (a consequence of their preferential exclusion from protein surfaces; *Section 2.2.1*) has additionally resulted in the development of a number of *in vivo* and *in vitro* biotechnological applications. An example of the former is the development of transgenic drought resistant plants. Heterologous expression of glycine betaine synthesis systems, such as the *E. coli betBA* genes, has facilitated the creation of desiccation resistant varieties of commercially important crops such as tobacco, rice and potatoes (Holmstrøm *et al.*, 1994). *In vitro* biotechnological applications of compatible solute function has, on the other hand, focused mainly on the development of improved buffer systems for optimal efficiency of commercially available restriction enzymes and PCR (polymerase chain reaction) reagents.

A number of potential medical applications for compatible solutes include the development of moisturisers, skin care products and possibly a role as protective compounds for healthy cells during chemotherapy (Sauer and Galinski, 1997). However, given the increasing incidence of multiple drug resistance amongst microbial pathogens, perhaps the most interesting application of compatible solutes is the development of novel drug delivery systems. Based on smugglin technology (Payne, 1986), the widespread ability of microorganisms to accumulate compatible solutes may be exploited for the delivery of structurally related compounds with anti-microbial activity (Peddie *et al.*, 1998).

In conclusion then, a detailed analysis of the molecular mechanisms governing the salt stress response of bacterial cells, provides us not only with a better understanding of the characteristics of bacterial growth and survival in the natural environment, but also facilitates the development of novel and innovative processes in food and biomedicine.

Chapter II

Identification and disruption of BetL, a secondary glycine betaine transport system linked to the salt tolerance of *Listeria monocytogenes* LO28

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ABSTRACT

The trimethylammonium compound glycine betaine (*N,N,N*-trimethylglycine) can be accumulated to high intracellular concentrations, conferring enhanced osmo- and cryotolerance upon *Listeria monocytogenes*. This chapter reports the identification of *betL*, a gene encoding a glycine betaine uptake system in *L. monocytogenes,* isolated by functional complementation of the betaine uptake mutant *Escherichia coli* MKH13. The *betL* gene is preceded by a consensus σ^B-dependent promoter and is predicted to encode a 55-kDa protein (507 amino acid residues) with 12 transmembrane regions. BetL exhibits significant sequence homologies to other glycine betaine transporters, including OpuD from *Bacillus subtilis* (57% identity) and BetP from *Corynebacterium glutamicum* (41% identity). These high affinity secondary transporters form a subset of the trimethylammonium transporter family specific for glycine betaine, whose substrates possess a fully methylated quaternary ammonium group. The observed K_m value of 7.9 μM for glycine betaine uptake after heterologous expression of *betL* in *E. coli* MKH13 is consistent with values obtained for *L. monocytogenes* in other studies. In addition, a *betL* knockout mutant which is significantly affected in its ability to accumulate glycine betaine in the presence or absence of NaCl has been constructed in *L. monocytogenes.* This mutant is also unable to withstand concentrations of salt as high as can the $BetL^+$ parent, signifying the role of the transporter in *Listeria* osmotolerance.

INTRODUCTION

In the early 1980s a number of major outbreaks of human listeriosis established *Listeria monocytogenes* as an important foodborne pathogen (Gill, 1988). Even allowing for improvements in diagnostic techniques and greater awareness, the incidence of listeriosis appears to be increasing (Low and Donachie, 1997). This is extremely significant given that mortality rates of 23% have been reported for the organism (Schucant *et al.*, 1991). *L. monocytogenes* can survive a variety of environmental stresses, growth having been reported at

NaCl concentrations as high as 10% (McClure *et al.*, 1989) and at temperatures as low as -0.1°C (Walker *et al.*, 1990). The ability of the organism to withstand hostile environments is illustrated by an outbreak of listeric septicaemia, which was linked to consumption of salted mushrooms (7.5% NaCl) stored at low temperatures (Junttila and Brander, 1989). The ability of the organism to survive both high salt concentrations and low temperatures is attributed mainly to the accumulation of the compatible solute glycine betaine. This trimethyl amino acid, which occurs at high concentrations in sugar beets and other foods of plant origin, has been shown to stimulate growth of *L. monocytogenes* at between 0.3 and 0.7 M NaCl (Amezaga *et al.*, 1995), resulting in a 2.1-fold increase in the growth rate at 0.7 M NaCl (Amezaga, 1996) and a 1.8-fold increase at 4°C (Ko *et al.*, 1994). Patchett *et al.* (1992) described glycine betaine uptake in *L. monocytogenes* as a highly specific, constitutive, energy dependent system which was subsequently shown to be $\Delta\psi$–driven *via* co-transport with Na^+ (Gerhardt *et al.*, 1996) and regulated at the protein level by a novel osmolyte-sensing mechanism (Schucant *et al.*, 1991). On the other hand, a recent report suggests that at least a component of the glycine betaine uptake system in *Listeria* is σ^B-dependent, since a σ^B-knockout mutant was affected in its ability to accumulate glycine betaine (Becker *et al.*, 1998).

While much information regarding the physiological characterisation of glycine betaine transport is available, genetic analysis of the uptake systems in *L. monocytogenes* has been largely ignored. In contrast, the genetic basis of glycine betaine uptake in other Gram-positive bacteria has been studied extensively. *Bacillus subtilis* has been shown to possess three transport systems for glycine betaine: the secondary uptake system OpuD (Kappes *et al.*, 1996) and two binding-protein-dependent transport systems OpuA (Kempf and Bremer, 1995) and OpuC (ProU) (Lin and Hansen, 1995). The secondary transport system BetP, isolated by Peter *et al.* (1996), is involved in glycine betaine accumulation in *Corynebacterium glutamicum*.

This chapter describes the isolation, characterisation and disruption of *betL*, a gene which plays an important role in glycine betaine uptake in *L.*

monocytogenes and which exhibits high homologies to the secondary glycine betaine uptake systems of other Gram-positive bacteria.

MATERIALS AND METHODS

Table. 1 Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or characteristic(s)[a]	Source or reference
Strains		
L. monocytogenes		
LO28	Serotype 1/2c	P. Cossart, Institut Pasteur
LO28G	LO28 containing pVE6007	This study
LO28B	LO28 *betL*::pCPL2, BetL$^-$	This study
E. coli		
DH5α	*supE44* Δ*lac U169*(ϕ80*lacZ*ΔM15)R17 *recA1 endA1 gyrA96 thi-1 relA1*	Gibco-BRL
MKH13	MC4100Δ(*putPA*)101Δ(*proP*)2Δ(*proU*)	Kempf and Bremer, 1995
Pasmids		
pUC18	Ap^r ColE1 *ori*	Vieria and Messing, 1982
pCPL1	pUC18 containing 2.5 Kb of *L. monocytogenes* genomic DNA	This study
pVE6007	Cm^r Ts derivative of pWV01	Maguin *et al.*, 1992
pORI19	Em^r Ori$^+$ RepA$^-$ *lacZ'*	Law *et al.*, 1995
pCPL2	pORI19 containing DNA from *betL*	This study
pCPL3	pCPL1 cut with *Eco*RI	This study

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance

Media, chemicals, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α was grown at 37°C in Luria-Bertani (LB) medium (Maniatis *et al.*, 1992). *E. coli* MKH13 was grown at 37°C in either LB medium or M9 minimal medium (GIBCO/BRL, Eggenstein, Federal Republic of Germany

[FRG]) containing 0.5% glucose, 0.04% arginine, 0.04% isoleucine, and 0.04% valine. *L. monocytogenes* strains were grown in brain heart infusion (BHI) broth or in tryptone soy broth (Sigma Chemical Co., St. Louis, Mo.) supplemented with 0.6% yeast extract. Glycine betaine (Sigma) was added to M9 as a filter-sterilised solution to a final concentration of 1 mM. Radiolabelled [1-^{14}C]glycine betaine (55 mCi/mmol) was purchased from American Radiolabelled Chemicals Inc. (St. Louis, Mo.). Erythromycin, ampicillin, and chloramphenicol were made up as described by Maniatis *et al.* (1982) as concentrated stocks and added to media at the required levels. Where necessary, medium osmolarity was adjusted by the addition of NaCl.

DNA manipulations and sequence analysis

Restriction enzymes, RNase, Shrimp alkaline phosphatase and T4 DNA ligase were obtained from Boehringer GmbH (Mannheim, FRG) and were used according to the manufacturer's instructions. Genomic DNA was isolated from *L. monocytogenes* as described by Hoffman and Winston (1987). Plasmid DNA was isolated with the Qiagen QIAprep spin miniprep kit (Qiagen, Hilden, FRG). *E. coli* was transformed by standard methods (Maniatis *et al.*, 1982) while electrotransformation of *L. monocytogenes* was achieved by the protocol outlined by Park and Stewart (1990). Restriction fragments were isolated using the Qiaex II gel extraction kit (Qiagen, Hilden, FRG). Polymerase chain reaction (PCR) reagents (*Taq* polymerase and deoxynucleoside triphosphates dNTPs) were purchased from Boehringer and used according to the manufacturer's instructions with a Hybaid (Middlesex, United Kingdom) PCR express system. Oligonucleotide primers for PCR and sequence purposes were synthesised on a Beckman Oligo 1000M DNA synthesiser (Beckman Instruments, Inc., Fullerton, Calif.). Nucleotide sequence determination was performed on an ABI 373A automated sequencer with the Dye Terminator sequence kit (Applied Biosystems, Warrington, United Kingdom). Nucleotide and protein sequence analysis were done using Lasergene (DNASTAR Ltd., London, United Kingdom). Homology searches were performed with the BLAST program (Altschul *et al.*, 1990).

Construction of an *L. monocytogenes* genomic library

A genomic DNA preparation from *L. monocytogenes* was partially digested with *Sau*3A and ligated to plasmid pUC18 DNA, which had been digested with *Bam*HI and dephosphorylated with shrimp alkaline phosphatase. The resulting recombinant plasmids were transformed in restriction deficient *E. coli* DH5α, and colonies were selected on LB plates containing ampicillin (50 μg/ml), IPTG (isopropyl-1-thio-β-D-galactopyranoside) (1 mM), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 μg/ml). Approximately 70% of the plasmids in the bank (30,000 CFU) carried inserts, as judged from their LacZ$^-$ phenotypes. Transformants were pooled and grown for 2 h in LB medium with ampicillin and stocked at -80°C. Plasmid DNA was extracted and used to transform the glycine betaine uptake mutant *E. coli* MKH13. Transformants were selected on M9 minimal medium containing 4% NaCl and 1 mM glycine betaine.

Restriction deletion analysis

pCPL3 (Table 1) was constructed by digestion of pCPL1 with *Eco*RI, followed by religation (Fig. 1). The pCPL1 insert contains one *Eco*RI site (nucleotide [nt] 1379 [Fig. 1]), and a second is located in the multiple cloning site. The larger *Eco*RI fragment of pCPL1 was gel extracted, religated, and transformed into MKH13. Removal of the smaller *Eco*RI fragment resulted in inactivation of *betL* by removing a 350-bp region (not counting the TAA stop codon) from the 3' end of the gene. The loss of the *Eco*RI fragment in pCPL3 was confirmed by restriction analysis. Gene inactivation was confirmed by failure of MKH13 to grow following transformation and selection on minimal medium containing 4% NaCl and 1 mM glycine betaine.

Construction of an *L. monocytogenes betL* mutant

A *betL* mutant was constructed by gene disruption with a single crossover event, as described by Law *et al.* (1995). This system relies upon the lactococcal pWV01-derived Ori$^+$ RepA$^-$ vector pORI19. Maintenance of pORI19 is dependent on the temperature sensitive pGhost plasmid pVE6007 to supply RepA *in trans*. A 552-bp fragment (nt 702 to 1253 [Fig. 1]) from the centre of the *betL* gene was generated by PCR with primers *Xba*IKO 5′ TAAGCGCCACTCTAGACC 3′ (nt 702 to 719 [Fig. 1]) and *Eco*R1KO 5′ GCACGAATTCACCAAGTA 3′ (nt 1236 to 1253 [Fig. 1]), modified to contain the restriction sites *Xba*I and *Eco*RI (underlined), respectively. The resulting PCR product, purified by gel extraction, was cut with *Xba*I and *Eco*R1 and ligated into similarly digested pORI19 to give pCPL2 (Fig. 1), which was then transformed into *L. monocytogenes* LO28G (LO28 harbouring pVE6007). A temperature up-shift from 30°C to the nonpermissive 42°C resulted in the loss of pVE6007. Plating on erythromycin selected for chromosomal integration of pCPL2 at the point of homology with *betL*. PCR with primers *betL* F (nt 401 to 422 [Fig. 1]; 5′ AGTCCGATTGGCTCGATTCGAC 3′) and *betL* R (nt 1790 to 1812 [Fig. 1]; 5′ TCGCGAAATAGTCGCGGCAAAGC 3′) was used to confirm the integration event in one mutant strain, designated LO28B. A 4.6 kb product (corresponding to the length of *betL* plus pCPL2) was obtained for LO28B while LO28 gave a 1.4 kb product (corresponding to *betL* alone).

Transport assays

E. coli cells grown overnight in minimal medium (Davis and Mingioli, 1950) were inoculated into fresh minimal medium to an optical density at 600 nm (OD_{600}) of 0.05. Cells were harvested in mid-log phase (OD_{600} between 0.4 to 0.6), washed twice, and suspended to an OD_{600} of 1.0 in minimal medium. Subsequently, the cells were incubated with shaking for 5 min at 37°C, and transport was initiated by the addition of [1-^{14}C]glycine betaine. For K_m determination, the glycine betaine concentration was varied from 0.2 to 10 μM. Radioactivity was measured with a liquid scintillation counter (model 1600TR; Packard Instruments Co., Downers Grove, Ill.). To determine the ability of LO28 and LO28B to accumulate [1-^{14}C]glycine betaine, log phase cells grown in BHI broth were harvested by centrifugation, washed twice, and resuspended in 50 mM potassium phosphate buffer (pH 6.8) to an OD_{600} of 1.0. Glucose was added to a final concentration of 5 mM to energise the cells and where indicated, 3% NaCl was added to subject the cells to osmotic up-shock. After 20 min of incubation at 30°C, assays were initiated by the addition of [1-^{14}C]glycine betaine (at a final concentration of 10 μM). Cells were collected on 0.45 μm-pore-size cellulose nitrate filters (Schleicher & Schuell GmbH, Dassell, FRG) under vacuum. Filters were then washed with 3 ml of buffer (same osmolarity as the assay buffer), and the radioactivity trapped in the cells was measured by liquid scintillation counting as described above. In the cases of both *E. coli* and *Listeria*, protein concentrations of cell suspensions were derived from standard curves relating OD_{600} to protein concentration.

Nucleotide sequence accession number

The nucleotide sequence data reported in this chapter have been submitted to GenBank and assigned accession number AF102174.

RESULTS

Cloning of the *betL* gene by functional complementation of *E. coli* MKH13

In contrast to the parental strain MC4100, the mutant *E. coli* MKH13 is unable to synthesise glycine betaine from its precursor, choline, and lacks the transport systems PutP, ProP, and ProU, rendering it unable to grow on high osmolarity (3 to 4% NaCl) minimal media containing glycine betaine. The pUC18::LO28 genome library (see Materials and Methods) was transformed into MKH13, and transformants were selected on minimal medium containing 4% NaCl and 1 mM glycine betaine. No colonies appeared following a control transformation with pUC18 alone, while transformation efficiencies of approximately 80 CFU/μg of DNA were achieved from the plasmid bank, with colonies appearing after 36 h at 37°C. Plasmids isolated from 10 such colonies were retransformed into MKH13 to confirm complementation. Restriction analysis revealed that all 10 clones contained the same 2.5 kb insert. When clones were plated onto high osmolarity media containing either carnitine or proline no growth was observed, indicating that the cloned insert encodes a system specific for glycine betaine transport.

A representative plasmid, designated pCPL1, was chosen for further characterisation. Analysis revealed that if pCPL1 was deleted from the internal

*Eco*RI site to create pCPL3, no complementation of MKH13 was observed (Fig. 1). Approximately 1.9 kb of the insert was sequenced from both strands. Analysis of the sequenced region revealed a single large open reading frame spanning positions 209 to 1732. A TTG start codon was chosen as the initiation codon based on homology data. A long inverted repeat immediately downstream of *betL* probably functions as a *rho*-independent transcription termination signal with a ΔG of -28.2 kcal/mol (Platt, 1981). Upstream of the TTG start codon potential -10 and -35 regions (GTTA[16 nt]GGGAAA) which have considerable homology with the recently identified σ^B-dependent consensus promoter (GTTT[15/16 nt]GGGTAA) can be identified (Becker *et al.*, 1998). Upstream of the putative promoter site is a short inverted repeat with a ΔG of -13 kcal/mol which may act as a terminator for upstream sequences (Fig. 1). Sequencing upstream of this inverted repeat revealed the presence of a gene homologous to the L-argininosuccinate lyase gene from *Cyanobacterium synechocystis*.

The *betL* gene encodes a 507 residue protein (designated BetL) with a calculated molecular mass of 55.27 kDa. A search for related proteins in the databases revealed significant similarity to the Gram-negative choline transporter BetT (Lamark *et al.*, 1991) from *E. coli* (38% identity) and two Gram-positive secondary transporters, OpuD from *Bacillus subtilis* (57% identity) and BetP from *C. glutamicum* (41% identity). Both OpuD (Kappes *et al.*, 1996) and BetP (Peter *et al.*, 1996) are members of the trimethylammonium transporter family, whose substrates possess a fully methylated quaternary ammonium group. In the case of OpuD, BetP, and BetL, this substrate is glycine betaine. Hydropathy analysis of BetL, according to the method of Kyte and Doolittle (1982), predicts that BetL is an integral membrane bound protein containing 12 transmembrane domains. In fact, the entire hydropathy profile is very similar to that of OpuD (data not shown). Multiple alignments of the three proteins—BetL, OpuD and BetP—show a high degree of relatedness over the entire lengths of their sequences, but one region in particular, a 37-amino-acid segment stretching from amino acids 310 to 346, which includes the eighth transmembrane segment and the connecting

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                   [GATC]AAATTAAAGTCGCTCTCGAAAACGCGAAAAAAACTTTGTAAAACTAACTG   50
GGATGCTGAATGGCGTTCCAGTTTTTGCTGATTTCTCCCCCTTTTTCCTCTTAGCTGATTTGTCAGAGCACTACTTTT  129
                      -35                    -10                        RBS
TTTGTTACTATTAAAAAGAATGTTACCTTTTTGCTAACATGGGGAAATACATACAGAGAAAATAAAGGGAAGTGATGTA  208

TTG AAA AAA TTA ACA AAT GTC TTT TGG GGA TCG GGT TTT CTA GTT TTA TTA GCA GTT TTA  268
M   K   K   L   T   N   V   F   W   G   S   G   F   L   V   L   L   A   V   L     20
TTT GGG GCT TTT TTG CCA GAG CAA TTT GAG ACT TTT ACA AAC CAT ATC CAA AAA TTT CTA
F   G   A   F   L   P   E   Q   F   E   T   F   T   N   H   I   Q   K   F   L
ACA AGT AAT TTT GGT TGG TAT TAT TTA ATC GTT GTA GCG ATT ATT ATT ATC TTC TGC TTG  388
T   S   N   F   G   W   Y   Y   L   I   V   V   A   I   I   I   I   F   C   L     60
TTT TTA GTT TTA AGT CCG ATT GGC TCG ATT CGA CTC GGA AAA CCG GGT GAA GAA CCT GGT
F   L   V   L   S   P   I   G   S   I   R   L   G   K   P   G   E   E   P   G
TAT AGT AAT AAA TCT TGG TTT GCG ATG TTG TTT AGT GCT GGA ATG GGA ATT GGC CTC GTT  508
Y   S   N   K   S   W   F   A   M   L   F   S   A   G   M   G   I   G   L   V    100
TTC TGG GGT GCA GCT GAG CCG TTA TCT CAT TAT GCG GTC CAA GCT CCC GGA GGT GAA GTT
F   W   G   A   A   E   P   L   S   H   Y   A   V   Q   A   P   G   G   E   V
GGC ACG CAA GCA GCT ATG AAA GAT GCG CTT CGT TAT TCA TTC TTC CAC TGG GGA ATT TCT  628
G   T   Q   A   A   M   K   D   A   L   R   Y   S   F   F   H   W   G   I   S    140
GCT TGG TCG ATT TAT GCG ATT GTC GCT TTA GCA TTA GCT TAC TTC AAA TTC AGG AAA AAT
A   W   S   I   Y   A   I   V   A   L   A   L   A   Y   F   K   F   R   K   N
GCA CCT GGC TTA ATA AGC GCC ACA CTA TAC CCC ATT TTA GGC AAA CAT GCG AAA GGT CCT  748
A   P   G   L   I   S   A   T   L   Y   P   I   L   G   K   H   A   K   G   P    180
ATT GGA CAA TTG ATT GAT ATC ATT GCT GTT TTT GCG ACA GTC ATC GGT GTT GCA ACG ACA
I   G   Q   L   I   D   I   I   A   V   F   A   T   V   I   G   V   A   T   T
CTC GGT CTT GGC GCT CAA CAA ATT AAT GGT GGT CTT ACA TAC TTG TTT GGC GTT CCA AAC  868
L   G   L   G   A   Q   Q   I   N   G   G   L   T   Y   L   F   G   V   P   N    220
AAT TTT ACT GTC CAA TTT ACG ATT ATT GTT ATT GTC ACT ATT TTA TTT ATG TTA TCG GCT
N   F   T   V   Q   F   T   I   I   V   I   V   T   I   L   F   M   L   S   A
ATG TCC GGA CTT GAT AAA GGG ATT CAG CTT TTA AGT AAT GTA AAT ATT TAT GTT GCT GGT  988
M   S   G   L   D   K   G   I   Q   L   L   S   N   V   N   I   Y   V   A   G    260

GTT TTA TTA GTT TTA ACA CTT ATT CTT GGA CCT ACT CTA TTC ATT ATG AAT AAC TTC ACC
V   L   L   V   L   T   L   I   L   G   P   T   L   F   I   M   N   N   F   T
AAT TCA TTT GGT GAC TAC TTA CAA AAT ATC ATC CAA ATG AGT TTT CAG ACA GCA CCT GAT 1108
N   S   F   G   D   Y   L   Q   N   I   I   Q   M   S   F   Q   T   A   P   D    300
GCG CCT GAT GCA CGA AAA TGG ATT GAC TCA TGG ACT ATT TTT TAT TGG GCT TGG TGG CTT
A   P   D   A   R   K   W   I   D   S   W   T   I   F   Y   W   A   W   W   L
TCT TGG TCA CCG TTC GTC GGA ATT TTC ATT GCC AGA ATT TCA CGC GGT AGA ACG ATT CGC 1228
S   W   S   P   F   V   G   I   F   I   A   R   I   S   R   G   R   T   I   R    340
CAA TTC TTA CTT GGT GTA ATC GTG CTT CCC GCT TTA GTC AGT GTG TTT TGG TTT GCC GTA
Q   F   L   L   G   V   I   V   L   P   A   L   V   S   V   F   W   F   A   V
TTT GGC GGT TCG GCG ATT TTT GTC GAA CAA CAT GGT AAT TCT GGT CTT TCA AGT TTA GCG 1348
F   G   G   S   A   I   F   V   E   Q   H   G   N   S   G   L   S   S   L   A    380
ACA GAA CAG GTA CTC TTT GGG GTC TTT AAT GAA TTC CCA GGT GGC ATG ATG TTA TCG ATT
T   E   Q   V   L   F   G   V   F   N   E   F   P   G   G   M   M   L   S   I
GTT GCG ATG ATT TTA ATT GCA GTC TTC TTT ATT ACT TCA GCT GAC TCA GCC ACA TTT GTT 1468
V   A   M   I   L   I   A   V   F   F   I   T   S   A   D   S   A   T   F   V    420
CTC GGT ATG CAA ACG ACG GGC GGA TCT TTA AAT CCA CCG AAC TCC GTC AAA GTA ACA TGG
L   G   M   Q   T   T   G   G   S   L   N   P   P   N   S   V   K   V   T   W
GGA TTA CTC CAA GCG GGA ATA GCA AGT GTG CTA CTC TAT GCA GGC GGA CTG ACA GCG CTT 1588
G   L   L   Q   A   G   I   A   S   V   L   L   Y   A   G   G   L   T   A   L    460
CAA AAT GCG TCG ATT ATA GCA GCC TTT CCG TTT TCT ATC GTC ATC ATC TTA ATG ATT GTT
Q   N   A   S   I   I   A   A   F   P   F   S   I   V   I   I   L   M   I   V
TCC TTA TTC GTT TCG TTA ACG AGG GAA CAA GAA AAG CTA GGA TTA TAC GTT CGA CCG AAA 1708
S   L   F   V   S   L   T   R   E   Q   E   K   L   G   L   Y   V   R   P   K    500
AAA TCA CAA CGT TCT CAA CTA TAA TAAAAAGGGATGAAAATCTACAACTAGATTTTCATCCCTTTTTCAT 1779
K   S   Q   R   S   Q   L   *                                                    507

CCTTTTTTCTGCTTTGCCGCGACTATTTCGCGATAGCTACGAGTTTGTTTAGGATATTTTTTTCGAAAGAAATGTTGCG 1858

AAACAACTGCTAACACGAGAGAAATGGCGGTAATTGGAATAGAGC                                   1903
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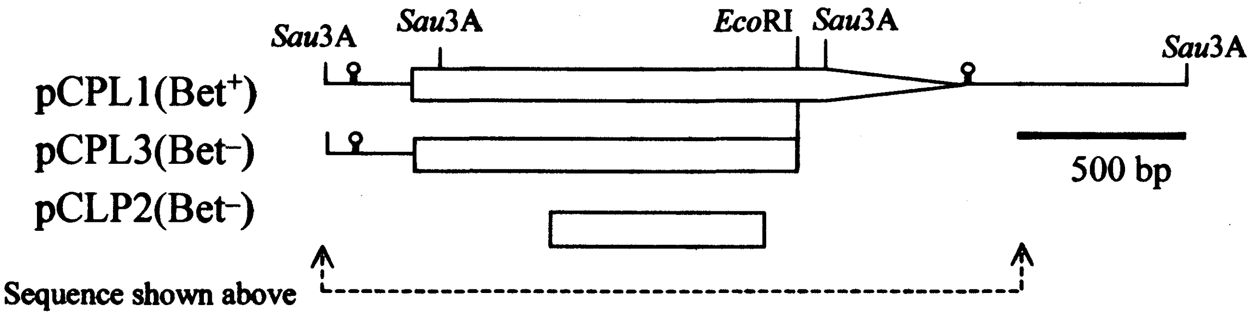

Fig. 1. DNA sequence of the *betL* gene and deduced amino acid sequence of the BetL protein. The likely ribosome-binding site (RBS) and the putative σ^B-dependent -10 and -35 sites are underlined. Inverted repeats are indicated by pairs of arrows. A graphic illustration of the cloned fragment of LO28 genomic DNA is also presented, together with constructs mentioned in the text.

cytoplasmic loop to the ninth transmembrane segment, is highly conserved. While it has been speculated that this region may function in substrate binding and membrane translocation in *B. subtilis* (Kappes *et al.*, 1996), its actual function is as yet unknown.

