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A single point mutation in the listerial *betL* σ^A-dependent promoter leads to improved osmo- and chill-tolerance and a morphological shift at elevated osmolarity

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Betaine uptake in *Listeria monocytogenes* is mediated by three independent transport systems, the simplest of which in genetic terms is the secondary transporter BetL. Using a random mutagenesis approach, based on the *E. coli* XL1 Red mutator strain, we identified a single point mutation in a putative promoter region upstream of the BetL coding region which leads to a significant increase in *betL* transcript levels under osmo- and chill-stress conditions and a concomitant increase in stress tolerance. Furthermore, the mutation appears to counter the heretofore unreported "twisted" cell morphology observed for *L. monocytogenes* grown at elevated osmolarities in tryptone soy broth.

Introduction

A characteristic feature of the intracellular foodborne pathogen *Listeria monocytogenes* is its ability to thrive in a variety of stressful environments.[1,2] This phenotypic robustness can be attributed at least in part to the ability of the organism to accumulate a variety of protective compounds, termed compatible solutes, which help to buffer the cell from the detrimental effects of a range of environmental insults.[3-5]

Previous work in our labs[6-8] (and others[9,10]) led to the identification and characterization of the principal compatible solute uptake/synthesis systems in *L. monocytogenes*. The first of these loci to be identified, *betL*, encodes a high affinity (Km 7.9 mM; Vmax 134 nmol/min/mg of protein) sodium-motive force dependent secondary betaine uptake system (BetL),[6] which is a member of the BCCT family of osmolyte transport systems.[3] Detailed in silico analysis revealed the presence of two putative promoter regions; a σ^A-like promoter and a σ^B-dependent promoter, respectively.[11] However, while transcriptional control through alternative sigma factors is important, the final yield of BetL protein product is also likely determined by translation efficiency.[4] In *E. coli*, and to a lesser extent *Bacillus subtilis*, for example, the use of non-ATG initiation codons has previously been shown to modulate expression at the translational level.[12] Given that *betL* is initiated with an alternative TTG start codon, it is likely that the locus is also regulated to some degree at the level of translation.[6,11]

Furthermore, in addition to transcriptional and translational control, detailed biochemical analyses revealed that the BetL protein is itself activated in response to changes in osmolarity. Rapid activation of pre-existing BetL protein (half-life [*t*1/2], 2 min) in response to relatively low NaCl concentrations (1 to 2% NaCl) suggests that BetL is one of the primary responders to rapid fluxes in external osmolarity.[11]

We outline the use of a random mutagenesis strategy, employing the *Epicurian coli*® mutator strain XLI-Red, to screen for mutations in the *betL* gene which result in improved stress resistance. The deletion of a single thymine residue (from a string of seven thymines), within the spacer region between the –10 and –35 binding sites of the σ^A-like promoter, resulted in a dramatically improved osmo- and chill-tolerance phenotype when expressed against both *E. coli* MKH13 and *L. monocytogenes* $LO28BCG_{SOE}$ backgrounds. Furthermore, we also report for the first time an unusual "twisted-cell" morphology exhibited by *L. monocytogenes* when grown at elevated osmolarity (>7% NaCl). Interestingly, this twisted cell morphology is not observed in *L. monocytogenes* $LO28BCG_{SOE}$ cells expressing the *betL** gene.

Results and Discussion

Shotgun cloning of the *L. monocytogenes* LO28 genome, followed by heterologous complementation of the compatible solute uptake mutant *Escherichia coli* MKH13, led to the identification of *betL*—the first genetic element linked to listerial osmotolerance.[6] In silico analysis revealed the presence of a consensus σ^B-dependent promoter-binding site downstream of a putative σ^A-like promoter, suggesting that in addition to being regulated

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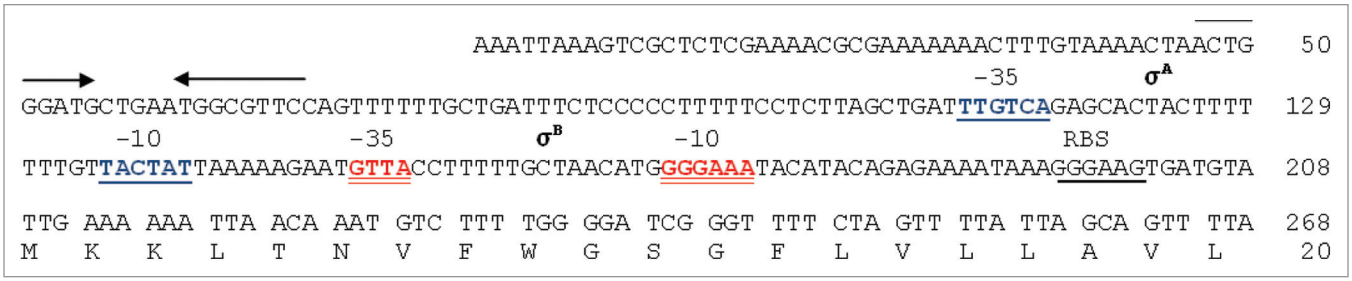

Figure 1. DNA sequence upstream of the *betL* coding region. Inverted repeats, indicated by pairs of arrows, delineate a likely rho-independent transcription terminal signal (ΔG -13 kcal). The predicted ribosome-binding site (RBS) and the putative σ^A and σ^B-dependent –10 and –35 sites are underlined. The first 20 amino acids of the predicted BetL protein are also presented; beginning with the alternative initiation codon TTG.