Analysis of BetL kinetics in *E. coli* MKH13

Uptake studies using [1-^{14}C]glycine betaine confirmed that growth of the strain carrying pCPL1 (BetL^{+}), when subjected to high osmolarity, was the direct result of glycine betaine accumulation mediated by BetL. Maximum uptake rates of 134 nmol/min/mg protein were determined by Michaelis-Menten kinetics. The K_m value of 7.9 μM observed following heterologous expression of *betL* in *E. coli* MKH13(pCPL1) correlates with the K_m value of 10 μM observed for *L. monocytogenes* in another study (Verheul *et al.*, 1997). Since no measurable uptake of [1-^{14}C]glycine betaine was observed for MKH13 clones carrying pUC18 alone (Fig. 2A), uptake of the compatible solute could be solely ascribed to the cloned insert on pCPL1. Given that the cloned gene is expressed, it is assumed that either the σ^B-dependent *Listeria* promoter is recognised in *E. coli* or transcription was initiated from another, undetermined site.

Analysis of a BetL$^-$ mutant of *L. monocytogenes* LO28

A BetL$^-$ mutant of *L. monocytogenes* LO28 (LO28B) was constructed by homologous recombination, as described in Materials and Methods. PCR analysis confirmed the disruption of the *betL* gene in strain LO28B (data not shown). The ability of LO28B to accumulate radiolabelled glycine betaine was significantly impaired in comparison with the parent strain (Fig. 2B). However, uptake was not completely abolished. In the presence of 3% NaCl, uptake of glycine betaine by LO28 was enhanced as expected but no increase in the level of uptake was observed for the mutant, suggesting that the enhanced uptake observed in the parent is due to activation of BetL rather than the induction of a separate system.

That glycine betaine uptake due to BetL may be linked to the salt tolerance of *L. monocytogenes* was confirmed in a simple plating experiment. LO28 and

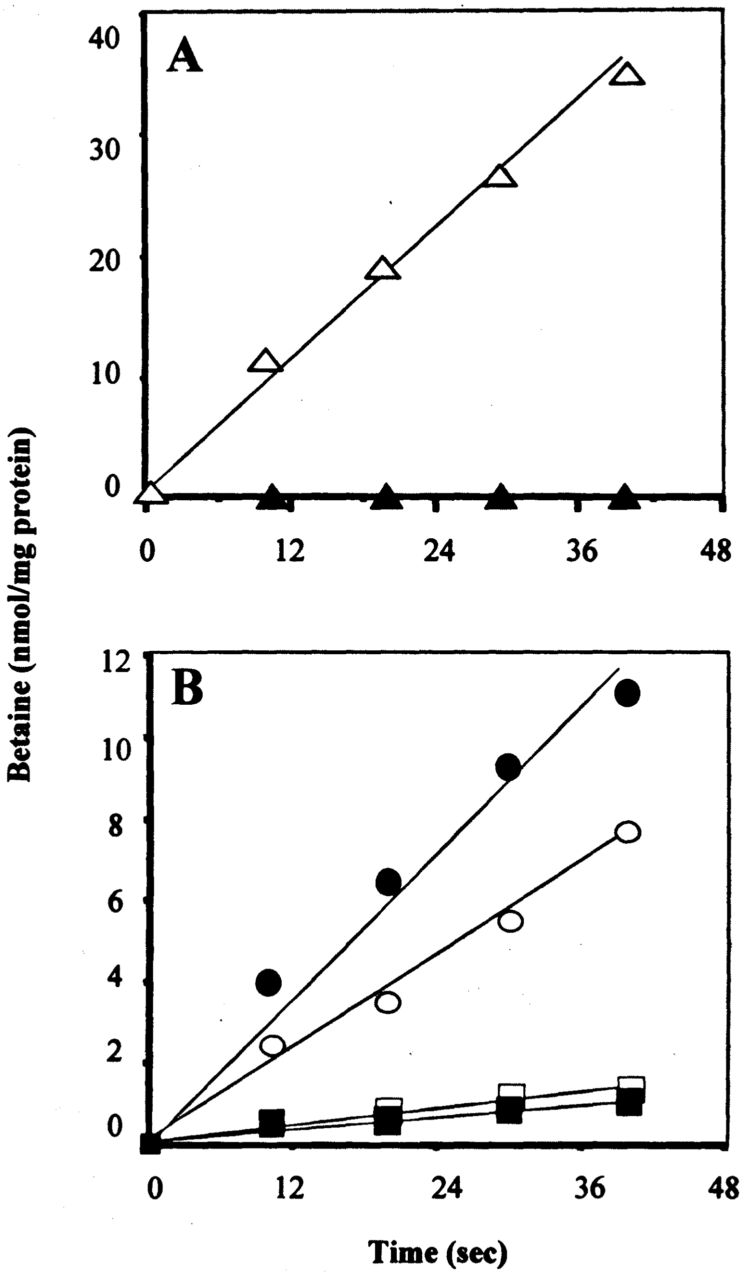

Fig. 2 (A) BetL-mediated glycine betaine uptake in *E. coli* MKH13. Uptake of [1-^{14}C]glycine betaine was assayed in low-osmolarity cultures at a final substrate concentration of 10 μM. *E. coli* MKH13(pCPL1) (BetL$^+$) was grown in M9 medium to mid-log phase and assayed for glycine betaine uptake (Δ). Strain MKH13(pUC18) (▲) was used as a control. Each point represents the mean value from at least two independent experiments. **(B)** Betaine accumulation in *L. monocytogenes* LO28 and the BetL$^-$ mutant LO28B. Mid-log phase cells (OD$_{600}$ 0.4 to 0.6) were harvested, washed twice, and resuspended in potassium phosphate buffer. Cells were energised by the addition of glucose and then divided into two equal volumes, and sodium chloride to a final concentration of 3% was added to one of the samples. After a 20-min incubation at 30°C, [1-^{14}C]glycine betaine (at a final concentration of 10 μM) was added to each sample and aliquots were removed at 10-s intervals, filtered through 0.45-μm filters, and counted by scintillation counting. ○, LO28; ●, LO28 plus 3% NaCl; □, LO28B; ■, LO28B plus 3% NaCl. Each point represents the mean value from at least two independent experiments.

LO28B were grown to stationary phase in BHI, serially diluted in Ringers, and plated on BHI agar containing an additional 4% NaCl. While LO28 gave large colonies within 48 hours at 37°C, LO28B was only able to form pinpoint colonies under the same conditions (Fig. 3).

DISCUSSION

Adaptation of bacteria to high solute concentrations involves intracellular accumulation of organic compounds called osmolytes (Booth *et al.*, 1994; Yancey *et al.*, 1982). Osmolytes (often referred to as compatible solutes because they can be accumulated to high intracellular concentrations without adversely affecting cellular processes) can be either taken up from the environment or synthesised *de novo,* and they act by counterbalancing external osmotic strength, thus preventing water loss from the cell and plasmolysis. Synthesised in relatively large quantities by plants (Hansen *et al.*, 1994), glycine betaine is the preferred compatible solute for the majority of bacteria (Csonka, 1989; Csonka and Hanson, 1991). While precursor molecules such as choline or glycine betaine aldehyde confer considerable osmotic stress tolerance to *B. subtilis* and *E. coli* in high-osmolarity media (Boch *et al.*, 1994; Landfald and Strøm, 1986), *L. monocytogenes* cannot synthesise glycine betaine from these molecules; thus, accumulation must occur *via* a transport system (Amezaga, 1996).

Many microorganisms possess two or more glycine betaine transport systems. *Salmonella typhimurium*, for example, possesses two genetically distinct pathways, a constitutive low-affinity system (ProP) and an osmotically induced high-affinity system (ProU) (Cairney *et al.*, 1985a; 1985b), while *B. subtilis* has three glycine betaine transport systems, OpuD, OpuA, and OpuC (Kappes *et al.*, 1996; Kempf and Bremer, 1995; Lin and Hansen, 1995). Generally these transport systems can be divided into two groups. The first of these are the multicomponent, binding-protein-dependent transport systems which belong to the superfamily of prokaryotic and eukaryotic ATP-binding cassette transporters or traffic ATPases (Higgins, 1992). Members of this family, including OpuA

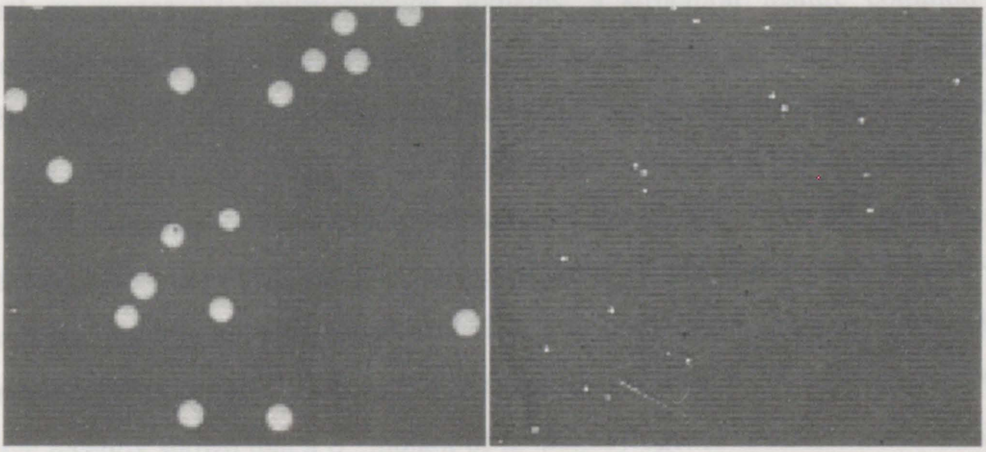

Fig. 3. Growth of *L. monocytogenes* LO28 (left) and the BetL⁻ mutant LO28B (right) on BHI agar containing an additional 4% NaCl after 48 h at 37°C.

(Kempf and Bremer, 1995) and OpuC (Lin and Hansen, 1995) of *B. subtilis* and ProU of *E. coli* (Lucht and Bremer, 1994), couple hydrolysis of ATP to substrate translocation across biological membranes. The second group belongs to a family of secondary transporters involved in the uptake of trimethylammonium compounds. Members of this family, including OpuD of *B. subtilis* and BetP of *C. glutamicum*, form single component mechanisms which couple proton motive force to solute transport across the membrane.

The *betL* gene isolated in this study encodes a 507-residue protein (BetL). BetL possesses 12 transmembrane domains, a structural feature common in secondary transport systems (Saier, 1994). The BetL protein thus represents the newest member of the prokaryotic secondary trimethylammonium transporter family. As with OpuD and BetP, BetL is highly specific for glycine betaine and fails to transport other trimethylammonium compounds such as carnitine or choline. An interesting feature of the *betL* gene is the presence of -10 and -35 promoter binding sites showing similarity to recently characterised σ^B-dependent promoters (Becker *et al.*, 1998). This is significant given that Becker *et al.* (1998) have recently shown that a σ^B mutant of *L. monocytogenes* is affected in its ability to accumulate glycine betaine. BetL thus may represent this predicted σ^B-mediated sodium or osmotically inducible component of glycine betaine transport in *L. moncoytogenes*. While it has been proposed that glycine betaine uptake in *L. monocytogenes* is controlled by activation of a constitutive enzyme (Ko *et al.*, 1994) regulated by a novel osmolyte-sensing mechanism (Verheul *et al.*, 1997), the presence of putative σ^B-dependent promoter binding sites suggests that BetL-mediated uptake of glycine betaine may be regulated, at least in part, at the level of transcription. As with the OpuD system in *B. subtilis*, maximal uptake activity by BetL thus may result from a combination of *de novo* synthesis of BetL and activation of pre-existing BetL (Kappes *et al.*, 1996).

The K_m value of 7.9 μM for BetL synthesised in *E. coli* MKH13 is similar to the value of 10 μM observed in *L. monocytogenes* (Verheul *et al.*, 1997) and is indicative of a high-affinity uptake system, allowing *Listeria* to scavenge glycine betaine from the environment. BetL thus may represent an important component of the glycine betaine-mediated salt and chill stress response in *Listeria* (Ko *et al.*, 1994). This is further evidenced by the dramatic decrease in the rate of glycine betaine uptake observed following disruption of *betL*. While non-specific uptake or passive diffusion cannot be ruled out, uptake rates of approximately 19% of that of the wild type observed for the BetL$^-$ mutant LO28B may suggest the presence of at least one other glycine betaine transporter in *L. monocytogenes*. Nonetheless, the important role of BetL in *Listeria* salt tolerance was established by a simple plate assay. Even though this assay was performed on a complex medium (and thus presumably in the presence of both carnitine and peptides which could act as osmolytes), the growth of LO28B was severely restricted. This preliminary confirmation of the importance of BetL will have to be characterised in more detail in further experiments.

In conclusion, while previous physiological investigations established the existence of a constitutive, highly specific mechanism for glycine betaine uptake in *Listeria* (Gerhardt *et al.*, 1996; Ko *et al.*, 1994; Verheul *et al.*, 1997), this study represents the first genetic analysis of compatible solute transport in *Listeria*. Interestingly, the presence of a putative σ^B-dependent promoter suggests that high osmolarity may stimulate increased transcription of *betL*, in addition to the activation of already synthesised BetL proteins.

ACKNOWLEDGEMENTS

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Chapter III

Analysis of the role of *betL* in contributing to the growth and survival of *Listeria monocytogenes* LO28

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Int. J. Food Microbiol. **60:** 261-268. (2000).

ABSTRACT

Survival of the foodborne pathogen *Listeria monocytogenes* in environments of elevated osmolarity and reduced temperature is attributed, at least in part, to the accumulation of the trimethylammonium compound glycine betaine. While the previous chapter describes the identification of *betL*, a gene encoding the secondary glycine betaine transporter BetL, which is linked to the salt tolerance of *Listeria*, the present study demonstrates that *betL*, preceded by a consensus σ^B-dependent promoter, is regulated by osmotic up-shock, at least in part at the level of transcription. Allelic exchange mutagenesis was used to construct an in-frame deletion in *betL* and the resulting mutant, designated BSOE, was used to determine the role of BetL in contributing to the growth and survival of *L. monocytogenes*, both in a high risk food (Camembert cheese) and animal model. Results indicate that while BetL plays an important role in glycine betaine mediated osmoprotection, mutating the gene does not significantly affect either the cryotolerance or virulence potential of the organism.

INTRODUCTION

Consumer demand for high quality, minimally processed foods, has in recent times favoured the introduction of milder preservation techniques (less acid, salt and chemical preservatives) creating a greater reliance on refrigeration as a method of food storage, both from a microbiological and quality standpoint (Abee and Wouters, 1999). This continuing trend towards minimal food processing and preservation has in turn been accompanied by a steady increase in the incidence of food poisoning, with emerging pathogens such as *Escherichia coli* O157 H7 and *Listeria monocytogenes* establishing themselves as significant agents of foodborne illness. *L. monocytogenes* (the causative agent of listeriosis, a potentially fatal disease with a reported mortality rate of 23% (Schucant *et al.*, 1991)) is of particular concern in minimally processed foods.

One of the major factors contributing to the recent ascent to prominence of *L. monocytogenes* as a foodborne pathogen is its robust physiology, growth being reported at temperatures as low as –0.1°C (Walker *et al.*, 1990) and at NaCl concentrations as high as 10% (McClure *et al.*, 1989). Survival of *L. monocytogenes* both at high salt concentrations and low temperatures is attributed mainly to the uptake of the trimethylammonium compound, glycine betaine. Accumulated to high intracellular concentrations without adversely affecting cellular processes, this highly effective and ubiquitous compatible solute (Csonka, 1989) has previously been shown to confer enhanced osmo- and cryotolerance upon *L. monocytogenes* (Ko *et al.*, 1994).

While a previous study reported the identification of *betL,* a gene encoding a betaine uptake system in *L. monocytogenes*, isolated by functional complementation of a glycine betaine uptake mutant, *E. coli* MKH13 (Sleator *et al.,* 1999a), this chapter investigates the effect of mutating BetL on the growth and survival of *L. monocytogenes*, both at high salt concentrations and low temperature environments, in a high-risk food (Camembert cheese) and on the virulence potential of the organism.

MATERIALS AND METHODS

Media, chemicals, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown in Luria-Bertani (LB) medium (Maniatis *et al.*, 1982), while *L. monocytogenes* strains were cultured either in tryptone soy broth (TSB) or agar (TSA;TSB plus 1.5% agar)(Sigma Chemical Co., St. Louis, Mo.) supplemented with 0.6% yeast extract (TSB-YE), or on *Listeria* selective agar (LSA) (Sigma). When a defined medium was required, the medium (DM) described by Premaratne *et al.* (1991) was used. Erythromycin (Em) and chloramphenicol (Cm) were made up as described in Maniatis *et al.* (1982) as concentrated stocks, and added to the media at the required levels. Where necessary, medium osmolarity was adjusted by the addition of NaCl.

Table 1. Bacterial strains and plasmids

Strain or Plasmid	Relevant properties[a]	Source or Reference
Strains		
E. coli		
DH5α	*supE* 44 Δ*lac U169*(ϕ80*lac* ZΔM15)R17 *recA1 endA1 gyrA96 thi*-1 *relA1*	Gibco-BRL
L. monocytogenes		
LO28	Serotype 1/2c	P. Cossart, Institut Pasteur
BSOE	Δ*betL*, *L. monocytogenes* LO28	This study
Plasmids		
pKSV7	Cm^r, temperature sensitive	Smith and Youngman, 1992
pCPL6	pKSV7 containing DNA from *betL*	This study

[a] Cm^r, chloramphenicol resistance

DNA manipulations

Restriction enzymes, RNase, shrimp alkaline phosphatase and T4 DNA ligase were obtained from Boehringer GmbH (Mannheim, FRG) and were used according to the manufacturer's instructions. Routine DNA manipulations were performed as described by Maniatis *et al.* (1982). Plasmid DNA was isolated with the Qiagen QIAprep spin miniprep kit (Qiagen). *E. coli* was transformed by standard methods (Maniatis *et al.*, 1982) while electrotransformation of *L. monocytogenes* was achieved using the protocol outlined by Park and Stewart (1990). Restriction fragments were isolated with the Qiaex II gel extraction kit (Qiagen). Polymerase chain reaction (PCR) reagents were purchased from Boehringer and used according to the manufacturer's instructions with a Hybaid (Middlesex, United Kingdom) PCR express system. Colony PCR was carried out following lysis of cells with Igepal CA-630 (Sigma).

RNA isolation and analysis

For studies on osmotic up-shock, overnight cultures of *L. monocytogenes*, grown at 37°C in TSB, were used to inoculate fresh media at a level of 1%. When the OD_{600} of the culture reached 0.5, cells were centrifuged, and salt stress was applied by re-suspension of the culture in TSB plus 4% added NaCl. Samples were taken at 0, 10, 15 and 30 min intervals and following centrifugation pellets were flash frozen in liquid nitrogen and stored at –70°C.

For RNA slot blots, total RNA was extracted from the frozen cell pellets using the hot-acid-phenol protocol described by Ripio *et al.* (1998). Samples of approximately 5 μg of total RNA were heated to 65°C in 1.3% dimethylsulfoxide (DMSO) for 10 min, before cooling on ice. The samples were then vacuum-blotted with a Bio-Rad slot-blot apparatus onto positively charged nylon membranes (Boehringer). RNA was crosslinked to the membranes with UV irradiation. Transcription of ***betL*** was monitored using an intragenic digoxigenin labelled probe generated by PCR using primers *Xba*IKO and *Eco*RIKO (Table 2). Detection of the labelled probe was mediated by the addition of an anti-DIG alkaline phosphate (AP) conjugated enzyme and CSPD substrate (Roche). Emission of light was captured by standard autoradiography (Hyperfilm, Amersham Life Sciences, England, HP7 9NA).

For reverse transcriptase (RT) PCR analysis of ***betL***, 5 μg of total RNA was diluted 1:10 in diethyl-pyrocarbonate (DEPC) treated water, samples were then cooled on ice for 5 min before being used as template for the RT reaction. Cooled template (8.5 μl) was added to an RT mix consisting of 4 μl of 5 x RT buffer (Boehringer), 2 μl 100 mM dithiothreitol, 0.5 μl of a 10 mM dNTP mix, 1 μl of RNAsin, and 100 ng of the random hexamer primer p(dN)6 (Roche). Finally 1 μl of Expand reverse transcriptase (Boehringer) was added and the reaction mixture was incubated for one hour at 37°C. The resultant cDNA was then used as template for PCR analysis using the *Xba*IKO and *Eco*RIKO primers (Table 2).

All glass and plastic-ware used in RNA analysis was treated with 2% sodium dodecyl sulphate (SDS) for 15 min, before rinsing with DEPC treated water.

Construction of a stable *L. monocytogenes betL*$^-$ mutant

The splicing by overlap extension (SOE) PCR procedure described by Horton *et al.* (1990) was used to create BSOE, a mutant with an internal 681 bp deletion in ***betL*** from nucleotide 623 to 1303 bp. Two 300 bp PCR products (nucleotides [nt] 323 to 622, amplified by primers SOEA and SOEB [Table 2], and nt 1304 to 1603 amplified by SOEC and SOED [Table 2]) 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 *E. coli* DH5α. The resulting plasmid designated pCPL6 was electroporated into LO28 and transformants were selected on TSA plates containing 10 μg/ml Cm. Forced chromosomal integration of pCPL6 at 42°C, followed by sequential passaging in TSB-YE at 30°C in the absence of Cm, facilitated allelic exchange between the intact *betL* gene and the 600 bp insert on pCPL6. The successful mutation event was confirmed by PCR using the BetL F and BetL R primers (Table 2).

Table 2. PCR primers used in this study

Primer	**Sequence (5'-3')**
SOEA (*betL*)	TTTCTAGAAAGTAATTTTGGTTGGTAT*
SOEB (*betL*)	TCCCCAGTGGAAGAATGA
SOEC (*betL*)	TCATTCTTCCACTGGGGAATTTTTGTCGAACAACATGGTAAT†
SOED (*betL*)	AATCGAAGCTTTTTGAAGCGCTGT*
BetL F	AGTCCGATTGGCTCGATTCGAC
BetL R	TCGCGAAATAGTCGCGGCAAAGC
*Xba*IKO	TAAGCGCCACTCTAGACC*
*Eco*RIKO	GCACGAATTCACCAAGTA*
p(dN)6	Random hexamer

*Nucleotides introduced to create restriction sites are underlined
†Overhang complementary to SOEB is underlined

Camembert cheese manufacture

Bovine milk (2.8% milk fat) was heat treated at 64-68°C for 15-20 s and cooled to 8-14°C. 0.002-0.005% F-DVS (Chr. Hansens) was added to the milk and held for 15-16 h at 12°C. Following pre-ripening the pH was 6.5-6.6. Subsequently the milk was pasteurised at 72°C for 15-20 s and cooled to 33-34°C prior to addition of 9-21 ml/100L milk of $CaCl_2$. For the production of traditional French Camembert, freeze dried DVS (Chr. Hansens) was added to portions (200 ml) of the cheese milk at a level of 10 mg/L. Log phase *L. monocytogenes* strains

(LO28 and BSOE) were also added at this point. The cheese was allowed to rest for 45-60 min and 10 μL/L standard cheese rennet (Chr. Hansens) was added to each milk sample when a pH of 6.3 was obtained. Following incubation, coagula were cut into 5-7 mm cubes and allowed to stand for 30-50 min with occasional gentle stirring. At this stage approximately 30% of the whey was siphoned off and the curd was ladled into moulds when the pH reached 5.6. The cheeses were turned after 1, 3 and 9 h, during which time the temperature of the cheese dropped by 11°C/h to 18-20°C. Following this the cheese was removed from the moulds and immersed in a 20% brine solution for 20 min. Cheeses were then sprayed with *Penicillium candidum* PCA FD (0.001 u/50g cheese) and ripened at 14-15°C and 85% relative humidity (RH) for one day followed by 8-9 days at 12-13°C and 95% RH. When satisfactory mould growth was obtained, the cheese was packed and stored at 4°C. Enumeration of listerial strains was performed by diluting duplicate samples in Ringers and surface plating in duplicate on LSA plates. Moisture levels in the cheese was determined using the dry-oven method described by Kosikowski (1982), while pH was determined using a WTW portable pH meter.

Virulence assays

Groups of 8 to 12-week old BALB/c mice were inoculated intraperitoneally with overnight cultures of the LO28 parent and mutant (BSOE) strains, suspended in 0.2 ml of phosphate-buffered saline (containing [per litre] 0.2 g of KH_2PO_4, 1.5 g of Na_2HPO_4, 0.2 g of KCl, 8.0 g of NaCl; pH 7.2), to a final concentration of 1.5 x 10^6 CFU/ml. Mice were sacrificed 3 days post infection, and numbers of viable organisms in the spleens of infected animals were determined by plating serial 10-fold dilutions of organ homogenates on TSA-YE.

RESULTS

Generation of BSOE, an *L. monocytogenes betL$^-$* mutant

To evaluate the role of BetL in contributing to the growth and survival of *L. monocytogenes,* allelic exchange mutagenesis was used to construct a *betL$^-$*

mutant with an internal 681 bp deletion (Fig. 1). This mutant, designated BSOE possesses a truncated form of the BetL protein, which lacks 6 of its 12 transmembrane domains. The 227 amino-acid deletion includes the highly conserved 37-aa domain (stretching from amino acids 310 to 346), which Kappes *et al.* (1996) postulated to function either as an important structural domain, specific to this family of secondary transporters, or in substrate binding and translocation across the membrane.