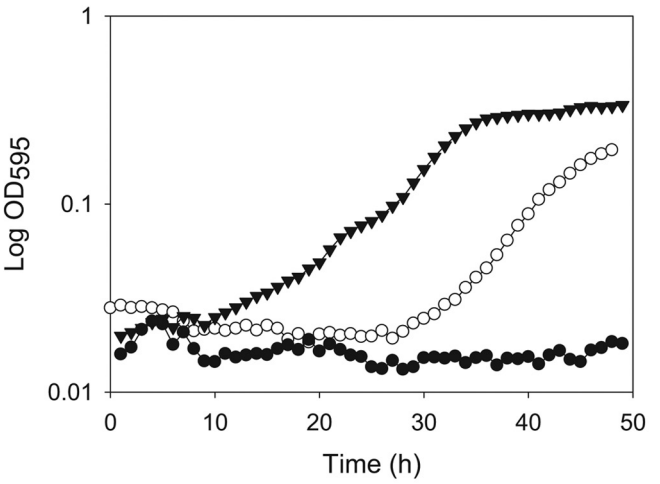

Figure 2. Growth of *E. coli* MKH13::pRS2 (▼; *betL**), *E. coli* MKH13::pRS3 (○; *betL*), and *E. coli* MKH13::pPL2 (●; negative control) in M9 minimal medium containing 2 mM betaine and 5% added NaCl.

at the protein level, *betL* is also likely regulated at the level of transcription.[11] RNA slot blot and reverse transcription analysis proved that this is indeed the case, with the gene showing a 1.6-fold increase in the level of transcription following 15 min exposure to 4% NaCl.[13] Furthermore, deleting *betL* from the listerial chromosome resulted in a dramatic reduction in the ability of the mutant to accumulate betaine (~19% that of the wild type), with an associated drop in the growth rate of the mutant at elevated osmolarities.[6]

In the current study, the complete *betL* gene (under the transcriptional control of its native promoters) was amplified from pCPL1 (using the primer pair *betL*F*Pst*I/*betL*R*Xba*I) and cloned into pCI372, a shuttle vector capable of replicating in both *E. coli* and *L. monocytogenes*. As expected, the resulting construct, designated pRS1, reversed the salt sensitive phenotypes of the osmotically challenged strains *E. coli* MKH13 and *L. monocytogenes* $LO28BCG_{SOE}$[14] (data not shown).

In an effort to improve BetL mediated osmotolerance, a random mutagenesis strategy was employed to introduce point mutations into the cloned listerial *betL* gene. Plasmid pRS1 (harbouring *betL*) was transformed into the *E. coli* mutator strain XL1-Red. Mutations in three of the primary DNA repair pathways of XL1-Red result in a mutation rate which is ~5,000-fold higher than that of the wild type; hence, pRS1 replication within this strain led to the introduction of random point mutations throughout the plasmid, including the target *betL* gene. The randomly mutated pRS1 "bank", designated $pRS1^{mut}$, was subsequently transformed into *E. coli* MKH13, and transformants were selected on LB agar plates containing 7% added NaCl (a salt concentration above the growth limit for MKH13::pRS1). No colonies were obtained following a control transformation with unmutated pRS1, but transformation efficiencies of 55 CFU/μg of DNA were achieved from $pRS1^{mut}$; with colonies appearing after 48 h at 37 °C. Following overnight growth at elevated osmolarities, plasmids from selected osmotolerant transformants were extracted, and the cloned insert sequenced. In each case, the same mutation was observed; i.e., a deletion of one of a string of seven thymines within the spacer region between the –10 and –35 binding sites of the *betL* σ^A-like promoter (**Fig. 1**). Proof that the observed phenotype was the result of the single point mutation in the cloned *betL* gene (as opposed to random mutations in the pCI372 plasmid backbone) was obtained by re-complementation studies, in which the mutated *betL* (designated *betL**) was cloned into a pPL2 backbone, creating pRS2. Further proof that the observed "hyper-osmotolerance" phenotype was the result of the *betL** mutation was obtained using the QuikChange®XL Site-Directed Mutagenesis Kit—replacing the missing thymine reversed the observed phenotype from "hyper-osmotolerance" to normal. Confirmation that the increased osmotolerance phenotype of *betL** is the direct result of improved betaine mediated osmoprotection was obtained following growth in defined medium at elevated osmolarity (5% added NaCl) in the presence and absence of betaine. While no growth was observed for any of the *E. coli* strains in the absence of betaine (data not shown), the strain expressing *betL** grew significantly better than the control strains in the presence of betaine (**Fig. 2**).

In order to assess the effect of the *betL** mutation against the native listerial background, the constructs were transformed into $LO28\Delta BCG_{SOE}$; a strain which is devoid of betaine uptake.[15] When expressed against the *Listeria* background, a significant advantage of pPL2 (the pRS2 and pRS3 backbone) over the multi-copy cytoplasmic pCI372 plasmid is that pPL2 integrates as a single copy in the listerial chromosome (specifically the

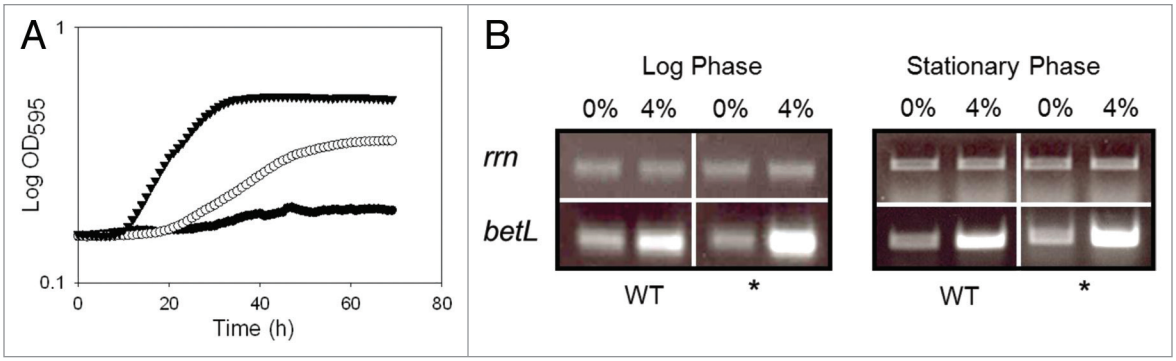

Figure 3. (**A**) Growth of *L. monocytogenes* LO28BCG$_{SOE}$::pRS2 (▼; *betL**), LO28BCG$_{SOE}$::pRS3 (○; *betL*), and LO28BCG$_{SOE}$::pPL2 (●; negative control) in TSB with 7% added NaCl. (**B**) Transcript levels of *betL* and *betL** against the *L. monocytogenes* LO28BCG$_{SOE}$ background, following exposure to 4% NaCl for 15 min. Control PCRs were performed with the *rrn* primers U141 and L142. For each RT-PCR, results are presented after 22 cycles and are representative of three biological replicates.

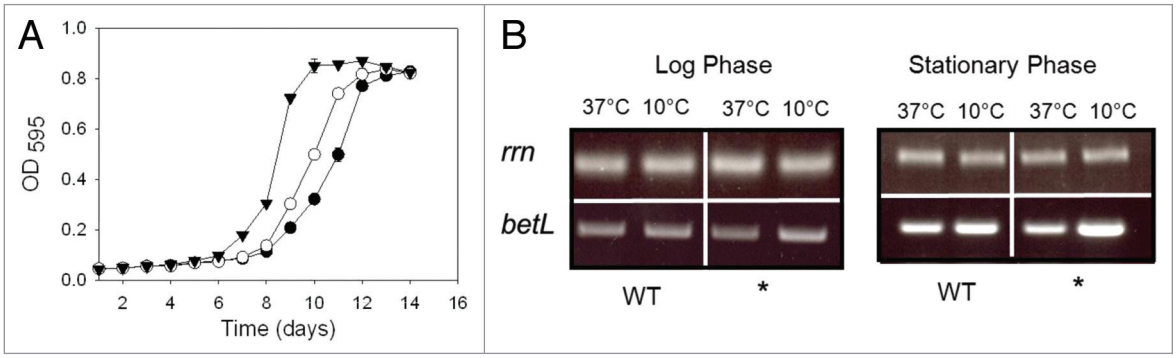

Figure 4. (**A**) Growth of *L. monocytogenes* LO28BCG$_{SOE}$::pRS2 (▼; *betL**), LO28BCG$_{SOE}$::pRS3 (○; *betL*), and LO28BCG$_{SOE}$::pPL2 (●; negative control) in TSB at 4 °C. (**B**) Transcript levels of *betL* and *betL** against the *L. monocytogenes* LO28BCG$_{SOE}$ background, following exposure to 10 °C for 30 min. Control PCRs were performed with the *rrn* primers U141 and L142. For each RT-PCR, results are presented after 22 cycles and are representative of three biological replicates.

tRNA^{Arg}-*attBB'* in both serotype 1/2 and 4b strains of *Listeria*[16,17]), thereby removing plasmid copy number as a variable in assessing the true contribution of *betL** to *L. monocytogenes* osmotolerance. Given the location of the thymine deletion, between the –10 and –35 promoter binding sites, it seemed plausible that the increased osmotolerance (**Fig. 3A**) was the result of increased transcription in *betL** relative to the *betL* wild type. RT-PCR analysis of the mutant and wild type genes suggests that this is indeed the case (**Fig. 3B**). While no significant difference in transcript levels were observed between *betL* and *betL** at 0% NaCl, at both log and stationary phase growth, expression levels do appear to differ at 4% NaCl, in both growth phases. Similarly, *betL** appears to exhibit slightly higher transcript levels under cold stress conditions (particularly at stationary phase **Fig. 4B**) and a concomitant increase in growth at refrigeration temperatures (**Fig. 4A**); a finding which is not altogether surprising given that BetL has previously been linked to chill tolerance in *L. monocytogenes*.[18] Promoter variants in genes encoding compatible solute uptake is not a new phenomenon and has been reported previously in the literature for a number of microorganisms; including *E. coli*,[19] *Salmonella typhimurium*,[20] and *B. subtilis*.[21,22] While the mechanism of promoter activation in the current study is still unclear, it is tempting to speculate that the deleted thymine may affect DNA topology, thereby facilitating improved promoter binding and activation. In support of this hypothesis, promoters of osmotolerance genes in different organisms have previously been shown to be induced only when DNA is highly supercoiled.[23,24] Interestingly, *proU* (encoding betaine uptake in *E. coli*) is controlled by changes in DNA topology;[25] while the osmoregulated promoter for *opuA*, encoding a betaine uptake system in *B. subtilis*, is likely subject to similar control. Indeed, both the osmoregulated *proU* and *opuA* promoters deviate from the consensus 17-bp in the length of their –10 and –35 spacer regions; with sub-optimal spacing of 16 and 18-bp respectively[24,26,27]—the latter being the predicted distance between the –10 and –35 binding sites of the *betL* σ^A-like promoter (**Fig. 1**). Given that RNA polymerase makes specific contacts with both the –10 and –35 regions; the relative orientation of these sequences is likely an important determinant for efficient transcription initiation.[28] Promoters with sub-optimal spacer regions, like the *betL* σ^A-like promoter, are thus likely to respond sensitively to environmentally controlled alterations in DNA topology and as such belong to a special class of DNA twist-sensitive promoters.[29] Indeed, Alice and Sanchez-Rivas[30] observed a direct link between osmotolerance and DNA supercoiling in