Physiological characterisation of BSOE

Growth of BSOE versus LO28 in TSB-YE (as determined by turbidity using a Spectra max 340 spectrophotometer, Molecular Devices) was measured over a range of salt concentrations (0-10% NaCl) at temperatures of 37°C (Fig. 2) and 4°C (Fig. 3). Results indicate that while BSOE grows significantly more slowly than its wild type parent at physiological temperatures and elevated osmolarities, no significant differences between parent and mutant were observed when grown at 4°C.

Consistent with these findings is the lack of any significant difference in the survival potential of both mutant and wild type, isolated from Camembert cheese, stored at 4°C (Fig. 4). The BetL mutant was further characterised with models of listeriosis by determining recovery rates of wild type and mutant strains from spleens following intraperitoneal infection. Mutant and wild type recovery rates three days post infection were almost identical (Fig. 5).

Transcriptional analysis of *betL*

Analysis of *betL* transcription was carried out using a combination of both RNA slot blots (Fig. 6A) and RT-PCR (Fig. 6B). RT-PCR analysis indicated that under normal growth conditions (TSB-YE in the absence of any added NaCl), constitutive expression of *betL* was observed, with a significant increase in the level of transcription occurring following osmotic up-shock. 15 min exposure to 4% NaCl resulted in a 1.6-fold increase in the level of transcription (as determined by densitometric analysis of the amplified cDNA product). Similar

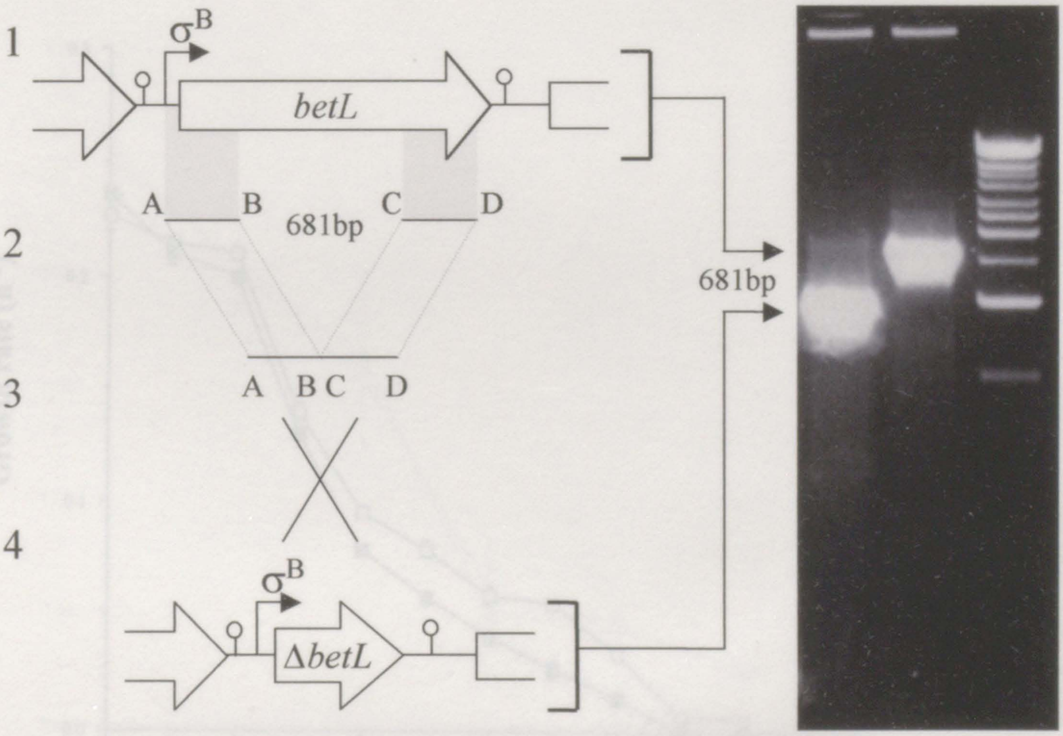

Fig. 1. Creation of the *betL* deletion mutant, BSOE. Part of the coding region of *betL* was eliminated using the splicing by overlap extension (SOEing) procedure (see Materials and Methods).

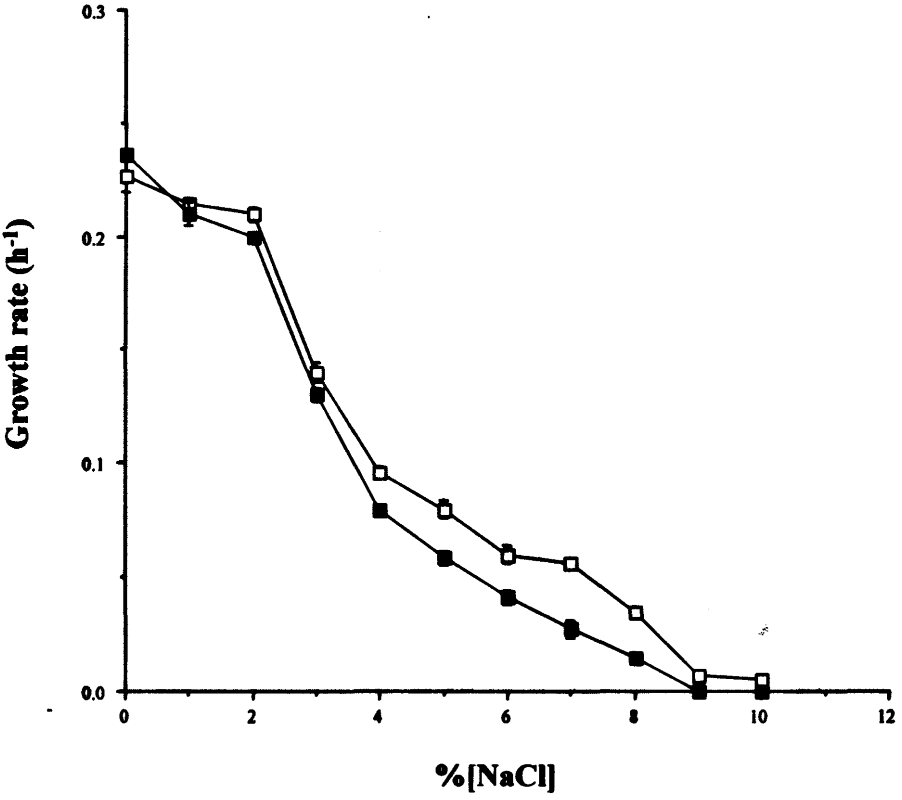

Fig. 2. Growth rates of LO28 (□) and BSOE (■) as a function of NaCl added to the medium. Overnight cultures of *L. monocytogenes* LO28 and BSOE (Δ*betL*) were cultured in the appropriate medium and the specific growth rates (μ) were determined during exponential growth at 37°C . Each point represents the mean value of three independent experiments.

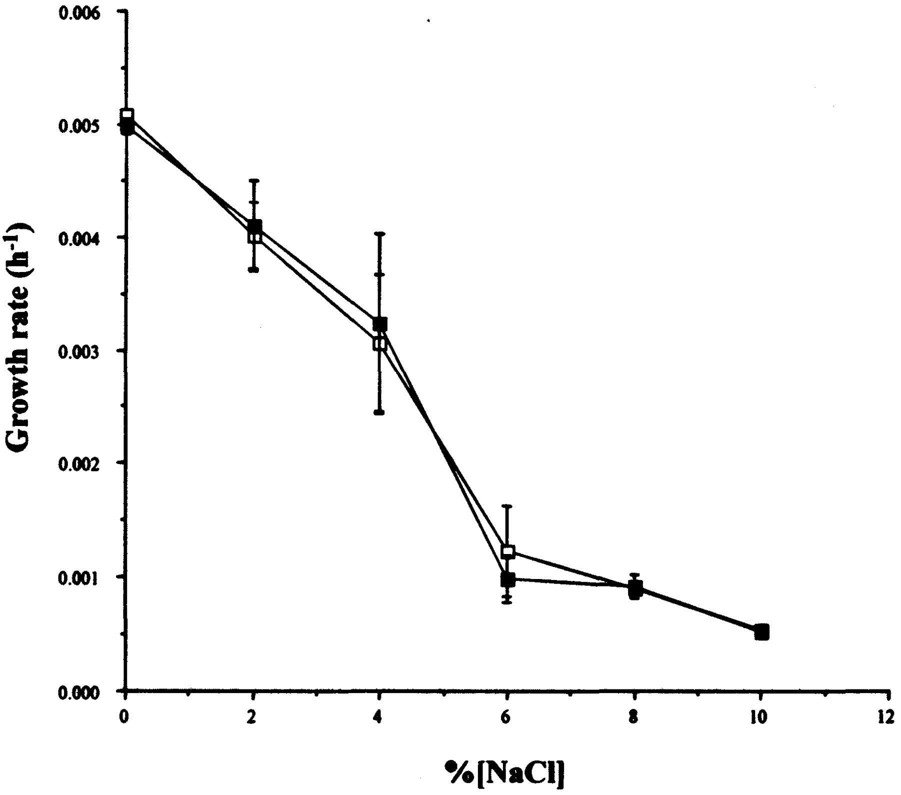

Fig. 3. Effect of high osmolarity at reduced temperatures on the survival of *L. monocytogenes* LO28 (□) and BSOE (Δ*betL*) (■) incubated at 4°C in TSB-YE at different concentrations of NaCl. Specific growth rates (μ) were determined during exponential growth and plotted against the %NaCl present in the growth medium. Each point represents the mean value of three independent experiments.

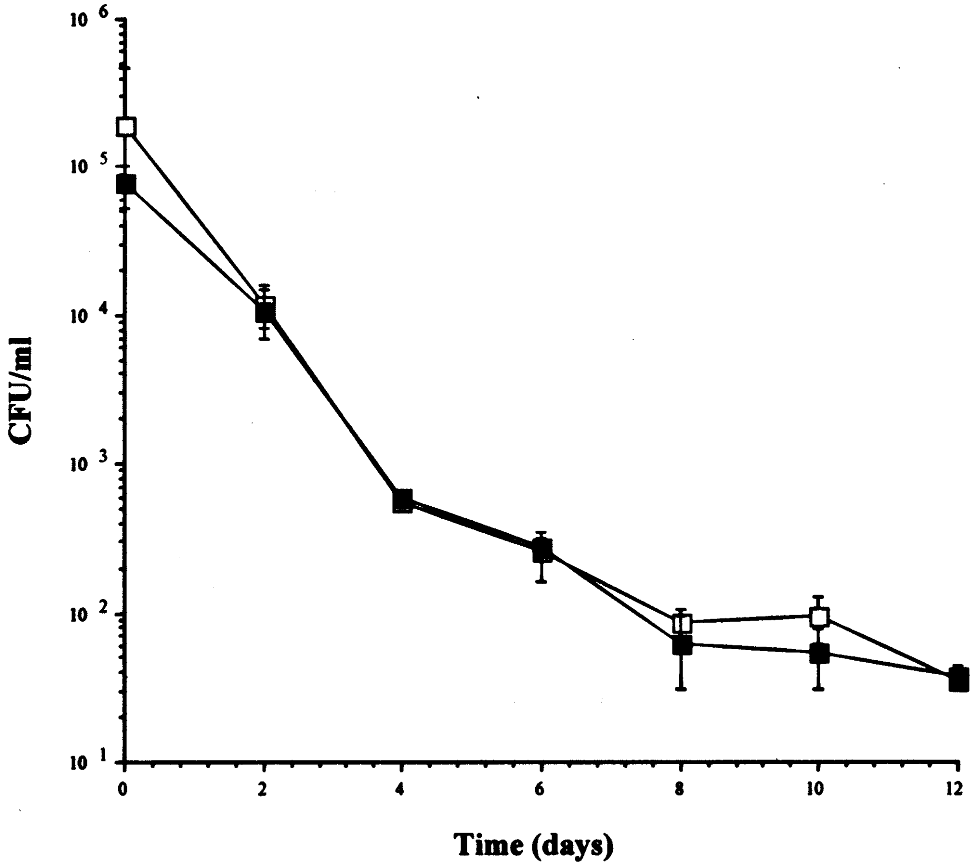

Fig. 4. Effect of deleting *betL* on the survival of *L. monocytogenes* during the manufacture and storage of Camembert cheese. LO28 (□), BSOE (Δ*betL*) (■). Each point represents the mean value of three independent experiments.

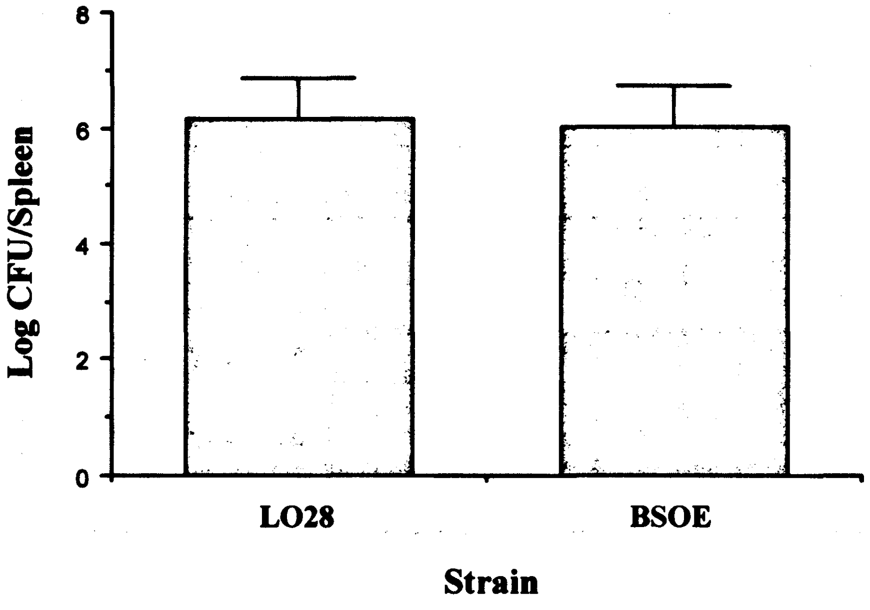

Fig. 5. Effect of deleting *betL* on the virulence of *L. monocytogenes*. Levels of *Listeria* in the spleens of infected mice three days post-infection are shown (*n* = 3). Repeat experiments showed similar results.

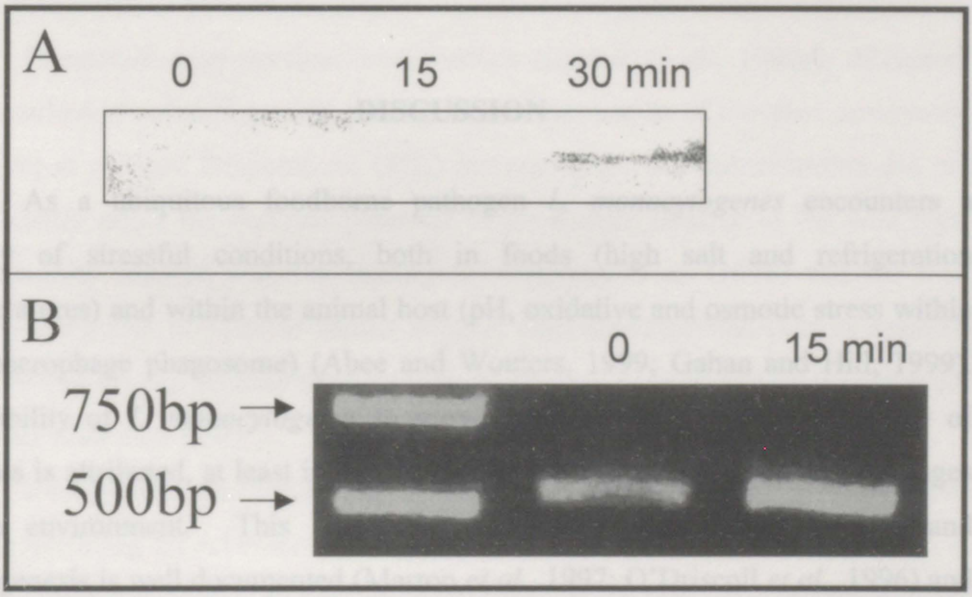

Fig. 6. **(A)** RNA slot blot transcription analysis of *betL* in *L. monocytogenes* LO28. A significant increase in the level of mRNA was observed following 30 min exposure to 4% NaCl. **(B)** RT-PCR transcription analysis of *betL* expression, both in the presence and absence of salt stress. While *betL* is constitutively expressed in the absence of a salt stress (lane 2), 15 min exposure to 4% NaCl (lane 3) resulted in a 1.6-fold increase in the intensity of the RT-PCR product.

results were obtained for RNA slot blots, which show a distinct increase in the level of mRNA, from initially undetectable levels, 30 min post exposure to a similar salt stress.

DISCUSSION

As a ubiquitous foodborne pathogen *L. monocytogenes* encounters a variety of stressful conditions, both in foods (high salt and refrigeration temperatures) and within the animal host (pH, oxidative and osmotic stress within the macrophage phagosome) (Abee and Wouters, 1999; Gahan and Hill, 1999). The ability of *L. monocytogenes* to grow and survive under such a variety of stresses is attributed, at least in part, to its ability to sense and respond to changes in its environment. This adaptive physiological response to survival and pathogenesis is well documented (Marron *et al.*, 1997; O'Driscoll *et al.*, 1996) and is believed to be coordinately regulated, mainly at the level of transcription (Mekalanos, 1992). A possible candidate for mediating stress adaptive responses in *L. monocytogenes* is the alternative sigma factor σ^B. This secondary subunit of RNA polymerase, governs a stress regulon comprising over 40 genes in the related bacterium *Bacillus subtilis* (Völker *et al.*, 1994). Recently Becker *et al.* (1998) identified and mutated the gene encoding the σ^B homologue in *L. monocytogenes*. This mutation exhibited a reduced ability to accumulate glycine betaine, consequently leading to reduced growth at high salt concentrations and low temperatures.

Recently, the identification of *betL*, a gene predicted to encode a secondary glycine betaine transporter (BetL), linked to the salt tolerance of *L. monocytogenes* LO28, was reported (Chapter II, this thesis). In the present communication it is shown that *betL* (preceded by a consensus σ^B-dependent promoter), is regulated at least in part, at the level of transcription, and as such forms an integral component of the listerial σ^B regulon. The effects of mutating *betL* on the survival of *Listeria* in complex environments of elevated osmolarity and reduced temperature were also investigated. Allelic exchange mutagenesis was used to construct an in-frame deletion in *betL*. This mutant designated BSOE, was chosen for further analysis in favour of the previously constructed LO28B strain, which was generated by plasmid insertion (Sleator *et al.*, 1999a), a technique which can lead to phenotypic reversion as well as polar mutations.

Consistent with previous observations (Sleator *et al.*, 1999a), disrupting *betL* resulted in reduced growth at 37°C in complex media of elevated osmolarity. However at reduced temperatures (4°C) increasing the salt concentration did not significantly affect the survival potential of the mutant relative to the wild type. These results coupled with the findings of Gerhardt *et al.* (1996) that the Na^+-betaine symporter of *Listeria* cannot support chill-activated transport in vesicles, indicate that glycine betaine mediated cryoprotection is governed by a system (or systems) other than BetL.

Recently Ko and Smith (1999) cloned the *gbuABC* system, a three-gene operon encoding an ATP-driven, osmoregulated glycine betaine transporter in *L. monocytogenes* 10403S. As well as contributing to the salt stress response of the organism, GbuABC (which is also present in *L. monocytogenes* LO28, unpublished results) was shown to be responsible for most of the chill-activated transport of glycine betaine in *Listeria*. Thus in the Camembert cheese trial, storage for prolonged periods at reduced temperatures, had no significant effect on the survival of the mutant relative to the wild type, since disrupting BetL does not inhibit the chill-activated glycine betaine uptake, and cryoprotection afforded by GbuABC. In addition, as outlined in chapter IV, recent work has led to the identification of OpuC, a carnitine uptake system, also capable of transporting glycine betaine. Given that the σ^B null mutant of Becker *et al.* (1998) was affected in its ability to transport carnitine as well as glycine betaine, it is predicted that *opuC*, like *betL* forms part of the σ^B regulon, a hypothesis currently under investigation. The existence of both OpuC and GbuABC, contributes further to the complexity of glycine betaine uptake in *Listeria*. The degeneracy of the systems underlines their importance, and may explain the lack of any significant differences observed between mutant and wild type, both in the food and animal model, *i.e.* deleting one transporter does not necessarily prevent glycine betaine mediated accumulation by the remaining systems.

Results obtained for the virulence study showed that mutating BetL does not significantly affect the virulence potential of the organism. Since carnitine, rather than betaine is the predominant osmolyte in animal tissues (Bieber, 1988), the effects if any, of mutating BetL may be masked by carnitine uptake (*via* OpuC), as well as osmolyte synthesis systems (such as the recently identified proline synthesis operon; *proBA* (Chapter V, this thesis)).

ACKNOWLEDGMENTS

I would like to thank Brid O'Driscoll for performing the Camembert cheese trial and Cormac Gahan for assistance with virulence studies.

Chapter IV

Analysis of the role of OpuC, an osmolyte transport system, in the salt tolerance and virulence potential of *Listeria monocytogenes*

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Colin Hill

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ABSTRACT

The success of *Listeria monocytogenes* as a foodborne pathogen owes much to its ability to survive a variety of stresses, both in the external environment prior to ingestion, and subsequently within the animal host. Growth at high salt concentrations and low temperatures is attributed mainly to the accumulation of organic solutes such as glycine betaine and carnitine. A novel system for generating chromosomal mutations (based on a lactococcal pWVO1-derived Ori^+ $RepA^-$ vector, pORI19) was utilised to identify a listerial OpuC homologue. Mutating the operon in two strains of *L. monocytogenes*, revealed significant strain variation in the observed activity of OpuC. Radiolabelled osmolyte uptake studies, together with growth experiments in defined media, linked OpuC to carnitine and glycine betaine uptake in *Listeria*. In addition the role of OpuC in contributing to the growth and survival of *Listeria* in an animal (murine) model of infection was investigated. Mutating OpuC resulted in a significant reduction in the ability of *Listeria* to colonise the upper small intestine and cause subsequent systemic infection following peroral inoculation. The multi-component OpuC transport system thus represents yet another addition to the arsenal of transporters used by *Listeria* for osmolyte acquisition during salt stress.

INTRODUCTION

Survival of the foodborne pathogen *Listeria monocytogenes* both at high salt concentrations (McClure *et al.*, 1989) and in low temperature environments (Walker *et al.*, 1990) is attributed mainly to the accumulation of the organic compounds, glycine betaine (*N,N,N*-trimethylglycine; Ko *et al.*, 1994) and carnitine (β-hydroxy-γ-*N*-trimethyl aminobutyrate; Beumer *et al.*, 1994). Accumulated to high intracellular concentrations without adversely affecting cellular processes, these compounds have previously been shown to function as effective compatible solutes (Csonka and Hanson, 1991; Yancey *et al.*, 1982) both in *Listeria* (Bayles and Wilkinson, 2000; Beumer *et al.*, 1994; Ko *et al.*, 1994) and other organisms (Graham and Wilkinson, 1992; Kets *et al.*, 1994).

The preferred compatible solute for the majority of bacteria (Csonka, 1989; Csonka and Hanson, 1991), and the most important osmolyte in *L. monocytogenes*, is the trimethylammonium compound, glycine betaine (Ko *et al.*, 1994). Present at relatively high concentrations in foods of plant origin (Hansen *et al.*, 1994), it has been shown to stimulate the growth of *L. monocytogenes* between 0.3 to 0.7 M NaCl (Amezaga *et al.*, 1995), and at temperatures as low as 4°C (Ko *et al.*, 1994). Recent studies identified genes encoding two glycine betaine transport systems in *Listeria*. The first of these, *betL* (Sleator *et al.*, 1999a; 2000), encodes a single component membrane bound protein, belonging to a family of secondary transporters of which OpuD of *Bacillus subtilis* (Kappes *et al.*, 1996) and BetP of *Corynebacterium glutamicum* (Peter *et al.*, 1996) are members. Transporters in this family couple ion motive force to solute transport across the cell membrane (Reizner *et al.*, 1994). The second system, encoded by the *gbuABC* operon (Ko and Smith, 1999), is a multi-component, binding-protein dependent transport system, forming part of a superfamily of prokaryotic and eukaryotic ATP-binding cassette transporters (Higgins, 1992). Members of this family, including OpuA (Kempf and Bremer, 1995) and OpuC (ProU) (Lin and Hansen, 1995) of *B. subtilis*, couple ATP hydrolysis to substrate translocation across biological membranes.

After glycine betaine, L-carnitine is regarded as the most effective osmolyte in *L. monocytogenes* (Ko *et al.*, 1994; Verheul *et al.*, 1997). Playing a role in fatty acid transport across the inner mitochondrial membrane (Idell-Wenger, 1981), carnitine can be accumulated to concentrations of up to 50 mM in some animal tissues (Bieber, 1988), approximately 5000-fold more than the previously calculated K_m value (10 μM) in *Listeria* (Verheul *et al.*, 1995). However, carnitine is not as effective as glycine betaine in contributing to either the salt or chill stress response of *L. monocytogenes* (Ko *et al.*, 1994). Nonetheless, the relative abundance of carnitine in mammalian tissues (Bieber, 1988) makes it the most readily available, and thus, possibly the most important osmolyte contributing to the survival of *L. monocytogenes*, both in foods of animal origin (Smith, 1996) and during subsequent intracellular growth following infection (Verheul *et al.*, 1995).

This report describes the isolation of mutants of *L. monocytogenes* unable to utilise carnitine as an osmoprotectant, using a modification of a system outlined by Law *et al.* (1995) for generating chromosomal mutations. The method is based on the conditional replication of the pWVO1–derived Ori^+ $RepA^-$ vector pORI19. The mutants were shown to carry a copy of pORI19 inserted into a region of the chromosome with extensive homology to the recently identified *opuC* operon of *L. monocytogenes* (Fraser *et al.*, 2000) and were used to determine the importance of OpuC-encoded osmolyte uptake in contributing to the growth and survival of *L. monocytogenes* in an animal (murine) model of infection.