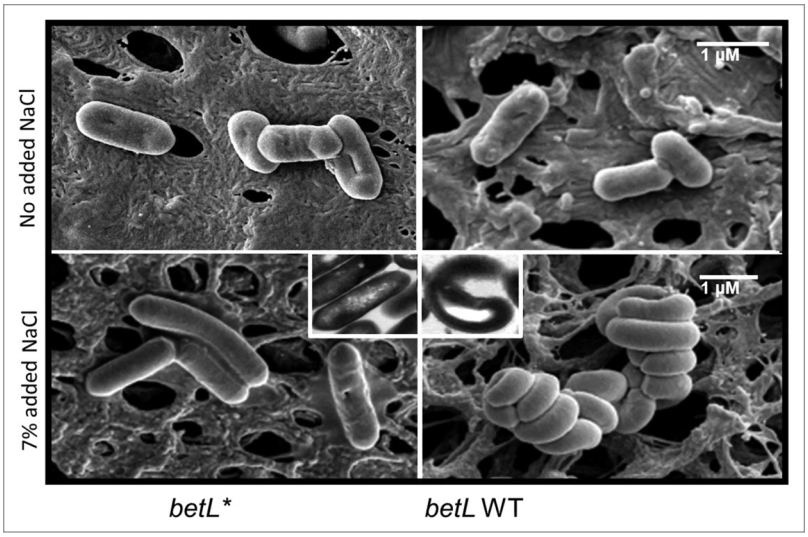

Figure 5. Scanning electron micrograph (SEM) at ×20k magnification of *L. monocytogenes* LO28BCG$_{SOE}$::pRS2 expressing *betL** (column 1) and LO28BCG$_{SOE}$::pRS3 expressing *betL* WT (column 2), with no added NaCl (row 1) and 7% added NaCl (row 2). Inset—transmission electron microscopy (TEM) of *L. monocytogenes* LO28BCG$_{SOE}$::pRS2 expressing *betL** (column 1) and LO28BCG$_{SOE}$::pRS3 expressing *betL* WT (column 2), with 7% added NaCl (row 2).

B. subtilis, while Grau et al.[31] noted similar fluctuations in DNA supercoiling during cold adaptation.

In addition to dramatically improving the growth of *L. monocytogenes* at elevated osmolarities, the *betL** mutation reverses a previously unreported "twisted-cell" morphology for *Listeria* grown in complex media (BHI or TSB) at 7% NaCl (**Fig. 5**). While the existence of elongated listerial cells at elevated osmolarities is not a new phenomenon;[32,33] the "twisted-cell" phenotype has not previously been reported. While the exact role of this twisted morphology is unclear, it may function as a survival strategy reducing the cellular surface area exposed to the bathing solution, thereby reducing the severity of the stress. Indeed, bacterial "huddling"—the observed close association of individual bacterial cells—has previously been reported by Corcoran et al.,[34] in probiotic lactobacilli subjected to in both spray and freeze-drying. The lack of a twisted morphology for *L. monocytogenes* LO28BCG$_{SOE}$ expressing *betL** suggests that the cells are less stressed and thus better equipped for growth and survival at elevated osmolarities.

Finally, the *betL** mutation in *L. monocytogenes* represents a double edged sword; from a food safety perspective, a single point mutation with the potential to induce such dramatic shifts in cell growth and survival at low a_w and temperatures—making an already dangerous pathogen even more formidable—raises significant food-safety concerns which need to be addressed.[1,35] However, from a synthetic biology point of view, a boosted-stress resistance locus, such as *betL**, represents a useful BioBrick[36] for the design of more physiologically robust pharmabiotic strains.[37-42]

Materials and Methods

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in **Table 1**. *E. coli* MKH13 was grown at 37 °C in either LB medium or M9 minimal medium (GIBCO/BRL, Eggenstein) containing 0.5% glucose, 0.04% arginine, 0.04% isoleucine, and 0.04% valine. Glycine betaine (Sigma) was added to M9 as a filter-sterilised solution to a final concentration of 1 mM. *L. monocytogenes* strains were grown in brain heart infusion (BHI) broth or in tryptone soy broth (TSB) supplemented with 0.6% yeast extract (Sigma Chemical Co.) or in chemically defined minimal medium[44] (DM). Antibiotics, when needed, were made up 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. Plasmid DNA was isolated using the Qiagen QIAprep Spin Miniprep Kit (Qiagen). *E. coli* was transformed by standard methods while electro-transformation of *L. monocytogenes* was achieved by the protocol outlined by Park and Stewart.[45] Polymerase chain reaction (PCR) reagents (*Taq* polymerase and deoxynucleoside triphosphates dNTPs) were purchased from Boehringer GmbH and used according to the manufacturer's instructions with a Hybaid PCR express system. Oligonucleotide primers, listed in **Table 2**, were synthesized on a Beckman oligo 1000M DNA synthesizer (Beckman Instruments Inc). Nucleotide sequence determination was performed on an ABI 373 automated sequencer using the BigDye™ Terminator sequence kit (Lark Technologies, Inc). Nucleotide sequence analysis was performed using BioMapper (nSilico Lifescience Ltd).

Generation and screening of *betL.** Random mutagenesis was performed using the strategy outlined previously.[46] Plasmid pRS1 harbouring the listerial *betL* gene 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 osmolyte uptake mutant *E. coli* MKH13. Mutations leading to enhanced osmotolerance were selected by plating transformants on LB medium containing 7% added NaCl (a salt concentration which does not permit the growth of MKH13 expressing pRS1). Plasmids isolated from the resultant osmotolerant MKH13 clones were then used to transform *L. monocytogenes* LO28BCG$_{SOE}$ (Δ*bet,* Δ*opuC,* Δ*gbu*).

Site directed mutagenesis. Single nucleotide additions and deletions within the putative *betL* σ^A-like promoter region were achieved using the QuikChange®XL Site-Directed Mutagenesis Kit (Stratagene) in accordance with the manufacturer's instructions.

Transcriptional analysis. Reverse transcriptase (RT)-PCR analysis was performed as previously described.[13] Essentially *L. monocytogenes* cells were grown, at 37 °C with shaking, to mid-exponential phase in BHI. Ten mililiters of culture were centrifuged and re-suspended in 1 ml of BHI with 4% added NaCl, for

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or characteristics[a]	Source or reference
Strains		
L. monocytogenes **LO28**	Serotype 1/2c	P. Cossart, Institut Pasteur
LO28ΔBCG$_{SOE}$	LO28 Δ*betL*, Δ*opuC*, Δ*gbu*	Wemekamp-Kamphuis et al.,[15]
E. coli **MKH13**	MC4100D(*putPA*)101D(*proP*)2D(*proU*)	Haardt et al.,[43]
XL1-Red	*endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT* Tn*10* (Tet^r)	Stratagene
Plasmids		
pUC18	ColE1 *ori*, Ap^r	Vieira and Messing[43]
pCPL1	pUC18 containing 2.5 kb of *L. monocytogenes* genomic DNA	Sleator et al.,[6]
pCI372	*E. coli/L. lactis* shuttle vector, 5.7 kb, Cm^r	Sleator et al.,[6]
pPL2	Site-specific listerial integrative vector, 6.1 kb, Cm^r	Lauer et al.,[16,17]
pRS1	pCI372::*betL*	This study
pRS1^{mut}	Randomly mutated pRS1 from *E. coli* XL1-Red	This study
pRS2	pPL2::*betL**	This study
pRS3	pPL2::*betL*	This study

[a]Ap^r ampicillin resistance, Cm^r chloramphenicol resistance, Tet^r tetracycline resistance.

analysis of salt stress, and BHI pre-chilled to 10 °C for chill stress. BHI at 37 °C with no added salt was used as a control. After 15 and 30 min incubation, cells were harvested by centrifugation and flash-frozen at –80 °C with liquid nitrogen. Total RNA was extracted using the hot acid phenol procedure described by Ripio et al.,[47] and cDNA was synthesized by adding 1 μg of total RNA to 4 μl of 5× RT buffer (Roche), 2 μl of 100 mM dithiothreitol, 0.5 μl of a deoxynucleoside triphosphate mix, 0.25 μl of RNasin, 100 ng of the random primer p(dN)$_6$, and 1 μl of Expand reverse transcriptase (Roche). The reaction mixture was incubated at 42 °C for 9 h.

In all cases control PCR reactions were used to ensure the complete removal of DNA from RNA preparations prior to reverse transcription and to ensure that levels of cDNA for samples to be compared were equal. Oligonucleotide primer pairs *Xba*IKO/EcoRIKO and U141/L142 were used for PCR amplification of a 551-bp fragment from the center of *betL* and a 806 bp *rrnA* DNA fragment respectively. Each (RT)-PCR reaction was performed in triplicate from three biological replicates.

All glass and plastic-ware used in RNA analysis was first treated with 2% sodium dodecyl sulfate (SDS) for 15 min, before rinsing with 1:10 in diethyl-pyrocarbonate (DEPC) treated water.