MATERIALS AND METHODS

Media, chemicals, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* EC101 was grown at 37°C in Luria-Bertani (LB) medium (Maniatis *et al.*, 1982). *L. monocytogenes* strains were grown either in Brain Heart Infusion (BHI) broth (Oxoid, Unipath Ltd. Basingstoke, United Kingdom) or on *Listeria* selective agar, LSA (Oxoid). Blood agar plates consisted of blood agar (Lab M) to which 5% sheep blood was added following autoclaving. When a defined medium was required, the medium (DM) described by Premaratne *et al.* (1991), was used. Where indicated, carnitine and glycine betaine (Sigma Chemical Co., St. Louis, Mo.) were added to DM as filter-sterilised solutions, to a final concentration of 1 mM. Radiolabelled L-[*N*-methyl-^{14}C]carnitine (50-62 mCi/mmol) and *N,N,N*-trimethylglycine [1-^{14}C] were purchased from NEN Life Sciences Products (Hoofddorp, The Netherlands) and Campo Scientific (Veenendaal, The Netherlands) respectively. Erythromycin (Em) and chloramphenicol (Cm) were made up as described by Maniatis *et al.* (1982) as concentrated stocks and added to media at the required levels. Where necessary the media osmolarity was adjusted by the addition of NaCl.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or Characteristic(s)*	Source or reference
Strains		
L. monocytogenes		
LO28	Serotype 1/2c	P. Cossart, Institut Pasteur
LO28G	LO28 containing pVE6007	Sleator *et al.*, 1999a
LO28C	LO28 *opuC*::pCPL5, $OpuC^-$	This study
ScottA	Wild type	T. Abee
ScottAG	ScottA containing pVE6007	This study
ScottAC	ScottA *opuC*::pCPL5, $OpuC^-$	This study
E. coli		
EC101	*E. coli* JM101 with *repA* from pWVO1 integrated in the chromosome	Law *et al.*, 1995
Plasmids		
pORI19	Em^r Ori^+ $RepA^-$ derivative of pORI28	Law *et al.*, 1995
pVE6007	Cm^r Ts derivative of pWVO1	Maguin *et al.*, 1992
pCPL5	pORI19 containing 1.1 kb of *L. monocytogenes* genomic DNA	This study

*Em^r, Erythromycin resistance; Cm^r chloramphenicol resistance

DNA manipulations and sequence analysis

Routine DNA manipulations were performed as described by Maniatis *et al.* (1982). Genomic DNA was isolated from *L. monocytogenes* by the method of Hoffman and Winston (1987). Plasmid DNA was isolated using the Qiagen QIAprep spin miniprep kit (Qiagen, Hilden, FRG). *E. coli* was transformed by standard methods (Maniatis *et al.*, 1982) while electrotransformation of *L. monocytogenes* was achieved by the protocol outlined by Park and Stewart (1990). Polymerase chain reaction (PCR) reagents (*Taq* polymerase and deoxynucleoside triphosphates dNTPs) were purchased from Boehringer GmbH (Mannheim, Germany) and used according to the manufacturer's instructions with a Hybaid (Middlesex, United Kingdom) PCR express system. Where mentioned, colony PCR was carried out following cell lysis with Igepal CA-630 (Sigma). Oligonucleotide primers for PCR and sequence purposes were synthesised on a

Beckman Oligo 1000M DNA synthesiser (Beckman Instruments Inc., Fullerton, California). Nucleotide sequence determination was performed on a Beckman CEQ 2000 DNA analysis system. Homology searches were performed against the GenBank database using the BLAST program (Altschul *et al.*, 1990).

Creation of a pORI19 integration bank in *L. monocytogenes* LO28

A bank of *L. monocytogenes* LO28::pORI19 insertion mutants was generated essentially as described by Law *et al.* (1995), with some minor modifications. A genomic DNA preparation from *L. monocytogenes* LO28 was partially digested with *Eco*RI and ligated to the ORI$^+$ RepA$^-$ plasmid pORI19, which had been digested with *Eco*RI and dephosphorylated with shrimp alkaline phosphatase. The resulting recombinant plasmids were transformed into *E. coli* EC101 (RepA$^+$) and colonies were selected on LB plates containing Em (250 μg/ml), IPTG (isopropyl-1-thio-β-D-galactopyranoside) (1 mM), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 μg/ml). Transformants were pooled and grown with shaking, for 2 h in LB broth containing Em (250 μg/ml). Plasmid DNA was then extracted and used to transform *L. monocytogenes* LO28G (LO28 harbouring the temperature sensitive, RepA$^+$ helper plasmid, pVE6007 (Sleator *et al.*, 1999a)). Immediately following transformation, cells were incubated in BHI broth containing Em (50 ng/ml) at 30°C for 180 min (to induce expression of Em^r-encoding genes). To induce loss of pVE6007 and force chromosomal integration of pORI19 at the points of homology with the cloned insert, 100 μl of the transformation mix was used to inoculate 10 ml BHI broth, pre-warmed to 42°C (the non-permissive temperature for pVE6007 replication in *Listeria*). Following overnight incubation at 42°C transformants were plated onto pre-warmed BHI-Em plates and incubated at 42°C for 48 h. Loss of pVE6007 was confirmed by lack of growth of the transformants on BHI Cm plates, coupled with an inability to isolate replicating plasmids from the cytosol.

Isolation of osmolyte uptake mutants of *L. monocytogenes* LO28

Putative osmolyte-deficient transport mutants were isolated by screening the pORI19 insertion mutant bank (by replica plating) on DM, DM + 3% NaCl

(DMS), DMS + 1 mM carnitine (DMSC), and DMS + 1 mM glycine betaine (DMSB). Mutants were confirmed by re-streaking onto DM agar plates to which either salt (3% w/v) or salt plus carnitine/glycine betaine (1 mM) was added.

Identification of disrupted genes

The isolated osmolyte uptake mutants were electroporated with the RepA$^+$ helper plasmid, pVE6007, recovered at 30°C on BHI-Em-Cm plates, and passaged subsequently in BHI-Em-Cm broth at 30°C. Inserts on the rescued plasmids, amplified by PCR with the Pharmacia (Upsala, Sweden) universal and reverse primers, were subjected to restriction analysis before a representative plasmid (designated pCPL5) was chosen for sequence determination and homology studies.

Generation of *L. monocytogenes* LO28 and ScottA::pCPL5 insertion mutants

L. monocytogenes strains LO28G and ScottAG (harbouring pVE6007) were transformed with pCPL5 and transformants were selected on BHI-Em-Cm plates at 30°C. As before, temperature up-shift from 30°C to 42°C, while selecting for Em resistance, resulted in loss of pVE6007 and targeted chromosomal integration of pCPL5. Loss of pVE6007 was established by sensitivity to Cm, while chromosomal integration of pCPL5 was confirmed by PCR.

Uptake studies

Cells grown overnight in defined media, were harvested by centrifugation (3000 x g, 15 min, 10°C), washed twice and re-suspended in 50 mM potassium phosphate (pH 6.8) containing 5 mM $MgSO_4$ and Cm 50 µg/ml (containing 3% NaCl when uptake experiments were performed in the presence of NaCl). Cells at an OD_{600} of 20 in this buffer were stored on ice until use. Cells (final OD_{600} of 1) were energised at 37°C with 10 mM glucose for 10 min prior to the addition of radiolabelled carnitine or betaine (final concentration of 18 µM). Where indicated, the buffer osmolarity was raised by the addition of 30% NaCl to a final concentration of 3%. Samples were withdrawn and uptake was stopped by the addition of 2 ml of 50 mM potassium phosphate (pH 6.8) buffer (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 (containing 3% NaCl when uptake experiments were performed in the presence of NaCl), and the radioactivity trapped in the cells was measured with a liquid scintillation counter (model 1600 TR, Pakard Instruments Co., Downers Grove, IL, USA). Uptake of osmolytes was normalised to the total cellular proteins, which was determined using the bicinchoninic acid method, as provided by the supplier (Sigma Chemicals, St. Louis, MO) with bovine serum albumin as a standard.

Virulence assays

Bacterial virulence was determined by intraperitoneal and peroral inoculation of 8 to 12-week old BALB/c mice. Intraperitoneal inoculations were carried out as described previously (Sleator *et al.*, 2000), using overnight cultures (6.5 x 10^5 cells) of mutant and wild type *Listeria*, suspended in 0.2 ml of phosphate buffered saline. For peroral inoculations, mutant and wild type strains, suspended in buffered saline with gelatin (BSG; 0.85% NaCl, 0.01% gelatin, 2.2 mM K_2HPO_4 and 4.2 mM Na_2HPO_4) were mixed at a ratio of LO28:LO28C and ScottA:ScottAC of 1:1. Mice were infected with approximately 1 x 10^{10} cells (total) using a micropipette tip placed immediately behind the incisors. Three days post infection mice were euthanised and listerial numbers were determined by spread plating homogenised samples onto BHI (for liver and spleen) and blood agar (for Peyer's patches and small intestine wall and contents) with and without added Em (5 μg/ml).

Nuceotide sequence accession number

The nucleotide sequence data reported in this chapter have been submitted to GenBank and assigned accession number AF211851.

RESULTS

Generation and screening of an *L. monocytogenes* LO28::pORI19 insertion bank

A genomic bank of *L. monocytogenes* LO28 was initially created in *E. coli* EC101 using the vector plasmid pORI19 as described in the Materials and Methods. Analysis of the bank of 25,000 clones indicated that over 90% contained inserts with an average insert size of 1.5 kb (range 500bp to 2.5 kb). This number of clones is estimated to give more than 10x coverage of the entire LO28 genome (using a value of 3 Mb for the genome (von Both *et al.*, 1999)). A plasmid bank was isolated from the EC101 clone set and electrotransformed into strain LO28G (a derivative of LO28 containing the helper plasmid pVE6007). A temperature up-shift from 30°C to the non-permissive 42°C, 180 min post electroporation, resulted in transformation efficiencies of approximately 10^3 CFU/μg of plasmid DNA. Random transformants were screened and proved to be Em resistant and Cm sensitive, indicating that the pORI19 clones had inserted in the chromosome at the point of homology.

The parental strain LO28 can grow on DM (defined medium), but not on DMS (DM containing 3% salt). However, the addition of osmolytes to create DMSC (DMS and 1 mM carnitine) or DMSB (DMS containing 1 mM betaine) permits the growth of LO28. Screening approximately 2000 colonies by replica plating led to the isolation of two isolates that grew on DM and DMSB, but were incapable of growth on DMS or DMSC. Thus, these two isolates have lost the parental ability to use carnitine to stimulate growth at high salt concentrations.

Restriction analysis of the pORI19 clones from both isolates, following plasmid rescue from the chromosome, revealed that both contained the same 1.1 kb insert; one such plasmid was chosen and designated pCPL5. Re-integration of pCPL5 into an LO28 wild type background generated the same mutant phenotype, thus confirming the role of the inserted fragment in the observed phenotype. A representative mutant, designated LO28C, was chosen for further characterisation. In addition, pCPL5 was used to create the corresponding mutant in *L. monocytogenes* ScottA, designated ScottAC. The stability of plasmid insertion in both mutants was confirmed by PCR analysis of cultures grown in the absence of Em at 30°C. No plasmid excision was observed, even after repeated subculture in the absence of antibiotic selection, thus confirming the stability of the mutant constructs (data not shown).

Genotypic analysis of the cloned insert on pCPL5

Sequence analysis of the 1.1 kb insert revealed two open reading frames oriented in the same direction and separated by three nucleotides, not including the TAA stop codon. The location of a putative ribosomal binding site (GAAG) for the second coding region (with no obvious upstream promoter binding domains), 13 nt upstream from the stop codon of the previous gene is consistent with the tight genetic organisation of an operon.

Homology searches revealed significant similarity both at the nucleotide (99% identity) and protein level to the recently identified OpuC multi-component osmolyte uptake system (comprising *opuCA-opuCB-opuCC-opuCD)* in *L. monocytogenes* EGD reported in the database (Fraser *et al*., 2000). Further analysis of the 1.1 kb insert and surrounding chromosomal DNA confirmed that pORI19 had inserted into the *opuCB* gene in LO28. A combination of sequencing and PCR analysis confirmed that the gene organisation reported for EGD is conserved in both LO28 and ScottA.

Physiological analysis of the listerial OpuC$^-$ mutants

Inactivation of the *Listeria opuC* operon following pCPL5 insertion dramatically reduced the osmoprotective effects of carnitine, but not glycine betaine, on the growth of *Listeria* (both LO28 and ScottA) in defined media of elevated osmolarity (Fig. 1). Radiolabelled uptake studies revealed a dramatic reduction in the observed rates of carnitine uptake for ScottAC as expected, both in the presence and absence of salt stress, relative to the wild type parent strain (Fig. 2). However, only very low levels of carnitine uptake were detected for the LO28 parent strain (~10 fold lower than for ScottA) under identical conditions.

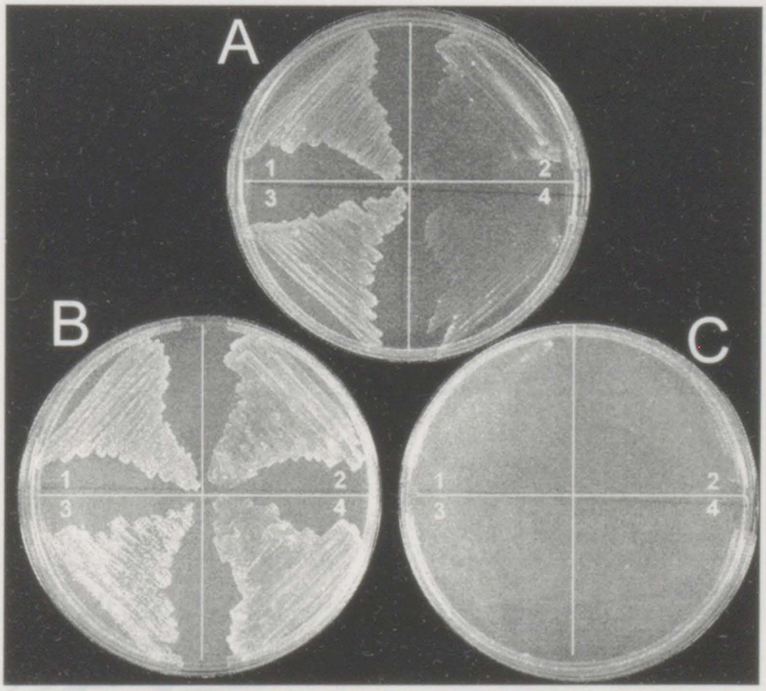

Fig 1. Growth of the OpuC⁻ mutants, LO28C and ScottAC relative to the parental wild type strains on defined media (DM) of elevated osmolarity. Plate **(A)** consists of DM containing 3% added NaCl plus 1 mM carnitine (DMSC), **(B)** consists of DMS plus 1 mM glycine betaine (DMSB) and **(C)** consists of DMS with no added osmolytes. For each plate 1: ScottA wild type, 2: ScottAC, 3: LO28 wild type, 4: LO28C. Clones were grown overnight in DM before being streaked onto the appropriate test plate. The photograph represents growth after 24 h.

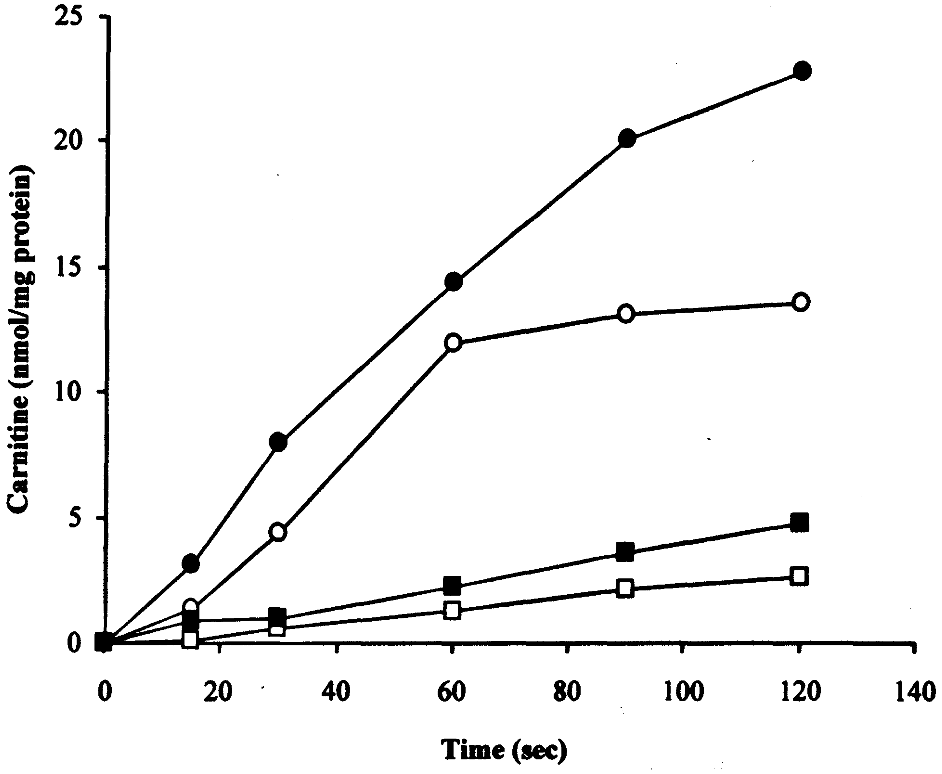

Fig. 2. L-carnitine transport. ScottA (○, ●) and ScottAC (□, ■) were assayed for L-[*N*-methyl-^{14}C]carnitine uptake, both in the presence (closed symbols) and absence (open symbols) of 3% NaCl.

While this low level uptake did not permit a proper assessment of the effects of the insertion event, a simple plate assay (Fig. 1) was used as confirmation of the phenotypic consequence for LO28C.

Since the OpuC homologue in *B. subtilis* is known to function in betaine uptake (Kappes *et al.*, 1996; Lin and Hansen, 1995), both LO28C and ScottAC were analysed for their ability to transport glycine betaine. In this instance, betaine uptake was observed in both strains. While LO28C exhibited reduced glycine betaine uptake relative to the parent strain, both at reduced and elevated osmolarities (Fig. 3A), the ScottAC mutant appeared only affected in its ability to transport glycine betaine at high salt concentrations (Fig. 3B). These findings not only stress the importance of OpuC in contributing to osmolyte uptake, but also serve to highlight significant strain variation in relation to osmolyte utilisation in *Listeria*, a phenomenon previously observed by Dykes and Moorehead (2000).

Virulence studies

Given the original premise that carnitine may prove an important osmolyte for *Listeria* during infection, strains were subjected to mouse virulence assays. Mice were inoculated intraperitoneally with either the mutant or wild type strains and the number of bacteria in the livers and spleens was determined three days post infection. The LO28C mutant strain reached significantly ($P<0.05$) lower levels than the wild type in the livers and spleens of infected animals. Numbers of the mutant in infected spleens were more than 3-fold lower than the wild type whilst numbers in the liver were over 20-fold lower than the parent strain (Fig. 4). These results indicate an important role for OpuC in *Listeria* virulence. However, in contrast, mutating OpuC in *L. monocytogenes* ScottA had no significant effect on virulence following intraperitoneal infection, again possibly reflecting strain variation.

Since the lumen of the gastrointestinal tract (previously suggested to function as the human resevoir of the organism, (Marco *et al.*, 1997)) has an osmolarity approximately equal to 0.3 M NaCl (Chowdhury *et al.*, 1996), the ability of the osmolyte uptake mutants LO28C and ScottAC to colonise the upper

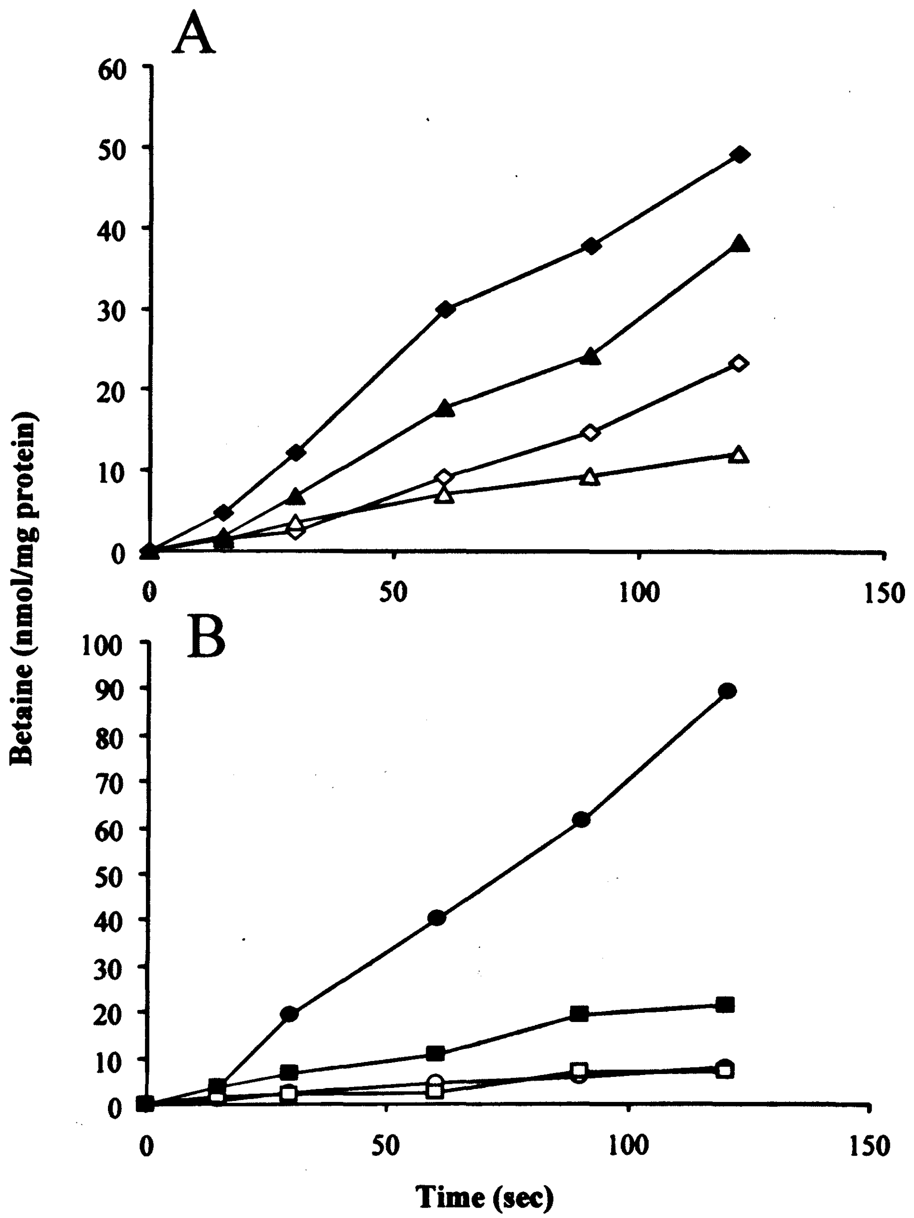

Fig. 3. Glycine betaine transport. **(A)** LO28C (Δ,▲) and **(B)** ScottAC (□,■) were assayed for *N,N,N*-trimethylglycine [1-^{14}C] uptake either in the presence (closed symbols) or absence (open symbols) of 3% NaCl. The parental wild type strains; LO28 and ScottA are represented by (◊,♦) and (○,●) respectively.

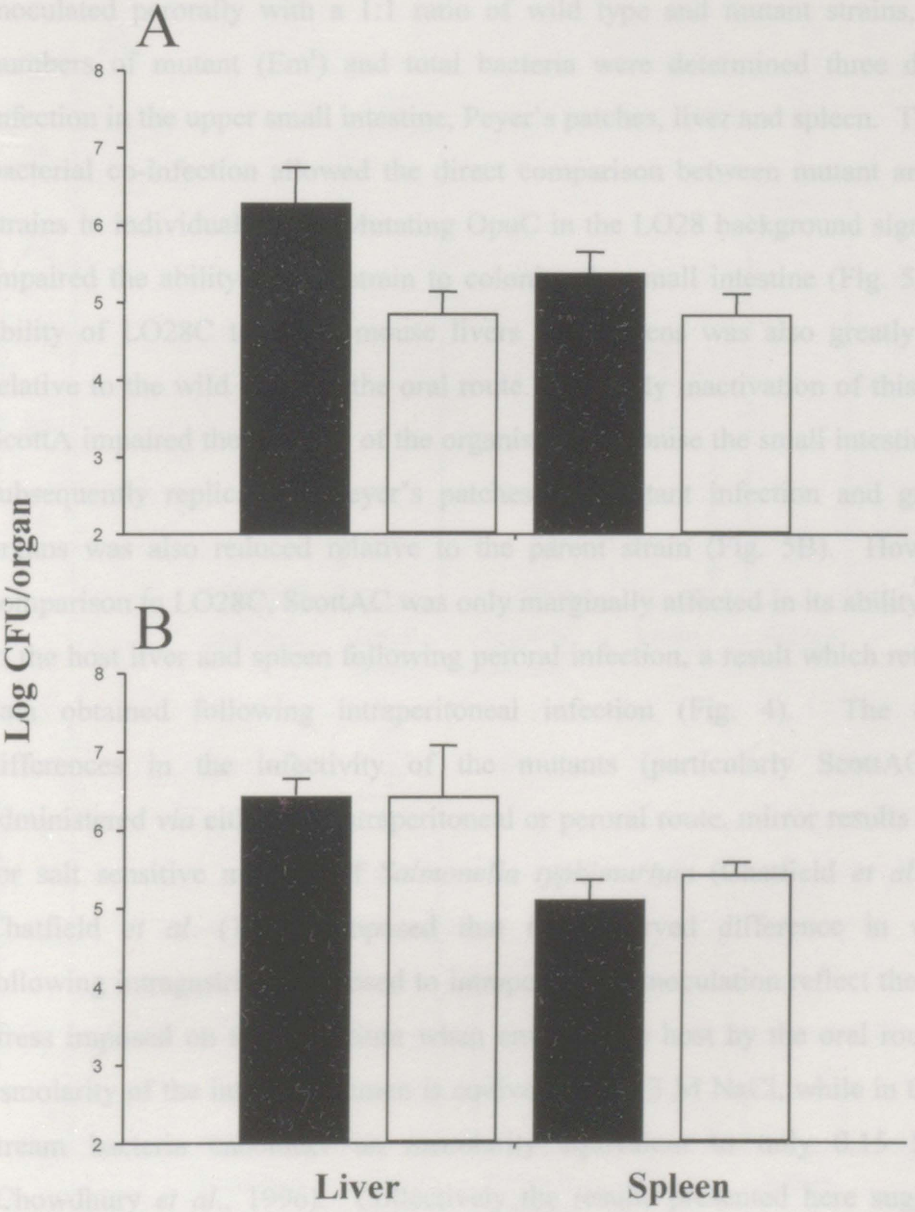

Fig. 4. Effect of mutating OpuC on the survival of **(A)** LO28C and **(B)** ScottAC relative to the parent wild type strains, following intraperitoneal inoculation. Levels of *Listeria* in the livers and spleens of infected mice three days post infection are shown (n = 4). Symbols: ■, wild type; □, mutant strains.

small intestine and cause systemic infection was examined. Mice were co-inoculated perorally with a 1:1 ratio of wild type and mutant strains, and the numbers of mutant (Em^r) and total bacteria were determined three days post infection in the upper small intestine, Peyer's patches, liver and spleen. The use of bacterial co-infection allowed the direct comparison between mutant and parent strains in individual mice. Mutating OpuC in the LO28 background significantly impaired the ability of this strain to colonise the small intestine (Fig. 5A). The ability of LO28C to infect mouse livers and spleens was also greatly reduced relative to the wild type *via* the oral route. Similarly inactivation of this locus in ScottA impaired the capacity of the organism to colonise the small intestine and to subsequently replicate in Peyer's patches. Resultant infection and growth in organs was also reduced relative to the parent strain (Fig. 5B). However, in comparison to LO28C, ScottAC was only marginally affected in its ability to grow in the host liver and spleen following peroral infection, a result which reflects the data obtained following intraperitoneal infection (Fig. 4). The observed differences in the infectivity of the mutants (particularly ScottAC) when administered *via* either the intraperitoneal or peroral route, mirror results obtained for salt sensitive mutants of *Salmonella typhimurium* (Chatfield *et al.*, 1991). Chatfield *et al.* (1991) proposed that the observed difference in virulence following intragastric as opposed to intraperitoneal inoculation reflect the osmotic stress imposed on the bacterium when entering the host by the oral route. The osmolarity of the intestinal lumen is equivalent to 0.3 M NaCl, while in the blood stream bacteria encounter an osmolarity equivalent to only 0.15 M NaCl (Chowdhury *et al.*, 1996). Collectively the results presented here suggest that OpuC is essential for efficient colonisation of the small intestine and resulting systemic infection by *L. monocytogenes*.