Microscopy. Microscopy was performed essentially as described by Considine et al.[48] Safranin (0.25%) stained samples of individual strains were viewed using Bright-field light microscopy at 1000× magnification to determine cell morphology. For scanning electron microscopy (SEM), overnight cultures in TSB ± 7% added NaCl (5 mL in a 30 mL round-bottomed tube) were centrifuged at 5000 rpm × 10 min. Pellets were immediately re-suspended in 2% glutaraldehyde, 2.5% paraformaldehyde in 0.165 M phosphate buffer (pH 7.3), and left to fix overnight at 4 °C. After fixation, the cells were subjected to three 10 min washes with 0.165 M phosphate buffer. The cells were post-fixed with 2% osmium tetroxide for 2 h before being washed again in buffer. Cells were dehydrated by to successive 25%, 50%, 75%, and 100% (100% × four times) solutions of acetone for 10 min, before being transferred to tetramethylsilane (TMS) for 15 min and air-dried in a fume hood. The samples were mounted on metal stubs using double-sided carbon tape and sputter coated with ~28 nm layer of gold using a BioRAD Polaron Sputter Coating System. The samples were viewed with a Joel JSM-5510 scanning electron microscope. For transmission electron microscopy (TEM), overnight cultures were fixed and post-fixed as for the SEM preparation. Following a buffer rinse, the cells were dehydrated in a series of solutions of ethanol: 10% for 15 min (three times), 50% for 30 min, 100% for 15 min (three times), and finally in epoxy propane for 30 min. The dehydrated cells were infiltrated with an epoxypropane:araldite mixture (3:1) overnight and then embedded in araldite and cured for 48 h in molds at 50 °C. Thin (70 nm) sections were obtained from the polymerized blocks using a Reichert-Jung Ultracut E ultramicrotome and collected on formvar-coated copper grids (100 mesh). The sections were stained with 2% uranyl acetate and Reynolds lead citrate stain and examined using a Joel JEM-2000FXII electron microscope.

Table 2. PCR primers used in this study

Primer	Sequence (5'-3')
betLFPstI	CAT CTG CAG GCT TTC TCC CCC TTT TTC CTC
betLRXbaI	CAT TCT AGA GCT CTA TTC CAA TTA CCG CCA TTT C
XbaIKO	TAA GCG CCA CTC TAG ACC
EcoRIKO	GCA CGA ATT CAC CAA GTA
U141	TTG CTC TTC CAA TGT TAG
L142	GAG TGC TTA ATG CGT TAG

[a]Nucleotides introduced to create restriction sites are underlined.