DISCUSSION

Molecular characterisation of the salt tolerance of *L. monocytogenes* has been the focus of much attention in recent times (Ko and Smith, 1999; Sleator *et*

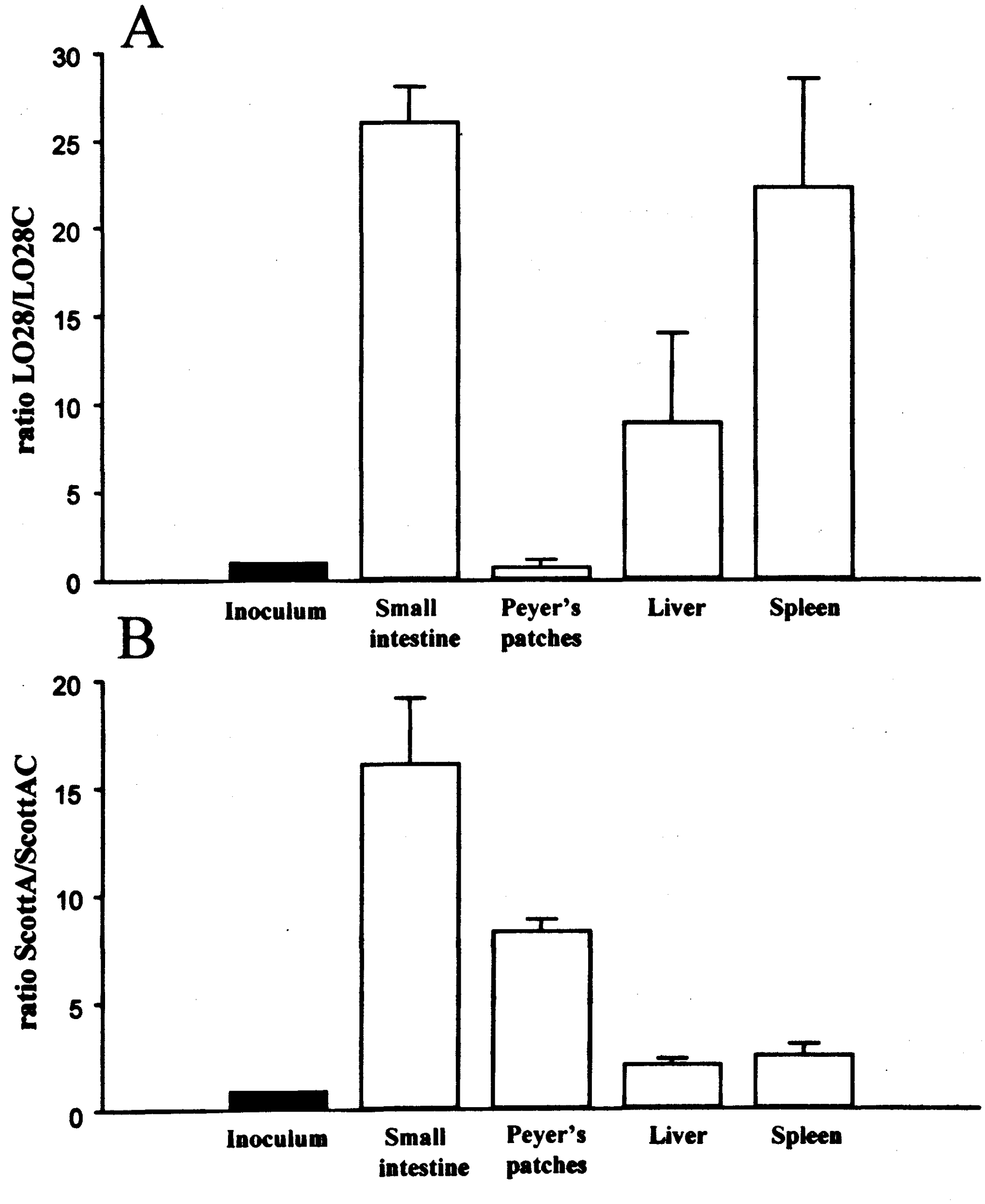

Fig. 5. (A) Survival of LO28 relative to LO28C and (B) ScottA relative to ScottAC following peroral co-inoculation of BALB/c mice. The ratio of the strains was determined both for the inoculum (■) and the relevant tissues and organs (□) three days post infection (*n* = 4).

al., 1999a; 1999b; Smith *et al.*, 1998). Combined with previous physiological investigations, genetic analysis has provided new insights into the mechanisms of listerial osmotolerance. Glycine betaine for example, previously assumed to be accumulated only by a single uptake system (Patchett *et al.*, 1994) is now known to be transported by at least three independent systems (Ko and Smith, 1999; Sleator *et al.*, 1999a). In addition, contrary to previous reports (Ko *et al.*, 1994), Phan-Thanh and Mahouin (1999) have recently provided evidence supporting the existence of a glycine betaine synthesis system in *L. monocytogenes.*

Heterologous complementation and transposon mutagenesis, techniques previously used for the successful isolation of genes encoding bacterial osmolyte transport systems (Ko and Smith, 1999; Sleator *et al.*, 1999a; 1999b) proved ineffectual in the search for the genetic elements governing carnitine uptake in *Listeria.* The osmolyte uptake mutants LO28C and ScottAC, and partial sequence of the disrupted listerial *opuC* operon, were eventually obtained using a modification of a system devised for lactococci by Law *et al.* (1995). This technique represents a novel strategy for generating listerial mutants. Unlike heterologous complementation, which requires functional cloning of the entire gene or operon (an important limitation when dealing with large multi-gene systems), DNA fragments as small as 200 bp can give rise to homologous recombination and successful chromosomal integration of pORI19. The system also lacks many of the shortcomings associated with transposon mutagenesis. Unlike transposons, which can possess 'hot spots' on the chromosome (Berg *et al.*, 1983; Lodge *et al.*, 1988), pORI19 target specificity is limited only by the completeness of the plasmid bank. Also the lack of transposable elements on the RepA$^-$ plasmid reduces the possibility of reversion, which can exist with transposon mutagenesis (Adler and Hofemeister, 1990; Marron *et al.*, 1997).

Originally identified as a chimeric *proU* operon conferring enhanced osmoprotection as a consequence of glycine betaine transport in *B. subtilis* LH45 (Lin and Hansen, 1995), the *opuC* operon also encodes the only osmotically significant carnitine transporter in this organism (Kappes and Bremer, 1998). Sequence analysis downstream of a recently constructed Tn*1545* adhesion mutant (Milohanic *et al.*, 2000) identified the *opuC* operon in *L. monocytogenes* EGD. In the present study, functional inactivation of this homologue in two distinct strains of *Listeria* namely LO28 and ScottA, resulted in mutants exhibiting reduced glycine betaine uptake, and an inability to use carnitine as an effective osmoprotectant. Uptake studies using radiolabelled substrate revealed significant variation in the observed rates of glycine betaine and carnitine transport, not only between the mutants, but also between the parental wild type strains. Not restricted to *Listeria* (Dykes and Moorhead, 2000), this phenomenon of strain variation in relation to osmolyte transport systems has previously been described in *Bacillus*. Disrupting the *opuC* operon in *B. subtilis* LH45 significantly reduces osmoprotection by glycine betaine (Lin and Hansen, 1995), whereas a similar mutation in *B. subtilis* JH642 has only a minor effect on glycine betaine uptake (Kappes *et al.*, 1996; 1999).

The low levels of carnitine uptake observed for LO28 wild type may reflect the absence of a dedicated carnitine transport system in this strain. The isolated *opuC* operon thus may encode a 'leaky' system, which although primarily dedicated to the uptake of glycine betaine, transports the structurally related trimethyl amino acid carnitine at a level, which, while too low to be detected under the conditions used in these assays, is nonetheless physiologically significant in terms of salt tolerance. Alternatively the effect of mutating OpuC on glycine betaine uptake may be indirect, and the low levels of carnitine uptake for LO28 may merely reflect strain specific differences in gene expression. While uptake studies revealed a possible role for OpuC in the transport of glycine betaine for both strains tested, disrupting the operon had no significant effect on glycine betaine mediated osmoprotection. This result was not altogether unexpected given that glycine betaine is known to be transported by at least two other high efficiency uptake systems (Ko and Smith, 1999; Sleator *et al.*, 1999a). Given that a number of nucleotide changes (one of which resulted in an amino acid substitution) were observed between the 1.1 kb insert of pCPL5 and the *opuC* sequence of EGD, it is tempting to speculate that the observed strain variation in the activity of OpuC is the consequence of strain specific point mutations within the operon.

Since carnitine is most likely the predominant osmolyte in animal tissues (Bieber, 1988), the ability of LO28C and ScottAC to survive and replicate in mouse tissues was investigated. For many foodborne 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 (Chowdhury *et al.*, 1996). 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 (Chowdhury *et al.*, 1996), the concentration at which maximum carnitine uptake occurs in *Listeria* (Smith, 1996). Given that glycine betaine occurs predominantly in plant tissues, growth and survival of bacteria during animal infection most likely relies on the presence of alternative osmolytes for maintenance of cell turgor. Having determined that *L. monocytogenes* mutants in OpuC survive poorly in the upper small intestine, it is proposed that carnitine may represent a key osmoprotectant facilitating growth in this otherwise limiting environment. The constant breakdown of the gastrointestinal epithelial layer (desquamation) may provide the source of carnitine for uptake by bacteria in this *milieu* of elevated osmolarity.

For OpuC$^-$ mutants in both LO28 and ScottA backgrounds the reduced ability to colonise the small intestine, is mirrored by lower bacterial levels in internal organs. This is especially evident for the OpuC$^-$ mutant in LO28 which demonstrates ~20 fold lower levels in infected spleens relative to the parent. Interestingly, LO28C but not ScottAC exhibits reduced virulence when administered by the intraperitoneal route. This suggests that the ScottA strain either possesses a carnitine transporter other than OpuC (evidenced by the NaCl inducible carnitine uptake observed against the OpuC$^-$ background of ScottAC (Fig. 2)) or relies on mechanisms other than carnitine uptake to maintain turgor pressure, during infection of internal organs. In contrast the role of OpuC in LO28 is of key importance for efficient survival and growth *in vivo*. Barbour *et al.* (1996) have previously shown significant variation in virulence of *L. monocytogenes* strains. The data presented in this chapter suggests that *L.*

monocytogenes strains may differ in their reliance on specific systems for maintaining homeostasis *in vivo*.

ACKNOWLEDGEMENTS

I would like to thank Jeroen Wouters and Tjakko Abee who contributed the radiolabelled osmolyte uptake studies, and also Cormac Gahan for assistance with the virulence work.

Chapter V

Identification and disruption of the *proBA* locus in *Listeria monocytogenes*: role of proline biosynthesis in salt tolerance and murine infection

Roy D. Sleator, Cormac G. M. Gahan and Colin Hill

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ABSTRACT

Intracellular accumulation of the amino acid proline has previously been linked to the salt tolerance and virulence potential of a number of bacteria. However, the contribution, if any, of proline synthesis from glutamate to both the salt tolerance and virulence potential of *Listeria monocytogenes* has, until now, remained largely undetermined. Complementation of the *proBA*$^-$ mutant *Escherichia coli* CSH26 led to the identification of the listerial *proBA* operon, which codes for enzymes functionally similar to the glutamyl kinase (GK) and glutamyl phosphate reductase (GPR) enzyme complex. These enzymes catalyse the first and second steps of proline biosynthesis in *E. coli*. The listerial *proBA* operon is flanked by stem loop structures, which probably function as *rho*-independent transcription termination signals, and is preceded by a presumptive σ^A-dependent promoter. The first gene of the operon, *proB*, is predicted to encode GK, a 276-residue protein with a calculated molecular mass of 30.03 kDa and pI of 5.2. Distal to the promoter and overlapping the 3' end of *proB* by 17 bp is *proA*, which encodes GPR, a 415-residue protein with a calculated molecular mass of 45.50 kDa (pI 5.3). Allelic exchange mutagenesis was used to create a chromosomal deletion mutant, which is auxotrophic for proline. This mutant was used to assess the contribution of proline anabolism to osmotolerance and virulence. While inactivation of *proBA* had no significant effect on virulence in mouse assays (either perorally or intraperitoneally), growth at high salt concentrations (>6% NaCl) was significantly reduced in the absence of efficient proline synthesis. Thus, it is proposed that while proline biosynthesis plays little, if any, role in the intracellular lifecycle and infectious nature of *L. monocytogenes*, it can play an important role in survival in osmolyte-depleted environments of elevated osmolarity.

INTRODUCTION

Survival of the foodborne pathogen *Listeria monocytogenes* in hyper-saline environments is attributed mainly to the accumulation of organic compounds termed osmolytes (Yancey *et al.*, 1982). Osmolytes, often referred to as compatible solutes (Brown, 1976) owing to their compatibility with cellular metabolism at high internal concentrations, can be either transported into the cell or synthesised *de novo*, and act by counterbalancing the external osmotic strength, thus preventing water loss and plasmolysis (Csonka, 1989; Csonka and Hanson, 1991).

Beumer *et al.* (1994) identified three principal compatible solutes in *Listeria*: proline, betaine and carnitine. While much information is available regarding uptake of these osmolytes from the external environment (Beumer *et al.*, 1994; Patchett *et al.*, 1994; Verheul *et al.*, 1997), a detailed analysis of osmolyte synthesis systems in *Listeria* has not been undertaken. Unlike the recently identified transport systems BetL (Sleator *et al.*, 1999a; 2000), OpuC (Fraser *et al.*, 2000; Chapter IV, this thesis) and GbuABC (Gerhardt *et al.*, 2000; Ko and Smith, 1999), osmolyte synthesis is not restricted by the availability of external osmolytes, a factor which may represent an important limitation for growth in hostile environments of elevated osmolarity such as the macrophage phagosome. Optimal growth of *L. monocytogenes* in low a_w environments thus may depend on osmolyte synthesis in combination with uptake.

Perhaps the best-characterised bacterial osmolyte synthesis system is that of proline (Baumberg and Klingel, 1993; Hayzer and Leisinger, 1981; Leisinger, 1996). For the majority of bacteria, proline is synthesised from glutamate *via* a four-step reaction catalysed by γ-glutamyl kinase (GK; *proB* product, EC 2.7.2.11), γ-glutamyl phosphate reductase (GPR; *proA* product, EC 1.2.1.41) and Δ^1-pyrroline-5-carboxylate (P5C) reductase (*proC* product, EC 1.5.1.2). The remaining step, third in the sequence, occurs spontaneously (Csonka and Baich, 1983). In other genera the *proB* and *proA* genes generally constitute an operon, which is distant from the *proC* gene on the chromosome. In addition to this pathway, a number of bacteria have been shown to synthesise proline *via* offshoots of the arginine biosynthetic pathway (Baumberg and Klingel, 1993).

As well as its role as an osmoprotectant, recent evidence suggests that proline biosynthesis may function as a virulence factor for certain pathogenic bacteria (Bayer *et al.*, 1999; Culham *et al.*, 1998; Schwan *et al.*, 1998). Marquis *et al.* (1993), using an uncharacterised listerial proline auxotroph obtained following transposon mutagenesis, concluded that while proline auxotrophy had no effect on virulence following intravenous inoculation, the possibility of reduced virulence during the intestinal phase of natural infection could not be ruled out. This chapter describes the isolation, characterisation and disruption of the listerial *proBA* operon, and investigates the role of this genetic element in contributing to the growth and survival of *L. monocytogenes* in environments of elevated osmolarity, and during subsequent infection (both intraperitoneal and peroral) of a murine model.

MATERIALS AND METHODS

Media, chemicals, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37°C either in Luria-Bertani (LB) medium (Maniatis *et al.*, 1982) or M9 minimal medium (GIBCO/BRL, Eggenstein, Federal Republic of Germany [FRG]) containing appropriate additional requirements. *L. monocytogenes* strains were grown either in Brain Heart Infusion (BHI) broth (Oxoid, Unipath Ltd. Basingstoke, United Kingdom) or in chemically defined minimal medium (DM; Premaratne *et al.*, 1991). Blood agar plates consisted of blood agar base (Lab M) to which 5% sheep blood was added following autoclaving. All experiments involving the selection of proline-prototrophic (Pro^+) derivatives of proline auxotrophic (Pro^-) strains were carried out using proline deficient minimal media, supplemented with 0.2 mM arginine to eliminate spontaneous Pro^+ phenotypic revertants carrying suppressor mutations, which may allow the arginine biosynthetic pathway to function in proline biosynthesis (Berg

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or Characteristic(s)[a]	Source or reference
Strains		
L. monocytogenes		
LO28	Serotype 1/2c	P. Cossart, Institut Pasteur
LO28(rif)	Rif^r *L. monocytogenes* LO28	This study
PSOE	*ΔproBA*, *L. monocytogenes* LO28, Pro$^-$	This study
PSOEC	PSOE(pCPL9), Pro$^+$	This study
E. coli		
DH5α	*supE44* Δ*lac* U169(ϕ80*lacZ*ΔM15)R17 *recA1 endA1 gyrA96 thi-1 relA1*	Gibco-BRL, 1995
CSH26	*ara*, Δ(*lac proBA*), *thi*	L. Csonka
MKH13	MC4100Δ(*putPA*)101Δ(*proP*)2Δ(*proU*)	E. Bremer
RC711	*proA23*, *lac-28*, *tsx-81*, *trp-30*, *his-51*	M. Berlyn
J5-3	*proB22*, *metF63*	M. Berlyn
JM240	*proC47*, *glnV42*(AS), λ$^-$, *cys-54*	M. Berlyn
DPWC	Carries Tn*1000* on the F factor	A. Coffey
BW26	Km^r, F$^-$ Tn*1000* recipient strain	A. Coffey
CSH26(A)	CSH26(pCPL10::Tn*1000*)A, Pro$^-$	This study
CSH26(B)	CSH26(pCPL10::Tn*1000*)B, Pro$^-$	This study
CSH26(C)	CSH26(pCPL10::Tn*1000*)C, Pro$^-$	This study
CSH26(D)	CSH26(pCPL10::Tn*1000*)D, Pro$^-$	This study
CSH26(E)	CSH26(pCPL10::Tn*1000*)E, Pro$^-$	This study
Plasmids		
pUC18	Ap^r ColE1 *ori*	Vieira and Messing, 1982
pCI372	Cm^r, 5.7 kb *E. coli*/*L. lactis* shuttle vector	Hayes, 1990
pKSV7	Cm^r, temperature sensitive	Smith and Youngman, 1992
pMOB	Ap^r, miniplasmid used for transposon targeted DNA sequence analysis	A. Coffey
pCPL7	pUC18 containing ~8.5 kb of the listerial chromosome	This study
pCPL8	pUC18 containing the 5.5 kb *Eco*RI fragment of the pCPL7 insert	This study
pCPL9	pCI372 containing the 5.5 kb *Eco*RI fragment of the pCPL7 insert	This study
pCPL10	pMOB containing the amplified PCR product of 5EF2*Eco*RI and 5ER2*Eco*RI	This study
pCPL11	pKSV7 containing DNA from *proBA*	This study

[a] Ap^r Ampicillin resistance, Cm^r chloramphenicol resistance, Km^r kanamycin resistance, Rif^r rifampicin resistance

and Rossi, 1974). Where necessary, proline (Sigma Chemical Co., St. Louis, Mo.) and 4-Nitropyridine 1-oxide (MERK-Schuchardt, Hohenbrunn, Germany) were added to the growth medium at the appropriate concentration, as filter-sterilised solutions. Radiolabelled L-[2,3,4,5-^{3}H]proline (100 Ci/mmol) was purchased from American Radiolabelled Chemicals Inc. (St. Louis, Mo.). Ampicillin (Ap), carbenicillin (Cb), chloramphenicol (Cm), kanamycin (Km) and rifampicin (Rif) were made up as described by Maniatis *et al.* (1982) as concentrated stocks and added to media at the required levels. Where indicated, media osmolarity was adjusted by the addition of NaCl.

DNA manipulations and sequence analysis

Restriction enzymes, RNase, Shrimp alkaline phosphatase and T4 DNA ligase were obtained from Boehringer GmbH (Mannheim, FRG) and were used according to the manufacturer's instructions. Genomic DNA was isolated from *L. monocytogenes* as described by Hoffman and Winston (1987). Plasmid DNA was isolated using the Qiagen QIAprep spin miniprep kit (Qiagen, Hilden, FRG). *E. coli* was transformed by standard methods (Maniatis *et al.*, 1982), while electrotransformation of *L. monocytogenes* was achieved by the protocol outlined by Park and Stewart (1990). Restriction fragments were isolated with the Qiaex II gel extraction kit (Qiagen). Polymerase chain reaction (PCR) reagents (*Taq* polymerase and deoxynucleoside triphosphates dNTPs) were purchased from Boehringer and used according to the manufacturer's instructions with a Hybaid (Middlesex, United Kingdom) PCR express system. Unless otherwise stated, PCR was carried out following lysis of cells with Igepal CA-630 (Sigma). PCR products were purified using the QIAquick PCR purification kit. Oligonucleotide primers (listed in Table 2) used for PCR and sequence purposes were synthesised on a Beckman Oligo 1000M DNA synthesiser (Beckman Instruments, Inc., Fullerton, Calif.). Nucleotide sequence determination was performed on an ABI 373A automated sequencer with the Dye Terminator sequence kit (Applied Biosystems, Warrington, United Kingdom). Nucleotide and protein sequence analysis were done using Lasergene (DNASTAR Ltd., London, United Kingdom). Protein secondary structure analysis was determined by using the PredictProtein program (EMBL Heidelberg, Germany) (Rost *et al.*, 1994). Homology searches were performed with the BLAST program (Altschul *et al.*, 1990).

Table 2. PCR primers used in this study

Primer	Sequence (5'-3')
SOEA (*proBA*)	TTTTAGTGAATTCTTGGCCAAA[*]
SOEB (*proBA*)	GAAAGGCATCTGCTACATCCCGGTAGGTCGCACTGGAAGTTG[†]
SOEC (*proBA*)	CCGGGATGTAGCAGATGCCTTTC
SOED (*proBA*)	GTTAATCTAGACTGCCGCAG[*]
SOEX (*proBA*)	CAGTCATCTCAGCTGCGAG
5EF2*Eco*RI	GCTTAAGGAGGGTTGATATGAATTCCAT[*]
5ER2*Eco*RI	CAGTGAAGGGAAAATGCAAGAAGAATTCA[*]
G186	ATATAAACAACGAATTATCTCC
G187	GTATTATAATCAATAAGTTATACC

[*] Nucleotides introduced to create restriction sites are underlined
[†] Overhang complementary to SOEC is underlined

Isolation of *proBA* from *L. monocytogenes*

A DNA library consisting of genomic DNA from *L. monocytogenes* LO28 partially digested with *Sau*3A and ligated to plasmid pUC18 DNA, digested with *Bam*HI and dephosphorylated with shrimp alkaline phosphatase, was constructed as described previously (Sleator *et al.*, 1999a). Plasmids were isolated and transformed into the proline synthesis mutant *E. coli* CSH26; transformants were then plated onto minimal medium containing no added proline to select for proline prototrophs. Plasmids isolated from complementing clones were tested for re-complementation of the proline auxotrophy, and analysed by agarose gel electrophoresis. Restriction deletion analysis (using enzymes whose recognition sites constitute the multiple cloning site of plasmid pUC18 (Vieira and Messing, 1982)), followed by re-complementation experiments, was used to isolate those plasmids with the smallest complementing insert.