Electron micrographs were obtained for areas of interest with a Megaview-III digital camera and analySIS software.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Hill C, Cotter PD, Sleator RD, Gahan CGM. Bacterial stress response in *Listeria monocytogenes*: jumping the hurdles imposed by minimal processing. Int Dairy J 2001; 12:273-83; http://dx.doi.orggvb/10.1016/S0958-6946(01)00125-X.
2. Sleator RD, Watson D, Hill C, Gahan CG. The interaction between Listeria monocytogenes and the host gastrointestinal tract. Microbiology 2009; 155:2463-75; PMID:19542009; http://dx.doi.org/10.1099/mic.0.030205-0.
3. Sleator RD, Hill C. Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. FEMS Microbiol Rev 2002; 26:49-71; PMID:12007642; http://dx.doi.org/10.1111/j.1574-6976.2002.tb00598.x.
4. Sleator RD, Gahan CG, Hill C. A postgenomic appraisal of osmotolerance in Listeria monocytogenes. Appl Environ Microbiol 2003; 69:1-9; PMID:12513970; http://dx.doi.org/10.1128/AEM.69.1.1-9.2003.
5. Sleator RD, Hill C. Compatible solutes: the key to Listeria's success as a versatile gastrointestinal pathogen? Gut Pathog 2010; 2:20; PMID:21143981; http://dx.doi.org/10.1186/1757-4749-2-20.
6. Sleator RD, Gahan CG, Abee T, Hill C. Identification and disruption of BetL, a secondary glycine betaine transport system linked to the salt tolerance of Listeria monocytogenes LO28. Appl Environ Microbiol 1999; 65:2078-83; PMID:10224004.
7. Sleator RD, Gahan CG, Hill C. Identification and disruption of the proBA locus in Listeria monocytogenes: role of proline biosynthesis in salt tolerance and murine infection. Appl Environ Microbiol 2001; 67:2571-7; PMID:11375165; http://dx.doi.org/10.1128/AEM.67.6.2571-2577.2001.
8. Sleator RD, Wouters J, Gahan CG, Abee T, Hill C. Analysis of the role of OpuC, an osmolyte transport system, in salt tolerance and virulence potential of Listeria monocytogenes. Appl Environ Microbiol 2001; 67:2692-8; PMID:11375182; http://dx.doi.org/10.1128/AEM.67.6.2692-2698.2001.
9. Ko R, Smith LT. Identification of an ATP-driven, osmoregulated glycine betaine transport system in Listeria monocytogenes. Appl Environ Microbiol 1999; 65:4040-8; PMID:10473414.
10. Fraser KR, Harvie D, Coote PJ, O'Byrne CP. Identification and characterization of an ATP binding cassette L-carnitine transporter in Listeria monocytogenes. Appl Environ Microbiol 2000; 66:4696-704; PMID:11055912; http://dx.doi.org/10.1128/AEM.66.11.4696-4704.2000.
11. Sleator RD, Wood JM, Hill C. Transcriptional regulation and posttranslational activity of the betaine transporter BetL in Listeria monocytogenes are controlled by environmental salinity. J Bacteriol 2003; 185:7140-4; PMID:14645273; http://dx.doi.org/10.1128/JB.185.24.7140-7144.2003.
12. Reddy P, Peterkofsky A, McKenney K. Translational efficiency of the Escherichia coli adenylate cyclase gene: mutating the UUG initiation codon to GUG or AUG results in increased gene expression. Proc Natl Acad Sci U S A 1985; 82:5656-60; PMID:3898067; http://dx.doi.org/10.1073/pnas.82.17.5656.
13. Sleator RD, Gahan CGM, O'Driscoll B, Hill C. Analysis of the role of betL in contributing to the growth and survival of Listeria monocytogenes LO28. Int J Food Microbiol 2000; 60:261-8; PMID:11016615; http://dx.doi.org/10.1016/S0168-1605(00)00316-0.
14. Wemekamp-Kamphuis HH, Wouters JA, Sleator RD, Gahan CG, Hill C, Abee T. Multiple deletions of the osmolyte transporters BetL, Gbu, and OpuC of Listeria monocytogenes affect virulence and growth at high osmolarity. Appl Environ Microbiol 2002; 68:4710-6; PMID:12324311; http://dx.doi.org/10.1128/AEM.68.10.4710-4716.2002.
15. Wemekamp-Kamphuis HH, Sleator RD, Wouters JA, Hill C, Abee T. Molecular and physiological analysis of the role of osmolyte transporters BetL, Gbu, and OpuC in growth of Listeria monocytogenes at low temperatures. Appl Environ Microbiol 2004; 70:2912-8; PMID:15128551; http://dx.doi.org/10.1128/AEM.70.5.2912-2918.2004.
16. Lauer P, Chow MYN, Loessner MJ, Portnoy DA, Calendar R. Construction, characterization, and use of two Listeria monocytogenes site-specific phage integration vectors. J Bacteriol 2002; 184:4177-86; PMID:12107135; http://dx.doi.org/10.1128/JB.184.15.4177-4186.2002.
17. Angelidis AS, Smith GM. Role of the glycine betaine and carnitine transporters in adaptation of Listeria monocytogenes to chill stress in defined medium. Appl Environ Microbiol 2003; 69:7492-8; PMID:14660402; http://dx.doi.org/10.1128/AEM.69.12.7492-7498.2003.
18. Mellies J, Wise A, Villarejo M. Two different Escherichia coli proP promoters respond to osmotic and growth phase signals. J Bacteriol 1995; 177:144-51; PMID:8002611.
19. Zhang X, Fletcher SA, Csonka LN. Site-directed mutational analysis of the osmotically regulated proU promoter of Salmonella typhimurium. J Bacteriol 1996; 178:3377-9; PMID:8655527.
20. Spiegelhalter F, Bremer E. Osmoregulation of the opuE proline transport gene from Bacillus subtilis: contributions of the sigma A- and sigma B-dependent stress-responsive promoters. Mol Microbiol 1998; 29:285-96; PMID:9701821; http://dx.doi.org/10.1046/j.1365-2958.1998.00929.x.
21. Hoffmann T, Wensing A, Brosius M, Steil L, Völker U, Bremer E. Osmotic control of opuA expression in Bacillus subtilis and its modulation in response to intracellular glycine betaine and proline pools. J Bacteriol 2013; 195:510-22; PMID:23175650; http://dx.doi.org/10.1128/JB.01505-12.
22. Graeme-Cook KA, May G, Bremer E, Higgins CF. Osmotic regulation of porin expression: a role for DNA supercoiling. Mol Microbiol 1989; 3:1287-94; PMID:2552265; http://dx.doi.org/10.1111/j.1365-2958.1989.tb00279.x.
23. Kempf B, Bremer E. OpuA, an osmotically regulated binding protein-dependent transport system for the osmoprotectant glycine betaine in Bacillus subtilis. J Biol Chem 1995; 270:16701-13; PMID:7622480; http://dx.doi.org/10.1074/jbc.270.28.16701.
24. Jordi BJAM, Owen-Hughes TA, Hulton CSJ, Higgins CF. DNA twist, flexibility and transcription of the osmoregulated proU promoter of Salmonella typhimurium. EMBO J 1995; 14:5690-700; PMID:8521826.
25. Higgins CF, Dorman CJ, Stirling DA, Waddell L, Booth IR, May G, et al. A physiological role for DNA supercoiling in the osmotic regulation of gene expression in S. typhimurium and E. coli. Cell 1988; 52:569-84; PMID:2830029; http://dx.doi.org/10.1016/0092-8674(88)90470-9.
26. Owen-Hughes TA, Pavitt GD, Santos DS, Sidebotham JM, Hulton CS, Hinton JC, et al. The chromatin-associated protein H-NS interacts with curved DNA to influence DNA topology and gene expression. Cell 1992; 71:255-65; PMID:1423593; http://dx.doi.org/10.1016/0092-8674(92)90354-F.
27. Auble DT, deHaseth PL. Promoter recognition by Escherichia coli RNA polymerase. Influence of DNA structure in the spacer separating the -10 and -35 regions. J Mol Biol 1988; 202:471-82; PMID:3050126; http://dx.doi.org/10.1016/0022-2836(88)90279-3.
28. Wang JY, Syvanen M. DNA twist as a transcriptional sensor for environmental changes. Mol Microbiol 1992; 6:1861-6; PMID:1508037; http://dx.doi.org/10.1111/j.1365-2958.1992.tb01358.x.
29. Alice AF, Sanchez-Rivas C. DNA supercoiling and osmoresistance in Bacillus subtilis 168. Curr Microbiol 1997; 35:309-15; PMID:9462962; http://dx.doi.org/10.1007/s002849900260.
30. Grau R, Gardiol D, Glikin GC, de Mendoza D. DNA supercoiling and thermal regulation of unsaturated fatty acid synthesis in Bacillus subtilis. Mol Microbiol 1994; 11:933-41; PMID:8022269; http://dx.doi.org/10.1111/j.1365-2958.1994.tb00372.x.
31. Hazeleger WC, Dalvoorde M, Beumer RR. Fluorescence microscopy of NaCl-stressed, elongated Salmonella and Listeria cells reveals the presence of septa in filaments. Int J Food Microbiol 2006; 112:288-90; PMID:16806551; http://dx.doi.org/10.1016/j.ijfoodmicro.2006.04.026.
32. Zaika LL, Fanelli JS. Growth kinetics and cell morphology of Listeria monocytogenes Scott A as affected by temperature, NaCl, and EDTA. J Food Prot 2003; 66:1208-15; PMID:12870754.
33. Corcoran BM, Ross RP, Fitzgerald GF, Dockery P, Stanton C. Enhanced survival of GroESL-overproducing Lactobacillus paracasei NFBC 338 under stressful conditions induced by drying. Appl Environ Microbiol 2006; 72:5104-7; PMID:16820516; http://dx.doi.org/10.1128/AEM.02626-05.
34. Sleator RD, Hill C. Food reformulations for improved health: A potential risk for microbial food safety? Med Hypotheses 2007; 69:1323-4; PMID:17452083; http://dx.doi.org/10.1016/j.mehy.2007.03.007.
35. Ho-Shing O, Lau KH, Vernon W, Eckdahl TT, Campbell AM. Assembly of standardized DNA parts using BioBrick ends in E. coli. Methods Mol Biol 2012; 852:61-76; PMID:22328426; http://dx.doi.org/10.1007/978-1-61779-564-0_6.
36. Sheehan VM, Sleator RD, Fitzgerald GF, Hill C. Heterologous expression of BetL, a betaine uptake system, enhances the stress tolerance of Lactobacillus salivarius UCC118. Appl Environ Microbiol 2006; 72:2170-7; PMID:16517668; http://dx.doi.org/10.1128/AEM.72.3.2170-2177.2006.
37. Sheehan VM, Sleator RD, Hill C, Fitzgerald GF. Improving gastric transit, gastrointestinal persistence and therapeutic efficacy of the probiotic strain Bifidobacterium breve UCC2003. Microbiology 2007; 153:3563-71; PMID:17906153; http://dx.doi.org/10.1099/mic.0.2007/006510-0.
38. Sleator RD, Hill C. Battle of the bugs. Science 2008; 321:1294-5; PMID:18772416; http://dx.doi.org/10.1126/science.321.5894.1294b.
39. Sleator RD, Hill C. New frontiers in probiotic research. Lett Appl Microbiol 2008; 46:143-7; PMID:18028323; http://dx.doi.org/10.1111/j.1472-765X.2007.02293.x.