Tn*1000* mutagenesis

Tn*1000* mutagenesis was carried out essentially as described by Strathmann *et al.* (1991), using *E. coli* DPWC as the Tn*1000* containing host strain and *E. coli* BW26 as the F^- recipient. The cloned insert on the smallest complementing plasmid (pCPL8) was amplified by PCR using primers 5EF2*Eco*RI and 5ER2*Eco*RI, digested with *Eco*RI and ligated to similarly digested pMOB. The resulting construct, designated pCPL10, was isolated by functional complementation of *E. coli* CSH26, selected on proline deficient minimal media. Plasmid pCPL10 was then transformed into *E. coli* DPWC, which carries Tn*1000* on the F factor. Following transformation, mobilisation of the transposon into pCPL10 occurred in *E. coli* DPWC, the transposition transiently fusing the F factor and pCPL10 in a co-integrated structure subsequently transferred to *E. coli* BW26 by bacterial mating. Following conjugation, resolution of the co-integrate in *E. coli* BW26 resulted in a single copy of Tn*1000* placed randomly within pCPL10. Since *E. coli* BW26 is Km resistant and pCPL10 codes for Cb resistance (pMOB carries the β-lactamase gene for Ap/Cb resistance), plating onto media with both antibiotics selected for *E. coli* BW26 cells harbouring pCPL10 mutated randomly with Tn*1000*. These cells were pooled and grown for 2 h in LB medium containing Cb 50 μg/ml. Plasmid DNA was extracted and used to transform *E. coli* CSH26, selected on LB medium containing 10 mM proline and 50 μg/ml ampicillin. Clones, in which the complementing insert was functionally inactivated, were isolated by replica plating, based on their lack of growth on proline deficient minimal media. Since the presence of the transposon places known sequencing primer sites adjacent to unknown, unsequenced regions of the target DNA, isolation of a set of clones in which Tn*1000* is situated 100-500 nucleotides [nt] apart, allowed the operon sequence to be assembled from overlapping DNA sequences generated using the Tn*1000* specific primers G186 and G187 in combination with the Pharmacia (Upsala, Sweden) universal and reverse primers.

Transport assays

Radiolabelled proline uptake was measured essentially as described by Culham *et al.* (1998).

Generation of an *L. monocytogenes proBA*$^-$ mutant

The splicing by overlap extension (SOEing) PCR procedure described by Horton *et al.* (1990) was used to create PSOE, a mutant with an internal 1394 bp deletion in *proBA*. SOE PCR primers were designed to amplify two ~300 bp DNA fragments, one comprising the 5' end of *proBA* (nucleotides [nt] 76 to 384, amplified by primers SOEA (*proBA*) and SOEB (*proBA*) [Table 2]) and the other comprising the 3' end of the operon (nt 1779 to 2082, amplified by primers SOEC (*proBA*) and SOED (*proBA*) [Table 2]). The resulting products were gel extracted, mixed in a 1:1 ratio and re-amplified using the SOEA (*proBA*) and SOED (*proBA*) primers. The amplified 613 bp product was digested with *Eco*RI and *Xba*I and cloned into the temperature sensitive shuttle vector pKSV7 (Smith and Youngman, 1992) before being transformed into *E. coli* DH5α. The resultant plasmid designated pCPL11 was electroporated into *L. monocytogenes* LO28 and transformants were selected on BHI agar plates containing 10 µg/ml Cm. Forced chromosomal integration of pCPL11 at 42°C, followed by sequential passaging in BHI at 30°C in the absence of Cm, facilitated allelic exchange between the intact *proBA* operon and the 613 bp insert on pCPL11. The successful mutation event was confirmed by PCR using the SOEX (*proBA*) and SOED (*proBA*) primers (Table 2).

Virulence assays

Bacterial virulence was determined by intraperitoneal and peroral inoculation of 8 to 12-week old BALB/c mice. Intraperitoneal inoculations were carried out as described previously (Sleator *et al.*, 2000), using overnight cultures of mutant and wild type *Listeria* (6×10^5 cells), suspended in 0.2 ml of phosphate buffered saline. For peroral inoculations, mutant and wild type strains suspended in buffered saline with gelatin, were mixed at a 1:1 ratio of LO28(Rif^r):PSOE. Mice were infected with approximately 1×10^9 cells (total) using a micropipette tip placed immediately behind the incisors. Three days post infection mice were euthanised and listerial numbers were determined by spread plating homogenised samples onto BHI (for liver and spleen) and blood agar (for Peyer's patches and small intestine wall and contents) with and without added rifampicin (50 μg/ml).

Nucleotide sequence accession number

The nucleotide sequence data reported in this chapter have been submitted to GenBank and assigned accession number AF282880.

RESULTS

Complementation of *E. coli* CSH26

The *proBA* mutant *E. coli* CSH26 is unable to synthesise proline, rendering it incapable of growth in proline deficient minimal medium. A pUC18::LO28 genome library (see Materials and Methods) was transformed into CSH26, and transformants were selected on minimal medium containing no added proline. While no transformants were obtained with pUC18 alone, transformation efficiencies of approximately 50 colony forming units (CFU)/μg of DNA were achieved from the plasmid bank, colonies appearing after 24 h at 37°C. Plasmids isolated from five random transformants were re-transformed into CSH26 to confirm complementation. Following analysis by gel electrophoresis, all five clones were shown to contain the same ~8.5 kb insert. A representative plasmid designated pCPL7 was chosen for further characterisation.

Restriction analysis of pCPL7 revealed that the cloned insert contained a single *Eco*RI cut site, with a second site located in the vector multiple cloning site. This was utilised to reduce the insert to a ~5.5 kb region which was still capable of complementing the lesion in CSH26. When a representative plasmid containing the 5.5-kb insert, designated pCPL8, was subjected to further restriction analysis, no smaller DNA fragment capable of complementation could be isolated.

Functional expression of a listerial proline synthesis system is the basis for complementation of *E. coli* CSH26

To confirm that complementation of *E. coli* CSH26 with pCPL8 was the result of proline biosynthesis; a number of growth experiments were performed. Both *E. coli* CSH26(pCPL8) and *E. coli* CSH26(pUC18) were inoculated into minimal medium with or without 10 mM proline. While growth of CSH26(pCPL8) was observed both in the presence and absence of proline, the control strain CSH26(pUC18) only grew in the media containing added proline (Fig. 1A). To further characterise the insert on pCPL8, the plasmid was introduced into *E. coli* strains (RC711 Δ*proA*, J5-3 Δ*proB*, JM240 Δ*proC*, MKH13 Δ*putPA*, Δ*proP*, Δ*proU*) with well-characterised mutations in various proline biosynthetic and uptake genes. For those strains with mutations in proline biosynthetic genes, the results indicated that the plasmid contained sufficient genetic information to restore the proline prototrophy in the Δ*proA* and Δ*proB*, but not Δ*proC* mutants. The plasmid was unable to complement the proline uptake deficiency in MKH13, and as expected, no measurable L-[2,3,4,5-^{3}H]proline uptake was observed for MKH13 containing pCPL8 (data not shown).

Sequence analysis of the complementing insert

Tn*1000* mutagenesis (Liu *et al.*, 1987; Strathmann *et al.*, 1991) facilitated rapid localisation and sequence determination of the complementing genes on pCPL10. Following transposon insertion, replica plating based on functional inactivation of the Pro$^+$ phenotype led to the isolation of approximately 50 Pro$^-$ mutants. In each Pro$^-$ mutant tested, the site of the Tn*1000* insertion mapped to a ~2 kb portion of the insert. Based on this analysis, 2707 bp of DNA sequence was generated by bi-directional sequencing from a set of five clones (Table 1) in which Tn*1000* insertions were positioned at approximately 300 bp intervals (Fig. 2).

Analysis of the sequenced region (the G+C content of 37.2% is characteristic of the genus *Listeria* (Farber and Peterkin, 1991)) revealed the presence of two complete open reading frames (ORFs), oriented in the same

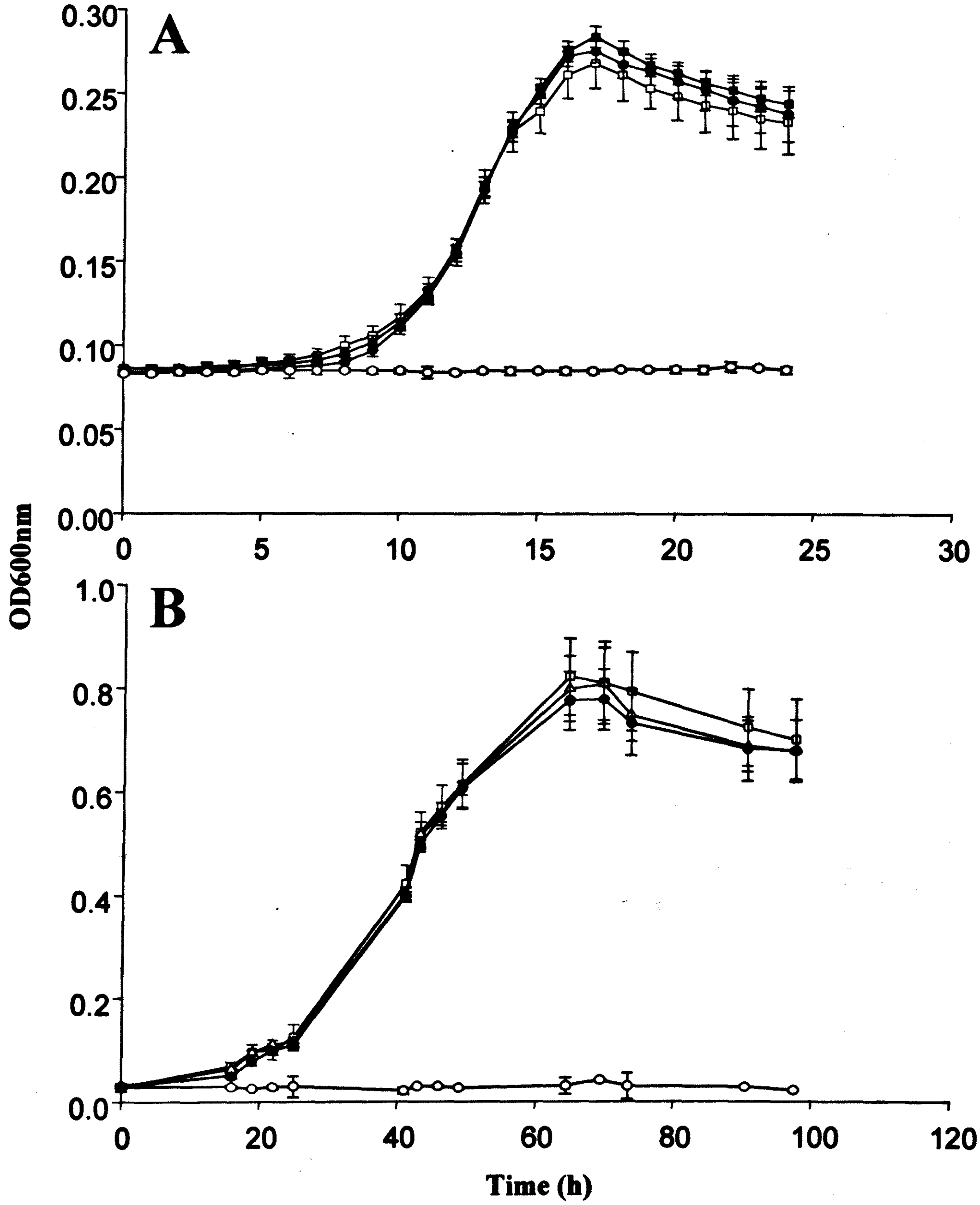

Fig. 1. **(A)** Growth of *E. coli* CSH26(pUC18) (○) and CSH26(pCPL8) (□) in M9 minimal media (as determined by turbidity using a spectra max 340 spectrophotometer, Molecular Devices), both in the presence (closed symbols) and absence (open symbols) of 10 mM proline. **(B)** Growth of *L. monocytogenes* LO28 (Δ), PSOE (○,●) and PSOEC (□), in the presence (closed symbols) and absence (open symbols) of 10 mM proline.

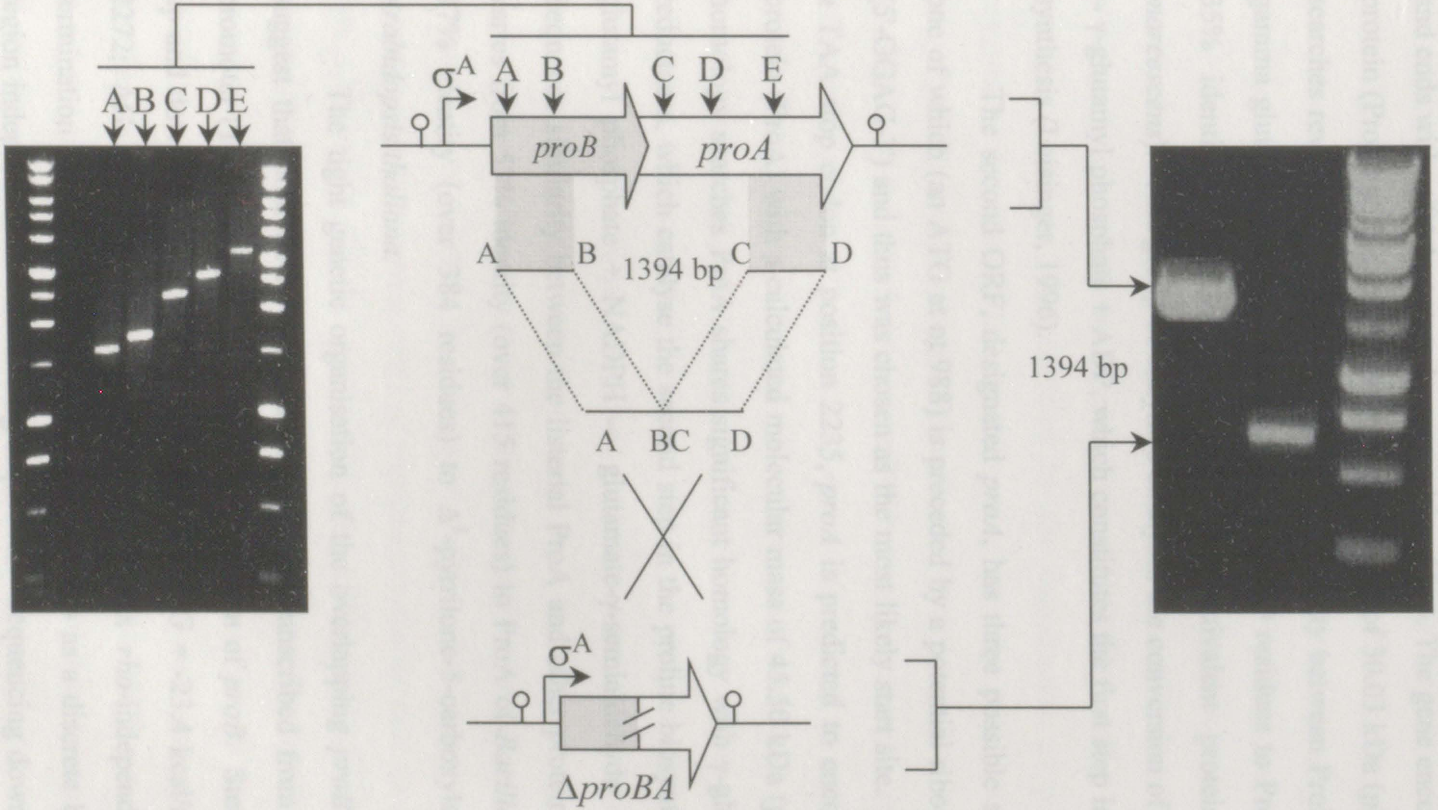

Fig. 2. Random Tn*1000* insertion within the pCPL10 plasmid of *E. coli* CSH26 clones A to E. The oligonucleotide combination used for PCR was the transposon specific primer G186 and the M13 forward primer. Creation of the *proBA* deletion mutant, PSOE, is also illustrated. Part of the coding region of *proBA* was eliminated using the splicing by overlap extension (SOEing) procedure (see Materials and Methods) and confirmed by PCR.

direction and overlapping by 17 nucleotides (Fig. 2). The first ORF, which was designated *proB* based on sequence homologies, starts at an ATG codon at nt 174, 10 nucleotides downstream of a potential ribosomal binding site (5'-GAGG-3'), and ends with a TAA stop codon at position 1004. The gene encodes a 276-residue protein (ProB) with a calculated molecular mass of 30.03 kDa (pI 5.2). Homology searches revealed a significant degree of similarity between ProB and a family of gamma glutamyl kinases (38% identity over 259 residues to ProB of *E. coli* and 35% identity over 259 residues to the equivalent protein from *Serratia marcescens*). This group of enzymes catalyses the conversion of glutamate + ATP → γ-glutamyl phosphate + ADP, which constitutes the first step in bacterial proline synthesis (Leisinger, 1996).

The second ORF, designated *proA*, has three possible start codons, only one of which (an ATG at nt 988) is preceded by a potential ribosome binding site (5'-GGAG-3') and thus was chosen as the most likely start site. Terminating with a TAA stop codon at position 2235, *proA* is predicted to encode a 415-residue protein (ProA) with a calculated molecular mass of 45.50 kDa (pI 5.3). Based on homology searches ProA shares significant homology with γ-glutamyl phosphate reductases, which catalyse the second step in the proline biosynthetic pathway (γ-glutamyl phosphate + NADPH → glutamate-γ-semialdehyde + $NADH^+$ + P_i). Sequence similarity between the listerial ProA and other proteins in the database varies from 53% identity (over 415 residues) to ProA of *Bacillus halodurans*, to 37% identity (over 384 residues) to Δ^1-pyrrilone-5-carboxylate synthetase of *Arabidopsis thaliana*.

The tight genetic organisation of the overlapping *proB* and *proA* genes suggest that both ORFs constitute an operon transcribed from a single σ^A like promoter (TAGACA [16 nt] TAAAAT) upstream of *proB*. Stem loop structures up and down stream of the operon (nt 39-79; ΔG = -23.4 kcal/mol and nt 2234-2272; ΔG = -17.2 kcal/mol) may function as *rho*-independent transcription termination signals, suggesting that *proBA* exists as a discrete bicistronic-coding region independent of surrounding sequences. Sequencing downstream of *proBA* revealed the 3' end of an incomplete ORF (*orf-3**), which would encode a protein with 31% identity (over 77 residues) to *ydfD*, a member of the GntR transcription regulator family of *Bacillus subtilis* (Kunst *et al.*, 1997).

Creation of an *L. monocytogenes proBA*⁻ mutant

In order to properly evaluate the role of *proBA* in contributing to the growth and survival of *L. monocytogenes*, allelic exchange mutagenesis was used to create a 1394 bp deletion in the *proBA* operon, designed to inactivate both genes (Fig. 2). The resulting mutant designated PSOE, exhibited complete proline auxotrophy, requiring upwards of 10 mM proline to restore growth to a level comparable to that of the parent strain in DM (Fig. 1B). As expected, this mutation could be complemented by the introduction of pCPL9, a plasmid constructed by cloning the *proBA* operon into the lactococcal vector pCI372 (Hayes, 1990), which is capable of replication in *Listeria* (Fig. 1B). In the absence of added salt, the growth rate of the PSOE mutant in complex media such as BHI was unaffected (Fig. 3), presumably due to high levels of both free proline and proline containing peptides in this environment (Amezaga *et al.*, 1995). A peculiar feature of *proBA*⁻ mutants of *E. coli* is their increased resistance to the compound 4-Nitropyridine 1-oxide. However this phenotype (the biochemical basis of which is as yet unknown) appears not to extend to the corresponding mutant in *Listeria* (data not shown).

Since proline is known to function as an effective osmolyte in *Listeria* (Beumer *et al.*, 1994) the effects of deleting *proBA* on the growth of *L. monocytogenes* in environments of elevated osmolarity were investigated (BHI; 0-10% added NaCl, Fig. 3). An unusual, but highly reproducible, phenomenon was observed in this experiment whereby growth rates differ significantly at low salt concentrations between 2 and 4% salt (with a lower growth rate observed for the PSOE mutant), converge in the range of 5-6% salt, and once again diverge significantly at higher salt concentrations (with the PSOE mutant again growing more slowly than the parent). It is proposed that these unusual data reflect the dual roles of proline in bacterial systems; on the one hand acting as an essential amino acid and on the other, as an important osmolyte. The reasoning is as

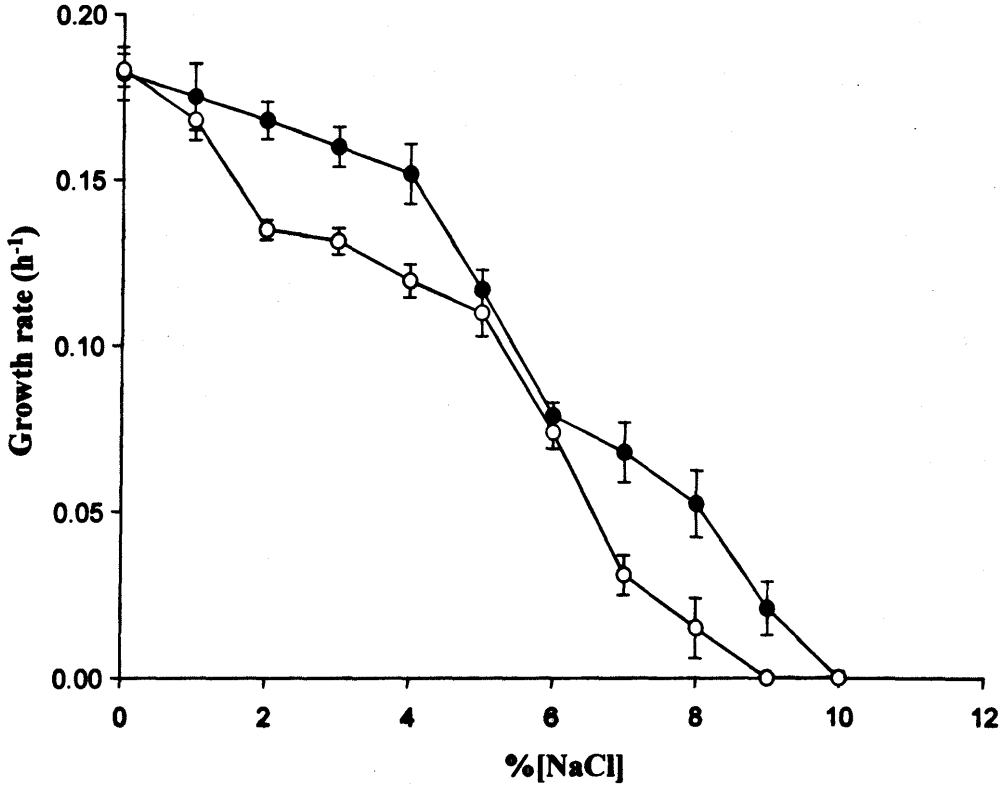

Fig. 3. Growth rates of LO28 (•) and PSOE (○) as a function of NaCl added to the medium (BHI). Overnight cultures of *L. monocytogenes* LO28 and PSOE (Δ*proBA*) were cultured in the appropriate medium and the specific growth rates (μ) were determined during exponential growth. Each point represents the mean value of three independent experiments.

follows: BHI contains approximately 2 mM glycine betaine (Smith, 1996) and glycine betaine uptake is known to suppress the accumulation of proline in other bacteria (Molenaar *et al.*, 1993; Pourkomailian and Booth, 1994). Given that it has previously been demonstrated that glycine betaine uptake is maximal in *Listeria* between 2-4% salt (Ko *et al.*, 1994), it is likely that the uptake of proline is maximally inhibited at these concentrations. In support of this proposal, it was demonstrated that the addition of glycine betaine dramatically affects the growth of PSOE in minimal media containing proline, confirming that proline uptake is inhibited in the presence of 1 mM glycine betaine (Fig. 4). Thus, it appears that insufficient proline is the principal reason for the slower growth rates in BHI at 2-4% salt observed in Fig. 3. In BHI containing between 4 and 6% salt, glycine betaine uptake is no longer operating at maximal efficiency and therefore permits the accumulation of sufficient proline from the medium to meet the cells' nutritional needs and thus allow the growth rates of parent and mutant to converge (Fig. 3). At the higher salt concentrations above 6% mutant growth rates again drop significantly relative to the wild type; at these concentrations most osmolyte transport systems are either saturated or no longer functional due to structural changes in the membrane (Patchett *et al.*, 1994). Thus, in the absence of effective osmolyte transport, ProBA appears to play a critical role in the growth of *L. monocytogenes* at elevated osmolarities by providing sufficient proline to act in its other role as an osmolyte.