40. Sleator RD, Hill C. Rational design of improved pharmabiotics. J Biomed Biotechnol 2009; 2009:275287; PMID:19753318; http://dx.doi.org/10.1155/2009/275287.
41. Sleator RD, Hill C. Patho-biotechnology: using bad bugs to do good things. Curr Opin Biotechnol 2006; 17:211-6; PMID:16459072; http://dx.doi.org/10.1016/j.copbio.2006.01.006.
42. Haardt M, Kempf B, Faatz E, Bremer E. The osmoprotectant proline betaine is a major substrate for the binding-protein-dependent transport system ProU of Escherichia coli K-12. Mol Gen Genet 1995; 246:783-6; PMID:7898450; http://dx.doi.org/10.1007/BF00290728.
43. Vieira J, Messing J. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 1982; 19:259-68; PMID:6295879; http://dx.doi.org/10.1016/0378-1119(82)90015-4.
44. Premaratne RJ, Lin WJ, Johnson EA. Development of an improved chemically defined minimal medium for Listeria monocytogenes. Appl Environ Microbiol 1991; 57:3046-8; PMID:1746963.
45. Park SF, Stewart GSAB. High-efficiency transformation of Listeria monocytogenes by electroporation of penicillin-treated cells. Gene 1990; 94:129-32; PMID:2121618; http://dx.doi.org/10.1016/0378-1119(90)90479-B.
46. Sleator RD, Gahan CG, Hill C. Mutations in the listerial proB gene leading to proline overproduction: effects on salt tolerance and murine infection. Appl Environ Microbiol 2001; 67:4560-5; PMID:11571156; http://dx.doi.org/10.1128/AEM.67.10.4560-4565.2001.
47. Ripio MT, Vázquez-Boland JA, Vega Y, Nair S, Berche P. Evidence for expressional crosstalk between the central virulence regulator PrfA and the stress response mediator ClpC in Listeria monocytogenes. FEMS Microbiol Lett 1998; 158:45-50; PMID:9453154; http://dx.doi.org/10.1111/j.1574-6968.1998.tb12798.x.
48. Considine KM, Sleator RD, Kelly AL, Fitzgerald GF, Hill C. Novel listerial genetic loci conferring enhanced barotolerance in Escherichia coli. J Appl Microbiol 2011; 110:618-30; PMID:21223465; http://dx.doi.org/10.1111/j.1365-2672.2010.04924.x.