Virulence studies

The effects of deleting *proBA* on the virulence of *L. monocytogenes* were analysed both by intraperitoneal and peroral inoculation of BALB/c mice. Consistent with the findings of Marquis *et al.* (1993), the proline auxotroph PSOE showed no obvious reduction in virulence when administered *via* the peritoneal route. Mutant and wild type strains were recovered at approximately equal levels from both livers and spleens of infected animals; three days post inoculation (Table 3). Given that the oral route of infection may represent a more osmotically stressful environment than the peritoneal cavity (the osmolarity of the

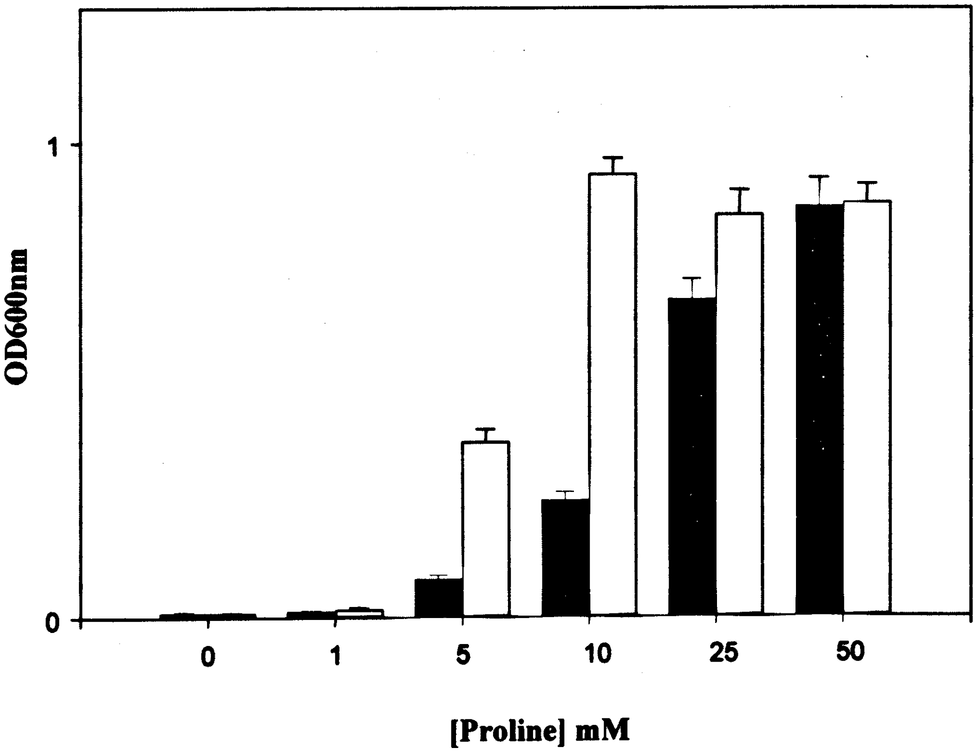

Fig. 4. Analysis of the effects of proline and glycine betaine on the growth of the *Listeria* proline auxotroph, PSOE. *L. monocytogenes* PSOE grown overnight in BHI, was washed twice in sterile Ringers before being inoculated (at 2%) into DM at various proline concentrations both in the presence (■) and absence (□) of 1 mM glycine betaine. The OD_{600} (after 60 h growth at 37°C) for each sample was taken in triplicate.

gastrointestinal tract is equivalent to 0.3 M NaCl, as opposed to 0.15 M NaCl for the blood stream (Chowdhury *et al.*, 1996)), the role of *proBA* in contributing to the intestinal phase of natural infection was analysed. Similar to the results obtained for intraperitoneal inoculation, mutating ProBA failed to inhibit colonisation of the upper small intestine or to disrupt the subsequent invasion and spread to internal organs (Table 3). Thus, it can be deduced that neither proline nor proline containing peptides are limiting during murine infection (Marquis *et al.*, 1993), nor is proline biosynthesis likely to function as a source of compatible solutes.

Table 3. Recovery of *L. monocytogenes* LO28 and the *proBA*$^-$ mutant PSOE, from the tissues of infected mice three days post intraperitoneal and peroral infection

Type of inoculation and organ/tissue	**Log$_{10}$ bacterial numbers per organ/tissue (± SD)^a**	
	LO28	**PSOE**
Intraperitoneal		
Liver	5.2 (0.4)	5.3 (0.2)
Spleen	5.5 (0.4)	5.5 (0.2)
Peroral		
Liver	3.9 (0.6)	4.1 (0.3)
Spleen	2.9 (0.3)	3.0 (0.3)
Small intestine wall and contents	3.7 (0.5)	3.8 (0.2)
Peyer's patches	2.8 (0.2)	2.8 (0.2)

^{a} Values are averages of four inoculated animals

DISCUSSION

The ubiquitous nature of the foodborne pathogen *L. monocytogenes* means that the organism is frequently exposed to a variety of environmental insults, both in foods prior to ingestion (Abee and Wouters, 1999) and subsequently within the infected host (where it is exposed, among other stresses, to the osmotic challenge of the gastrointestinal tract (Chowdhury *et al.*, 1996) and macrophage phagosomes (Gahan and Hill, 1999)). Survival of *Listeria* both at high salt concentrations and low temperature environments is attributed mainly to the accumulation of compatible solutes (Brown, 1976). Beumer *et al.* (1994) identified the three principal compatible solutes in *Listeria* as proline, betaine and carnitine. The isolation of genes encoding BetL (Sleator *et al.*, 1999a) and OpuC (Fraser *et al.*, 2000; Chapter IV, this thesis): osmolyte transport systems dedicated to the uptake of glycine betaine and carnitine, has previouly been reported. However, to date, no equivalent system has been described for the accumulation of proline in *Listeria.*

This chapter describes the identification and disruption of the listerial *proBA* operon encoding homologues of the glutamyl kinase (GK) and glutamyl phosphate reductase (GPR) complex of the *E. coli* proline biosynthetic pathway. The pathway from glutamate *via* glutamate-γ-semialdehyde (GSA) and its spontaneous cyclisation product Δ^1-pyrroline-5-carboxylate (P5C) to proline, was first proposed in 1952 (Vogel and Davis, 1952), and has since been described in other prokaryotes, both Gram-positive and Gram-negative (Baumberg and Klingel, 1993; Leisinger, 1996). However it wasn't until 1955 that the effectiveness of proline as a compatible solute was realised by Christian (1955a) who observed that accumulation of the osmolyte could relieve bacterial growth inhibition by osmotic stress.

Sequence analysis revealed that the physical organisation of the listerial *proBA* homologue is similar to that of *E. coli* (Deutch *et al.*, 1984), in which *proB* (coding for GK) and *proA* (coding for GPR) constitute an operon with a single σ^A-consensus promoter proximal to *proB*. While exhibiting a high degree of sequence similarity and functional compatibility, the two systems differ in a number of respects. Firstly, while the *E. coli* genes are separated by a 14 nt intergenic region, the listerial *proB* and *proA* genes overlap by 17 nt. This, together with the reduced size of the listerial *proB* gene (273 bp shorter than the equivalent gene in *E. coli*), leads to the formation of a tighter genetic domain, a feature that may reflect a degree of evolutionary divergence between the two systems. This is particularly relevant given that Hu *et al.* (1992) proposed that the evolutionary origins of the bi-functional plant enzyme Δ^1-pyrrilone-5-carboxylate synthetase might be linked to a genetic fusion of *proB* and *proA*.

The predicted secondary structure composition of ProBA (as determined with the PHD Email server and *e*motif search programmes) is a mixed class of *a*-

helix, β-sheet and loop structures. Conserved sequences at amino acid residue (aa) 103-110 and aa 321-342, of ProA correspond to the [AG]-X4-G-K [ST] sequence fingerprint of an ATP/GTP-binding site and γ-glutamyl phosphate signature sequence respectively (Rost *et al.*, 1994). As with *A. thaliana* (Savouré *et al.*, 1995) a $\beta\alpha\beta$ secondary structure near to the carboxy terminal domain of ProA (~aa 210-290), may function as a non-covalent NAD(P)H-binding domain. The existence of both ATP and putative NAD(P)H-binding sites on ProA alone, supports the formation of a GK /GPR enzyme complex (as previously described in *E. coli* (Leisinger, 1996)) for the catalysis of the ATP and NAD(P)H dependent first and second steps, respectively, of proline biosynthesis. Since prokaryotic proline synthesis is known to be regulated by proline-mediated inhibition of GK activity, multiple sequence alignments, in combination with *e*motif searches, were used to identify possible allosteric binding domains and adjacent or overlapping enzyme active sites. Two conserved regions of ProB (identified by *e*motif searches) extended from aa 77-109 and 120-144 respectively. These domains map closely to ProB mutations in *E. coli* (aa 107 Asp to Asn) (Csonka *et al.*, 1988) and *S. marcesens* (aa 117 Ala to Val) (Omari *et al.*, 1992), which result in proline overproduction due to reduced feedback repression, and thus may represent the allosteric binding domain of the enzyme.

The phenotypic consequences of eliminating the ProBA complex provided a unique opportunity to study proline transport in the absence of endogenous proline synthesis. Perhaps the most detailed knowledge of proline transport in Gram-positive bacteria involves the halotolerant foodborne pathogen *Staphylococcus aureus*. Proline uptake in *S. aureus* is mediated by two transport systems; a specific high affinity system (corresponding to PutP in *E. coli* (Wood, 1988)) and an osmotically inducible low affinity system, which is also dedicated to the uptake of glycine betaine (Bae and Miller, 1992; Pourkomailian and Booth, 1992; Townsend and Wilkinson, 1992) and thus resembles ProP and ProU of *E. coli* (Csonka, 1989). The relatively high concentration of proline (10 mM) required to complement PSOE suggests that *Listeria* may lack the scavenging capacity of a high affinity proline transporter, a hypothesis previously suggested by the findings of Patchett *et al.* (1992). The relatively high proline concentrations

(>10 mM), demonstrated by Beumer *et al.* (1994) to be osmotically significant, may be attributed to the presence of a low affinity uptake system, the activity of which appears to be inhibited by glycine betaine. Proline uptake in *Listeria* thus may resemble the situation in *Lactococcus lactis* in which proline appears to be transported by a single low affinity system which also transports glycine betaine (Molenaar *et al.*, 1993). Alternatively, the system may be specific for proline, but inhibited by pre-accumulated glycine betaine, as was described previously for proline transport in *S. aureus* (Pourkomailian and Booth, 1994). Since regulation of osmolyte uptake by feedback inhibition due to pre-accumulated solute has previously been described for both glycine betaine and carnitine uptake in *Listeria* (Verheul *et al.*, 1997), it is possible that this process may also extend to the accumulation of proline.

While the role of proline as an effective compatible solute is well documented (Csonka, 1989; Csonka and Hanson, 1991), recent evidence suggests that accumulation of the osmolyte may also be linked to the virulence potential of certain pathogenic bacteria (Bayer *et al.*, 1999; Culham *et al.*, 1998; Schwan *et al.*, 1998). Mutating the osmotically sensitive proline transporter ProP resulted in a 100-fold reduction in the colonisation of the murine urinary tract by uropathogenic *E. coli* (Culham *et al.*, 1998), while *putP*$^-$ mutants of *S. aureus* have been shown to demonstrate reduced virulence both in wound and murine abscess infection models, as well as in experimental endocarditis (a prototypical model of invasive *S. aureus* infection) (Bayer *et al.*, 1999; Schwan *et al.*, 1998). In the present study mouse virulence assays were conducted to investigate the effects of mutating ProBA (*i.e.* proline auxotrophy) on the colonisation of the murine gastrointestinal tract and subsequent growth within the intracellular *milieu* of the internal organs (liver and spleen). Consistent with previous observations by other workers (Marquis *et al.*, 1993) it was observed from intraperitoneal inoculations that proline auxotrophy has no significant effect on the growth of *Listeria* within the seemingly nutrient rich environment of the macrophage cytoplasm (Marquis *et al.*, 1993). The elevated osmolarity (0.3 M NaCl (Chowdhury *et al.*, 1996)) and otherwise limiting environment of the gastrointestinal tract also failed to inhibit growth and survival of PSOE, notwithstanding suggestions that proline biosynthesis may be important during the intestinal phase of natural infection (Marquis *et al.*, 1993). From these results it can be concluded that reduced proline synthesis has no significant effect on *Listeria* pathogenesis. Given the observed role of ProP and PutP in the virulence of uropathogenic *E. coli* and *S. aureus*, respectively, a detailed analysis of proline transport may be required to fully appreciate the importance (if any) of proline in contributing to the virulence potential of *L. monocytogenes*.

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Chapter VI

Mutations in the listerial *proB* gene leading to proline overproduction: effects on salt tolerance and murine infection.

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ABSTRACT

The observed sensitivity of *Listeria monocytogenes* to the toxic proline analogue L-azetidine-2-carboxylic acid (AZ), suggested that proline synthesis in *Listeria* may be regulated by feedback inhibition of γ-glutamyl kinase (GK); the first enzyme of the proline biosynthesis pathway, encoded by the *proB* gene. Taking advantage of the *Epicurian coli* mutator strain XL1-Red, random mutagenesis of the recently described *proBA* operon was performed, generating three independent mutations in the listerial *proB* homologue, leading to proline overproduction and salt tolerance when expressed in an *E. coli* (Δ*proBA*) background. While each of the mutations (located within a conserved 26 amino acid region of GK) was shown to confer AZ resistance (AZ^r) on an *L. monocytogenes proBA*$^-$ mutant, listerial transformants failed to exhibit the salt tolerant phenotype observed in *E. coli*. Since proline accumulation has previously been linked to the virulence potential of a number of pathogenic bacteria, the effect of proline overproduction on *Listeria* pathogenesis was analysed. However, the results suggest that, as previously described for proline auxotrophy, proline hyper-production has no apparent impact on the virulence potential of *Listeria*.

INTRODUCTION

Genetic and physiological analysis of proline accumulation in both prokaryotic and eukaryotic systems (Csonka and Hanson, 1991; Kavi Kishor *et al.*, 1995) has provided evidence that is consistent with diverse functions of proline; not only as a source of energy, carbon and nitrogen, but also as an effective osmolyte (Baumberg and Klingel, 1993; Csonka, 1989; Csonka and Hanson, 1991; Leisinger, 1996) and more recently as a potential virulence factor for a number of pathogenic bacteria (Bayer *et al.*, 1999; Culham *et al.*, 1998; Schwan *et al.*, 1998).

While proline can be synthesised from ornithine in both plants and animals (Hu *et al.*, 1992), glutamate is the primary precursor for proline biosynthesis in bacteria (Leisinger, 1996) and in osmotically stressed plant cells (Delauney *et al.*, 1993). Bacterial proline synthesis from glutamate occurs *via* three enzymatic reactions, catalysed by γ-glutamyl kinase (GK; *proB* product, EC 2.7.2.11), γ-glutamyl phosphate reductase (GPR; *proA* product, EC 1.2.1.41) and Δ^1-pyrroline-5-carboxylate (P5C) reductase (*proC* product, EC 1.5.1.2). For the majority of bacteria the *proB* and *proA* genes constitute an operon, which is distant from *proC* on the chromosome. In plants, e.g. *Vigna aconitifolia* and Arabidopsis, the first two steps of proline biosynthesis from glutamate are catalysed by Δ^1-pyrroline-5-carboxylate synthetase (P5CS), a bi-functional enzyme with both γ-glutamyl kinase and γ-glutamyl phosphate reductase activities at the N- and C-terminal domains respectively (Hu *et al.*, 1992).

For both prokaryotic and eukaryotic systems, proline synthesis from glutamate is regulated by feedback inhibition of the first enzyme in the pathway. Studies on purified enzymes suggest that in addition to proline mediated inhibition; the γ-glutamyl kinase activities of GK and P5CS are also modulated to a lesser extent by glutamate and ADP, thereby tuning proline synthesis to cellular substrate and energy availability (Smith *et al.*, 1984; Zhang *et al.*, 1995). Proline hyper-producing strains of bacteria, exhibiting reduced proline mediated feedback inhibition of γ-glutamyl kinase activity, (a result of single base pair substitutions in either the bacterial *proB* gene (Dandekar and Uratsu, 1988; Kosuge and Hoshino, 1998; Massarelli *et al.*, 2000; Omari *et al.*, 1992; Rushlow *et al.*, 1984) or the 5' domain of the plant P5CS coding region (Zhang *et al.*, 1995)), have been isolated based on their resistance to toxic proline analogues (AZT; L-Azetidine-2-carboxylic acid (Grant *et al.*, 1975) and DHP; 3, 4-dehydro-DL-proline (Sugiura and Kisumi, 1985); compounds which inhibit γ-glutamyl kinase activity, while not interfering with protein synthesis (Leisinger, 1996)).

In addition to the obvious advantages for commercial amino acid synthesis (Omari *et al.*, 1992), the osmoprotective properties of proline overproduction (Jakowec *et al.*, 1985) have led to the development of transgenic drought resistant plants (Hong *et al.*, 2000). However, since proline may function as a potential virulence factor (Bayer *et al.*, 1999; Culham *et al.*, 1998; Schwan *et al.*, 1998) and is known to facilitate the growth of certain pathogenic bacteria at elevated osmolarities (Csonka, 1981), the use of transmissible genetic elements encoding proline hyper-production may lead to undesirable consequences, if introduced prematurely into the natural environment.

The isolation and characterisation of the listerial *proBA* operon was described previously (Chapter V, this thesis). The present study describes the isolation of *proB* mutants which overproduce proline, and assesses the contribution of such overproduction to the growth and survival of *Listeria monocytogenes,* both in hyper-saline environments and during infection of an animal (murine) model.

MATERIALS AND METHODS

Media, chemicals, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37°C either in Luria-Bertani (LB) medium (Maniatis *et al*., 1982) or M9 minimal medium (GIBCO/BRL, Eggenstein, Federal Republic of Germany [FRG]) containing appropriate additional requirements. *L. monocytogenes* strains were grown either in Brain Heart Infusion (BHI) broth (Oxoid, Unipath Ltd. Basingstoke, United Kingdom) or in chemically defined minimal medium (DM; Premaratne *et al*., 1991). Blood agar plates consisted of blood agar base (Lab M) to which 5% sheep blood was added following autoclaving. Where necessary, proline and its analogues (AZT; L-Azetidine-2-carboxylic acid and DHP; 3, 4-dehydro-DL-proline) (Sigma Chemical Co., St. Louis, Mo.) were added to the growth medium at the appropriate concentration, as filter-sterilised solutions. Antibiotics when needed were made up as described by Maniatis *et al*. (1982) as concentrated stocks and added to media at the required levels. Where indicated, media osmolarity was adjusted by the addition of NaCl.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or characteristic(s)[a]	Source or reference
Strains		
L. monocytogenes		
LO28	Serotype 1/2c	P. Cossart, Institut Pasteur
PSOE	*L. monocytogenes* LO28 *ΔproBA*, Pro^{-}	Chapter V, this thesis
E. coli		
CSH26	*ara*, *Δ(lac proBA)*, *thi*, Pro^{-}	L. Csonka, Purdue
CSH26C	CSH26(pCPL9), Pro^{+}	This study
XL1-Red	*endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT* Tn*10* (Tet^{r})	Stratagene
Plasmids		
pCI372	Cm^{r}, 5.7 kb *E. coli*/*L. lactis* shuttle vector	Hayes, 1990
pCPL9	pCI372::5.5 kb *Eco*RI insert harbouring the LO28 *proBA* operon	Chapter V, this thesis
pCPL9^{mut}	Randomly mutated pCPL9 from *E. coli* XL1-Red	This study
pCPL12	pCPL9 ProB V121I; AZ^{r}	This study
pCPL13	pCPL9 ProB A144V; AZ^{r}	This study
pCPL14	pCPL9 ProB E146K; AZ^{r}	This study
pCPL15	pCPL9 ProB E146K; ProA I328V; AZ^{r}	This study
pCPL16	pCPL9 ProB V121I; AZ^{r}	This study

[a] AZ^{r} L-Azetidine-2-carboxylic acid resistance, Cm^{r} chloramphenicol resistance, Tet^{r} tetracycline resistance

DNA manipulations and sequence analysis

Routine DNA manipulations were performed as described by Maniatis *et al.* (1982). Plasmid DNA was isolated using the Qiagen QIAprep spin miniprep kit (Qiagen, Hilden, FRG). *E. coli* was transformed by standard methods (Maniatis *et al.*, 1982) while electrotransformation of *L. monocytogenes* was achieved by the protocol outlined by Park and Stewart (1990). Polymerase chain reaction (PCR) reagents (*Taq* polymerase and deoxynucleoside triphosphates dNTPs) were purchased from Boehringer GmbH (Mannheim, Germany) and used according to the manufacturer's instructions with a Hybaid (Middlesex, United Kingdom) PCR express system. Oligonucleotide primers for PCR and sequence purposes were synthesised on a Beckman Oligo 1000M DNA synthesiser

(Beckman Instruments, Inc., Fullerton, Calif.). Nucleotide sequence determination was performed on an ABI 373 automated sequencer using the BigDye™ Terminator sequence kit (Lark Technologies, Inc. Essex, UK). Nucleotide and protein sequence analysis were done using Lasergene (DNASTAR Ltd., London, UK). The nucleotide sequence of the *proBA* operon in *L. monocytogenes* can be accessed from the GenBank database (AF282880).

Generation of proline analogue resistant mutants

The plasmid pCPL9 harbouring the listerial *proBA* operon was transformed into the mutator strain *Epicurian coli*® XL1-Red (Stratagene), and transformants were selected on LB plates containing chloramphenicol (30 μg/ml). Transformants were then pooled and grown overnight at 37°C in LB broth. Randomly mutated plasmid DNA extracted from this culture was then used to transform the proline synthesis mutant *E. coli* CSH26. Mutations leading to proline overproduction were selected by plating transformants on M9 minimal medium containing 5 mM AZ. These transformants were then pooled and grown in M9 containing 4% added NaCl, to select for mutations encoding proline hyper-production leading to osmotolerance. Plasmids isolated from the resultant osmotolerant AZ^r CSH26 clones were then used to transform *L. monocytogenes* PSOE (Δ*proBA*), before screening for proline analogue resistance (AZ^r at 10 mM concentrations) and salt tolerance (growth in DM + 4% added NaCl).

Analysis of proline production

Proline hyper-production was assayed using a modification of the proline bioassay described by Kosuge and Hoshino (1998). The cell free extract from overnight cultures of proline producing strains, in proline deficient minimal media, was spotted (in 5 μl volumes) onto M9 plates without proline, and seeded with the *E. coli* proline auxotroph CSH26 indicator. Proline overproduction and excretion was confirmed by subsequent growth of the indicator cells. Quantitative analysis of the proline in the supernatant of putative proline overproducers was carried out using a Beckman 6300 amino acid analyser (Beckman Instruments Ltd., High Wycombe, UK).

Virulence assays

Bacterial virulence was determined by intraperitoneal and peroral inoculation of 8 to 12-week old BALB/c mice. Intraperitoneal inoculations were carried out as described previously (Sleator *et al.*, 2000), using overnight cultures of mutant and wild type *Listeria* (4×10^5 cells), suspended in 0.2 ml of phosphate buffered saline. For peroral inoculations, mutant and wild type strains suspended in buffered saline with gelatin were mixed at a ratio of 1:1. Mice were infected with approximately 2×10^9 cells (total) using a micropipette tip placed immediately behind the incisors. Three days post infection mice were euthanised and listerial numbers were determined by spread plating homogenised samples onto BHI (for liver and spleen) and blood agar (for Peyer's patches and small intestine wall and contents) with and without added chloramphenicol (10 μg/ml).

RESULTS AND DISCUSSION

Random mutagenesis of the listerial *proBA* operon

The observed AZ mediated growth inhibition of *L. monocytogenes* (Fig. 1) indicated that as with the majority of systems (both prokaryotic and eukaryotic), listerial proline biosynthesis from glutamate may be regulated by proline dependent feedback inhibition of the γ-glutamyl kinase activity. Mutations leading to proline analogue resistance (and consequential proline hyper-production) have been described for a number of organisms, and have in each case been linked to mutations in γ-glutamyl kinase, leading to a decreased sensitivity of the enzyme for its allosteric effector proline and its analogues (Dandekar and Uratsu, 1988; Kosuge and Hoshino, 1998; Massarelli *et al.*, 2000; Omari *et al.*, 1992; Rushlow *et al.*, 1984).

In an effort to generate proline hyper-producing strains of *L. monocytogenes* a random mutagenesis strategy was used to introduce point mutations into the cloned listerial *proBA* operon. Plasmid pCPL9 (harbouring the listerial *proBA* locus) was transformed into the *E. coli* mutator strain XL1-Red.

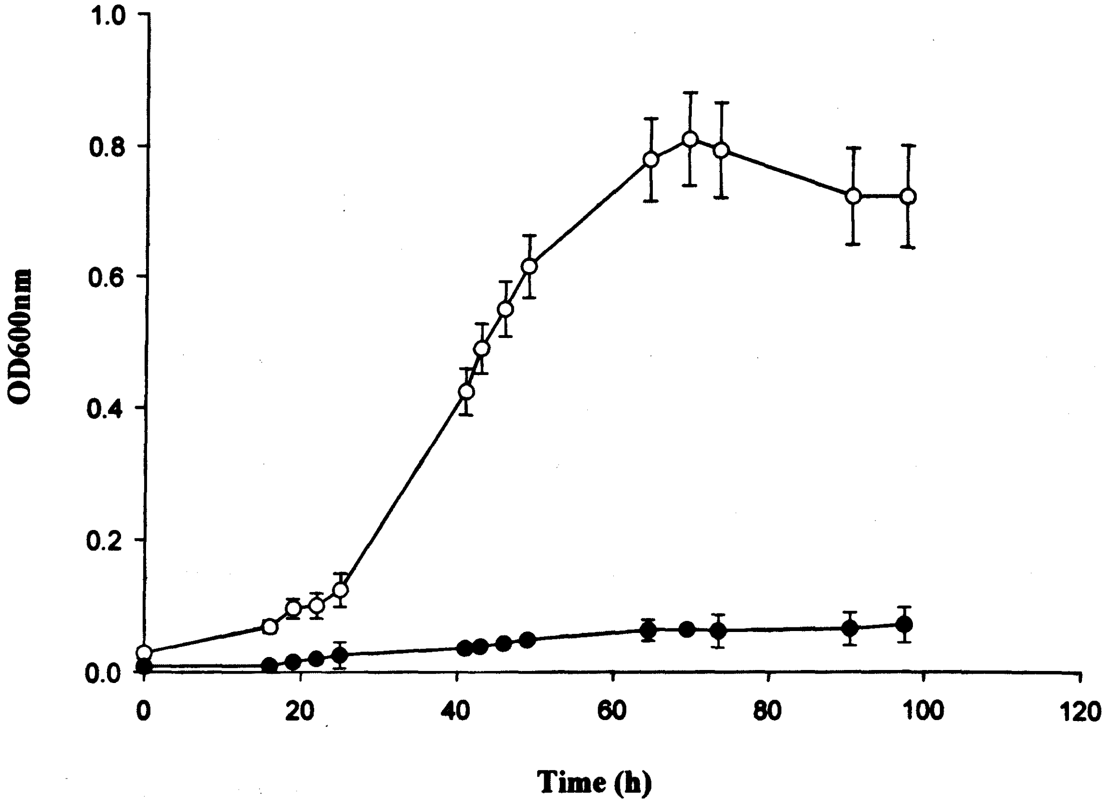

Fig. 1. Growth of *L. monocytogenes* LO28 (●) and PSOE(pCPL12) (○) in DM containing 10 mM L-Azetidine-2-carboxylic acid (AZ). Growth curves of *Listeria* containing plasmids with the other ProB mutations (pCPL13-16) described in the text were identical to that of PSOE (pCPL12), but for clarity are excluded from this graph.

Mutations in three of the primary DNA repair pathways of this strain results in a mutation rate which is ~5000–fold higher than that of the wild type; hence pCPL9 replication within XL1-Red led to the introduction of point mutations throughout the operon. The randomly mutated pCPL9 'bank', designated pCPL9^{mut} was subsequently transformed into the *E. coli* proline auxotroph CSH26, and transformants were selected on minimal medium containing 5 mM AZ. While no colonies were obtained following a control transformation with un-mutated pCPL9, transformation efficiencies of 75 colony forming units (CFU)/µg of DNA were achieved from pCPL9^{mut}; with colonies appearing after 36 h at 37°C. Following overnight growth at elevated osmolarities, five AZ^r transformants were chosen at random for further analysis. Proline production levels of the five analogue resistant strains were tested using the proline bioassay in combination with amino acid analysis (Fig. 2A). Complementation of the proline auxotrophic indicator strain showed that each clone exhibited proline overproduction and excretion as compared to the parent containing pCPL9. Proof that the observed phenotype was the result of mutations in the cloned listerial *proBA* operon, was obtained by re-complementation studies, in which plasmid isolated from each of the complementing clones once again conferred AZ^r, not only on the recipient *E. coli* CSH26 strain, but also on the listerial proline auxotroph PSOE (Fig. 1).

Sequence analysis of the mutated *proBA* genes

Plasmid DNA isolated from the five proline overproducing CSH26 clones (pCPL12-16, Table 1) was in each case, subjected to sequence analysis of the cloned listerial *proBA* operon. Nucleotide sequence comparisons with the wild-type *proBA* genes revealed a small number of base substitutions in the mutated operons (Fig. 3A). Interestingly the base changes, each of which results in an amino acid (aa) substitution within a defined (26 aa) region of the GK enzyme, map closely to previously isolated mutations leading to proline overproduction in other genera (Fig. 3B) (Dandekar and Uratsu, 1988; Kosuge and Hoshino, 1998; Massarelli *et al.*, 2000; Omari *et al.*, 1992; Rushlow *et al.*, 1984; Zhang *et al.*,

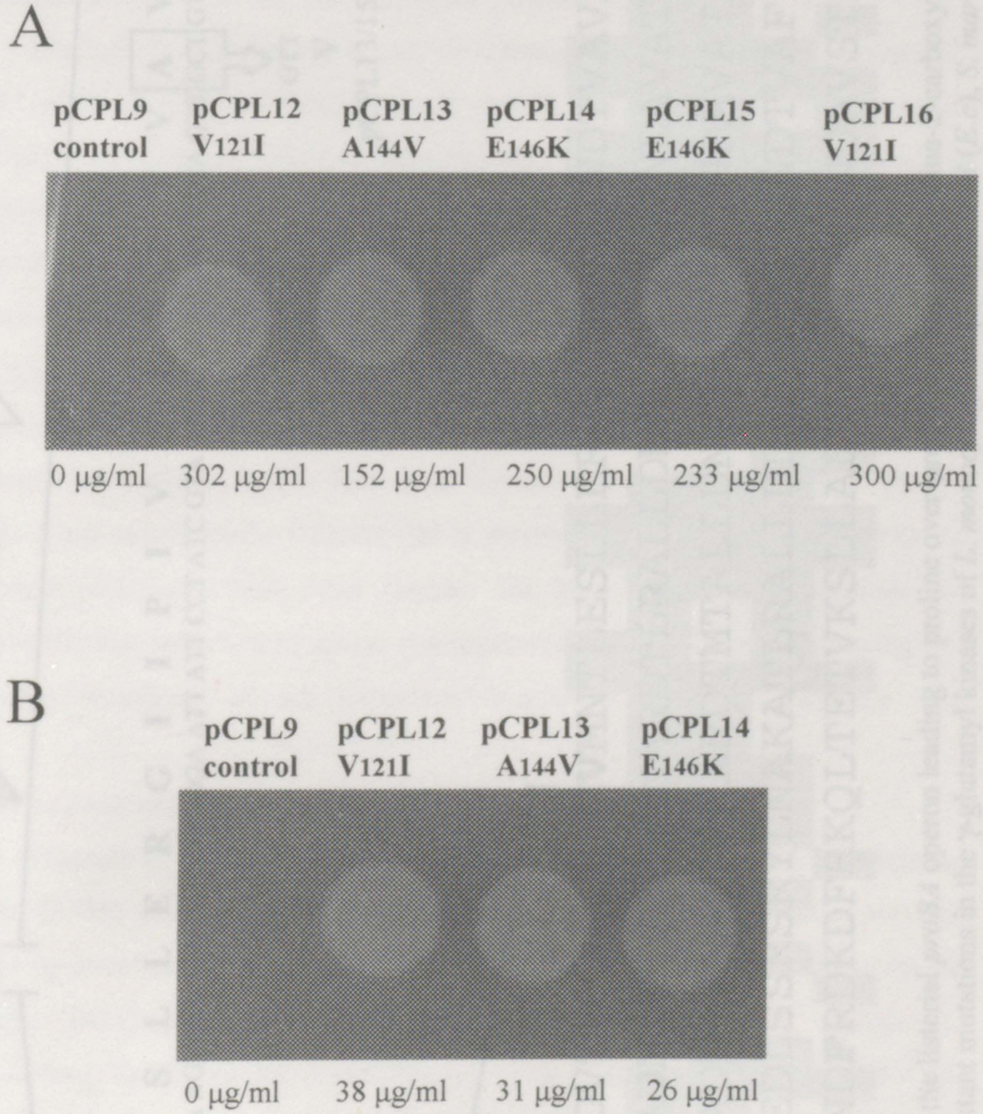

Fig. 2 Bioassay for proline overproduction, and concentrations of proline in the supernatant following transformation of **(A)** *E. coli* CSH26 and **(B)** *Listeria* PSOE, with mutated *proB* genes.

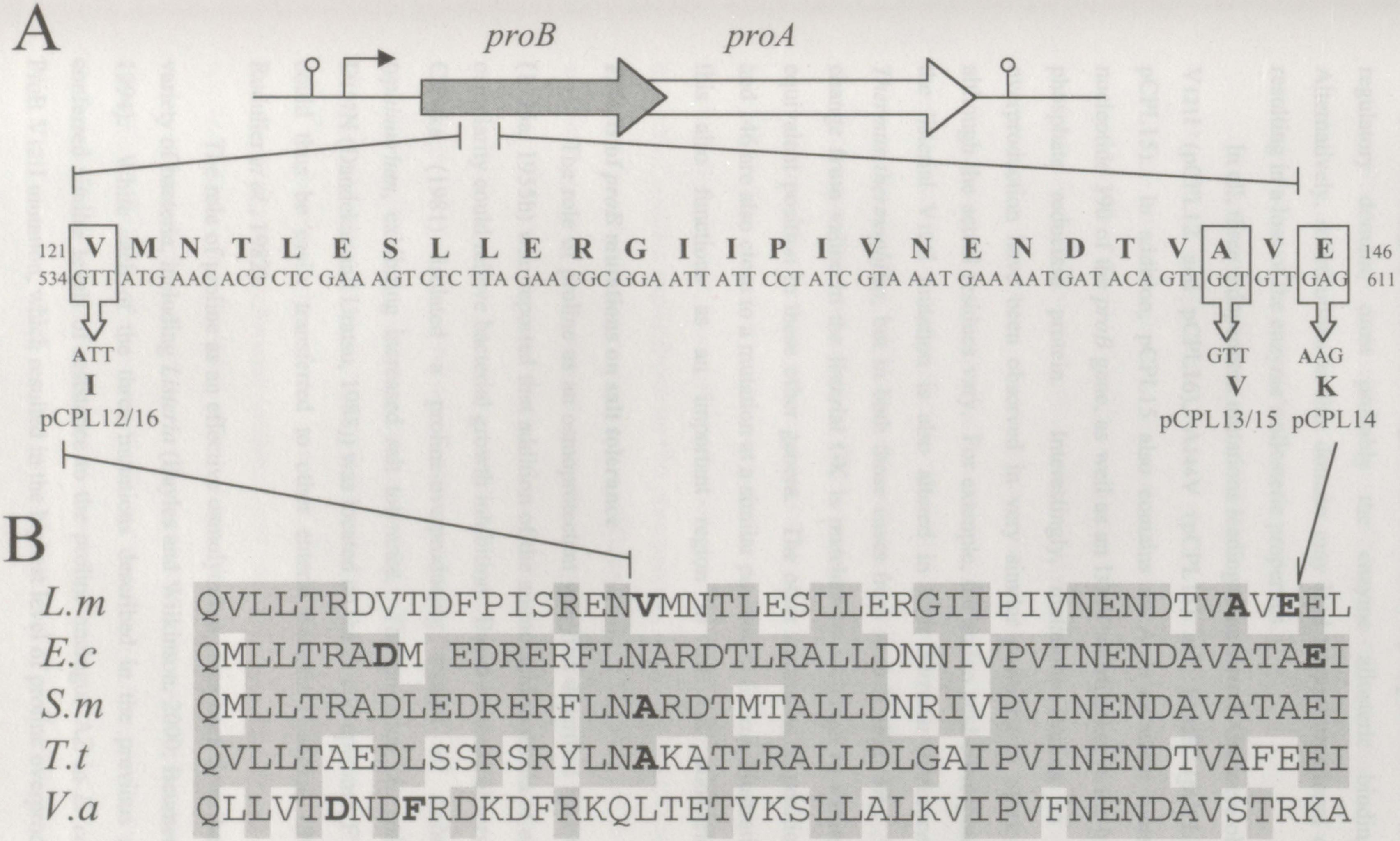

Fig. 3. (A) Point mutations in the listerial *proBA* operon leading to proline over-production and L-Azetidine-2-carboxylic acid resistance. **(B)** Feedback-resistant mutations in the γ-glutamyl kinases of *L. monocytogenes* (*L.m*), *E. coli* (*E.c*), *S. marcescens* (*S.m*), *T. thermophilus* (*T.t*) and *V. aconitifolia* (*V.a*). Residues affected by mutations conferring AZ resistance are in boldface. Conserved residues are shaded.

1995). This highly conserved region almost certainly represents an important regulatory domain, most probably the enzyme allosteric binding site. Alternatively, substitutions in this domain may lead to conformational changes resulting in a loss of the enzyme's allosteric properties.

In all, three independent mutations leading to an altered GK were obtained: V121I (pCPL12 and pCPL16), A144V (pCPL13) and E146K (pCPL14 and pCPL15). In addition, pCPL15 also contains an A to G silent mutation at nucleotide 390 of the *proB* gene, as well as an I328V substitution in the glutamyl phosphate reductase protein. Interestingly, mutations leading to proline overproduction have been observed in very similar positions in other genera, although the actual residues vary. For example, the amino acid corresponding to the listerial V121I mutation is also altered in both *Serratia marcescens* and *Thermus thermophilus*, but in both those cases from A to V (Fig. 3B). Thus, a change **from** valine in the listerial GK is matched by a change **to** valine at the equivalent position in these other genera. The other mutations at positions 144 and 146 are also close to a mutation at a similar position in *E. coli*, illustrating that this also functions as an important region in the GK allosteric site.

Effects of *proB* mutations on salt tolerance

The role of proline as an osmoprotectant was first described by Christian (1955a; 1955b) who reported that addition of the amino acid to media of elevated osmolarity could relieve bacterial growth inhibition. Based on these observations, Csonka (1981) isolated a proline-overproducing mutant of *Salmonella typhimurium*, exhibiting increased salt tolerance. The mutation (*E. coli* ProB D107N (Dandekar and Uratsu, 1988)) was located on the *E. coli* episome, F'_{128}, and could thus be easily transferred to other enteric bacteria (Csonka, 1981; Le Rudulier *et al.*, 1982).

The role of proline as an effective osmolyte has since been described for a variety of bacteria, including *Listeria* (Bayles and Wilkinson, 2000; Beumer *et al.*, 1994). While each of the three mutations described in the previous section conferred similar levels of resistance to the proline analogue AZ in *E. coli*, the ProB V121I mutation, which resulted in the highest level of proline overproduction and excretion (Fig. 2A), also conferred the highest level of osmotolerance at 4% NaCl, relative to the control strain (Fig. 4). The remaining mutations, while not as osmotolerant as ProB V121I, still showed significant increases in growth rate relative to the control at elevated osmolarities (Table 2).

The isolation and disruption of the listerial *proBA* operon was described recently, revealing a significant role for proline synthesis in contributing to the growth and survival of *L. monocytogenes* in environments of elevated osmolarity (Chapter V, this thesis). In order to further assess the importance of proline synthesis in *Listeria* the effect of overproducing proline on the same characteristics: osmotolerance and virulence, was analysed. All three independent *proB* mutations, leading to proline overproduction and analogue resistance, were introduced into the *Listeria* PSOE ($ProB^-$) background. While each of the mutated genes conferred AZ^r on PSOE, the observed levels of proline overproduction were found to be approximately ten-fold less than those in *E. coli* CSH26 (Fig. 2B).

While this evidence (AZ^r and proline overproduction, albeit at a reduced level) indicated a physiological consequence of the introduced mutations, none of the mutants exhibited an osmotolerant phenotype (data not shown). There are a number of possible explanations for this phenomenon, the most plausible of which concerns the extreme turgor requirement of Gram-positive bacteria, which can be as much as seven times that of their Gram-negative counterparts (Kempf and Bremer, 1998). Maintenance of elevated turgor requires the accumulation of high cytoplasmic concentrations of compatible solutes; e.g., while 0.5 mM proline is sufficient to promote maximal growth stimulation in *E. coli* at elevated osmolarities (Csonka, 1981), upwards of 10 mM proline is required to facilitate growth of *Listeria* at a similar salt concentration (Beumer *et al.*, 1994). Thus, the levels of proline overproduction observed (Fig. 2), while sufficient to permit growth of *E. coli* at otherwise inhibitory salt concentrations, appear too low to restore sufficient turgor to PSOE under salt stress conditions.

Increasing the capacity to produce proline, on its own, thus, may not be enough to confer osmotolerance to PSOE. In *S. marcescens* for example, maximal proline production (and consequential osmotolerance) resulted not only from

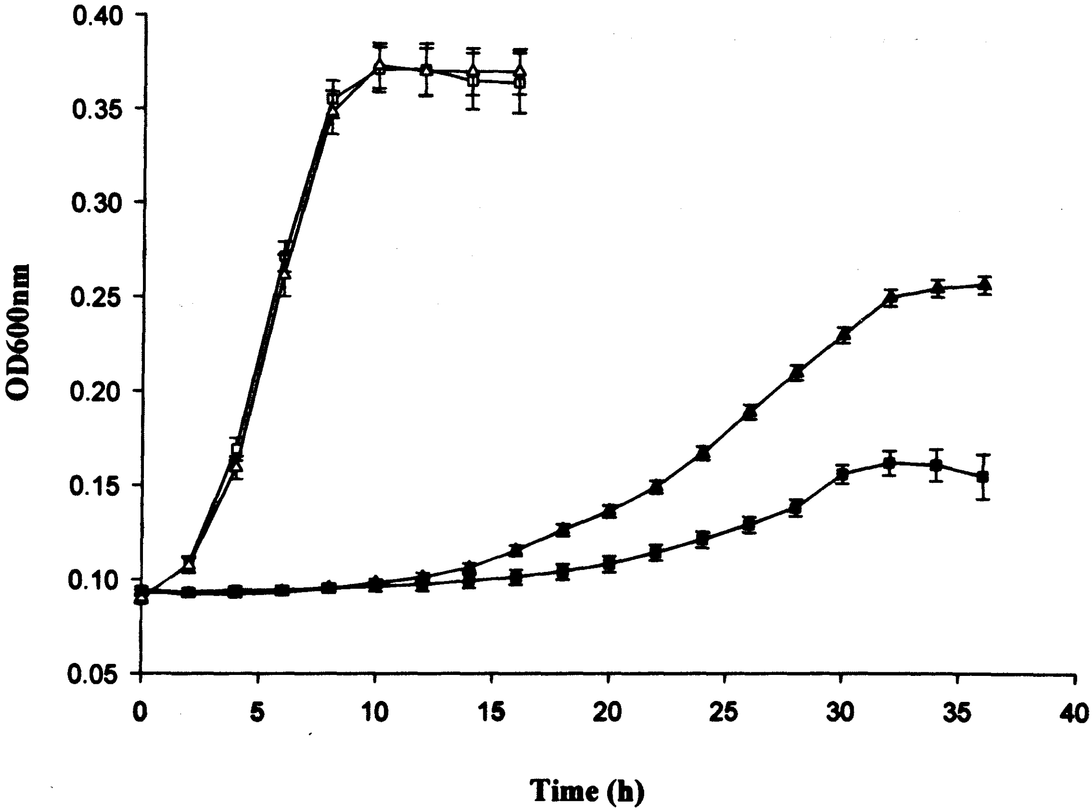

Fig. 4. Effect of the proline over-producing mutation, ProB V121I, on the growth of *E. coli* CSH26 in M9 minimal medium of elevated osmolarity. Growth (as determined by turbidity using a Spectra max 340 spectrophotometer, Molecular Devices), was measured both in the presence (closed symbols) and absence (open symbols) of 4% added NaCl. (□, ■) CSH26(pCPL9) control strain, (Δ, ▲) CSH26(pCPL12; ProB V121I). Each point represents the mean value of three independent experiments.

mutations in the *proB* gene leading to proline hyper-production (Omari *et al.*, 1992), but also an unknown mutation leading to an increased production of glutamate (the substrate for GK), in combination with mutations in the *putA* gene which result in a decreased rate of proline catabolism (Sugiura and Kisumi, 1985). Thus, the observed reduction in the levels of proline overproduction (Fig. 2) and consequential lack of a salt tolerance phenotype, when the *proB* mutations are transformed into PSOE, as opposed to the CSH26 background, may reflect either a limiting concentration of glutamate (and/or ATP) in *Listeria*, or degradation of excess proline by the listerial PutA equivalent. Strain specific effects may also contribute to the observed drop in proline production and excretion in *Listeria*, given that the *proB* mutations were originally isolated against an *E. coli* background and as such are presumably optimised for this environment.

Table 2. Growth rates in M9 minimal medium with 4% added NaCl, of *E. coli* CSH26 strains carrying listerial *proB* mutations leading to proline overproduction.

***E. coli* strains**	**Growth rate (h^{-1})**
Control strain:	
CSH26 (pCPL9)	0.022
L-Azetidine-2-carboxylic acid resistant strains:	
CSH26 (pCPL12; ProB V121I)	0.045
CSH26 (pCPL13; ProB A144V)	0.031
CSH26 (pCPL14; ProB E146K)	0.038
CSH26 (pCPL15; ProB E146K; ProA I328V)	0.035

Effects of proline overproduction on the virulence potential of *L. monocytogenes*

In addition to its role as an osmolyte, which in itself could potentially provide a distinct growth advantage to *Listeria* when exposed to the elevated osmolarity of the gastrointestinal tract (equivalent to 0.3 M NaCl, (Chowdhury *et al.*, 1996)), proline has also been suggested to function as a potential virulence factor in certain pathogenic bacteria (Bayer *et al.*, 1999; Culham *et al.*, 1998; Schwan *et al.*, 1998). Recent evidence suggests that, at least in plant cells, proline may also act as a free radical scavenger, protecting the cells from the damaging effects of oxidative stress (Hong *et al.*, 2000). Since an oxygen-dependent respiratory burst is one of the major mechanisms by which neutrophils and macrophages kill bacteria (Mahan *et al.*, 1996), proline hyper-production may shield *Listeria* from the oxidative stress encountered within the macrophage phagosome.

To analyse the effects of proline hyper-production on the virulence potential of *L. monocytogenes*, the plasmid carrying the ProB V121I mutation, which gave rise to the most pronounced osmotolerant phenotype in *E. coli*, and the highest levels of proline overproduction in *Listeria*, was used to transform *L. monocytogenes* PSOE. The resulting strain (ProB^{++}) was used to infect BALB/c mice, *via* the intraperitoneal and peroral routes. Similar to results obtained previously for proline auxotrophy (Marquis *et al.*, 1993; Chapter V, this thesis), proline hyper-production did not affect colonisation of the upper small intestine, nor did it disrupt invasion and spread to the internal organs (Table 3). Thus it is proposed that neither proline hyper-production nor inactivation of proline synthesis has any measurable effect on *Listeria* pathogenesis.

Table 3. Recovery of *L. monocytogenes* LO28 and the ProB V121I mutant from the tissues of infected mice three days post intraperitoneal and peroral infection

Type of inoculation and organ/tissue	**Log$_{10}$ bacterial numbers per organ/tissue (± SD)**[a]	
	ProB	**ProB V121I**
Intraperitoneal		
Liver	5.2 (0.4)	5.6 (0.4)
Spleen	5.5 (0.4)	5.8 (0.2)
Peroral		
Liver	4.5 (0.6)	4.6 (0.3)
Spleen	3.8 (0.2)	3.7 (0.3)
Small intestine wall and contents	4.1 (0.4)	4.0 (0.5)
Peyer's patches	2.6 (0.2)	2.6 (0.4)

[a] Values are averages of four inoculated animals

ACKNOWLEDGEMENTS

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Thesis Summary

In the autumn of 1997, at the outset of this study, analysis of the listerial osmostress response was mainly confined to physiological investigations. While Beumer *et al.* (1994) had identified the three principal compatible solutes in *Listeria* as proline, betaine, and carnitine, molecular characterisation of the mechanisms governing the accumulation of these compounds remained largely undetermined.

The object of this work was to redress the balance, using genetic approaches to gain a greater insight into the mechanisms of compatible solute accumulation, their role in osmotolerance and their contribution, if any, to the virulence potential of this ubiquitous foodborne pathogen.

Chapter II describes the isolation and disruption of *betL*, the first genetic element directly linked to the listerial salt stress response. Possessing twelve transmembrane domains (a structural feature common to secondary transporters) and exhibiting K_m and V_{max} values for betaine uptake of 7.9 µM and 134 nmol/min/mg protein, respectively, BetL at first appeared to conform with previous physiological investigations, which predicted that glycine betaine uptake in *Listeria* might be the result of a single highly specific secondary transporter (Ko *et al.*, 1994; Patchett *et al.*, 1994). However, residual betaine uptake in a BetL$^-$ background (approximately 19% of that of the wild type) provided the first indication that betaine uptake in *Listeria* is mediated by more than one system; a hypothesis later confirmed by the isolation of the GbuABC and OpuC systems (Ko *et al.*, 1999; Chapter IV, this thesis).

In Chapter III, transcriptional analysis of *betL*, which is preceded by a consensus σ^B-dependent promoter, proved that in addition to regulation at the protein level (Verheul *et al.*, 1997), betaine uptake mediated by BetL is also regulated at the level of transcription. In addition, allelic exchange mutagenesis was used to construct a stable in-frame deletion in *betL*, which was used to determine the role of the BetL protein in contributing to the growth and survival of *L. monocytogenes* both in a high risk food (Camembert cheese) and in an animal (murine) model. The results indicated that while BetL plays an important role in glycine betaine mediated osmoprotection, mutating the gene had no significant effect on either the cryotolerance or virulence potential of the organism. The observation that glycine betaine mediated cryoprotection is governed by a system or systems other than BetL was later confirmed by Ko *et al.* (1999) who demonstrated that the GbuABC system is responsible for most of the chill activated betaine uptake in *Listeria.*

Chapter IV documents the use of a novel system for generating chromosomal mutations (based on the lactococcal pWVO1-derived, Ori$^+$, RepA$^-$ suicide vector pORI19) to identify and disrupt the listerial OpuC homologue. Using a combination of growth experiments in defined medium and radiolabelled uptake studies, it was demonstrated that this multi-component ATP dependent system plays a role in both carnitine and betaine uptake in *Listeria.* Mutating OpuC resulted in a significant reduction in the ability of *L. monocytogenes* to colonise the upper small intestine and cause subsequent systemic infection following peroral inoculation of a murine model. The study thus provides the first direct link between osmotolerance and virulence in *L. monocytogenes.*

Chapter V, which describes the isolation and disruption of *proBA*, a two-gene operon encoding the first two enzymes of the listerial proline biosynthesis pathway, represents the first genetic analysis of osmolyte synthesis in *Listeria.* Allelic exchange mutagenesis was used to create a chromosomal deletion mutant in *proBA*, which is completely auxotrophic for proline. The observed lack of growth of this mutant, even in the presence of relatively high concentrations of proline (5 mM), indicates that, unlike the situation in *B. subtilis*, *Listeria* possesses only one proline biosynthesis system, and apparently lacks a functional high affinity proline transporter equivalent to either OpuE of *B. subtilis* or PutP of *E. coli.* In addition, while inactivation of *proBA* had no apparent effect on virulence in mouse assays (either perorally or intraperitoneally) growth at elevated osmolarity (>6% NaCl) was significantly reduced in the absence of efficient proline synthesis.

Chapter VI demonstrates that proline synthesis in *Listeria* is regulated by feedback inhibition of γ-glutamyl kinase (GK); the first enzyme of the proline biosynthesis pathway, encoded by the *proB* gene. While mutations in *proB* leading to proline overproduction were found to confer salt tolerance against an *E.*

coli background, listerial transformants failed to exhibit the same salt tolerant phenotype, possibly a consequence of the higher turgor requirement of Gram-positive over Gram-negative bacteria. In addition, as with proline auxotrophy, proline hyper-production was found to have no obvious effects on the virulence potential of *Listeria*.

While the work presented in this thesis represents a significant contribution to the field of listerial osmotolerance, the story is by no means complete. Having identified and mutated genes encoding individual osmolyte transport/synthesis systems, the next major challenge is to elucidate the complex interplay that exists between the individual systems, and provide additional information on the kinetics and regulation of each system against the listerial background. To this end, a 'bank' of osmosensitive mutants (Table 1) containing multiple deletions in the genes of interest has been constructed, using the techniques outlined in this thesis (*i.e.* SOEing deletions in combination with plasmid insertions), and has been sent to the laboratory of Tjakko Abee (Wageningen Agricultural University) for further analysis.

Table 1. Osmolyte uptake mutants of *L. monocytogenes* LO28

Mutant strain	Genotype
BSOE	Δ*betL*
GSOE	Δ*gbuABC*
LO28C	Δ*opuC*
BGSOE	Δ*betL*, Δ*gbuABC*
LO28BC	Δ*betL*, Δ*opuC*
LO28CG	Δ*opuC*, Δ*gbuABC*
LO28BCG	Δ*betL*, Δ*opuC*, Δ*gbuABC*

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