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

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Recommended Citation
Bioengineered. (2013). A single point mutation in the listerial betL σA-dependent promoter leads to improved osmo- and chill-tolerance and a morphological shift at elevated osmolarity. Available at: https://www.tandfonline.com/doi/full/10.4161/bioe.24094.

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To cite this article: Roland F Hoffmann, Susan McLernon, Audrey Feeney, Colin Hill & Roy D Sleator (2013) 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, Bioengineered, 4:6, 401-407, DOI: 10.4161/bioe.24094

To link to this article: https://doi.org/10.4161/bioe.24094

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Published online: 11 Mar 2013.
A single point mutation in the listerial betL σA-dependent promoter leads to improved osmo- and chill-tolerance and a morphological shift at elevated osmolarity

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Keywords: Listeria, osmotolerance, chill-tolerance, salt stress, twisted cells

Introduction

A characteristic feature of the intracellular foodborne pathogen Listeria monocytogenes is its ability to thrive in a variety of stressful environments. This phenotypic robustness can be attributed at least in part to 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.

Previous work in our lab and others 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 79 mM; Vmax 134 nmol/min/mg of protein) sodium-motive force dependent secondary betaine uptake system (BetL), which is a member of the BCC1 family of osmolyte transport systems. Detailed in silico analysis revealed the presence of two putative promoter regions; a σA-like promoter and a σB-dependent promoter, respectively. However, while transcriptional control through alternative sigma factors is important, the final yield of BetL protein is likely determined by translation efficiency. 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. 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.

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 [t1/2], 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.

We outline the use of a random mutagenesis strategy, employing the Epicurian coli® mutator strain XL1-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 σA-like promoter, resulted in a dramatically improved osmo- and chill-tolerance phenotype when expressed against both E. coli MKH13 and L. monocytogenes LO28BCG® 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).

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. In silico analysis revealed the presence of a consensus σA-dependent promoter-binding site downstream of a putative σA-like promoter, suggesting that in addition to being regulated
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In an effort to improve BetL-mediated osmotolerance, a random mutagenesis strategy was employed to introduce point mutations into the cloned listerial betL gene. The resulting construct, designated pRS1, was transformed into the mutator strain 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 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 pC372 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 QuickChange®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 LO28ΔBCGSOE; a strain which is devoid of betaine uptake. When expressed against the Listeria background, a significant advantage of pPL2 (the pRS2 and pRS3 backbone) over the multi-copy cytoplasmic pC372 plasmid is that pPL2 integrates as a single copy in the listerial chromosome (specifically the

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.15 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.4

In the current study, the complete betL gene (under the transcriptional control of its native promoters) was amplified from pCPL1 (using the primer pair betLPrl/FetLrLXLd) and cloned into pC372, 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 E. coli into pCI372, a shuttle vector capable of replicating in both E. coli and L. monocytogenes. LO28ΔBCGSOE; a strain which is devoid of betaine uptake. 15

**Figure 1.** DNA sequence upstream of the betL coding region. Inverted repeats, indicated by pairs of arrows, delineate a likely rho-independent transcriptional terminal signal (ΔG~13 kcal). The predicted ribosome-binding site (RBS) and the putative σ70- and σ28-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 (Δ betL), E. coli MKH13: pRS3 (Δ betL), and E. coli MKH13: pPL2 (negative control) in M9 minimal medium containing 2 mM betaine and 5% added NaCl. A set of data points is shown for each condition. The error bars represent the standard deviation of the mean.

**Figure 3.** Log OD/605 vs. Time (h) for E. coli MKH13: pRS2 (Δ betL), E. coli MKH13: pRS3 (Δ betL), and E. coli MKH13: pPL2 (negative control) in M9 minimal medium containing 2 mM betaine and 5% added NaCl. A set of data points is shown for each condition. The error bars represent the standard deviation of the mean.
rRNA \( ^{+}\)-attB in both serotype 1/2 and 4b strains of \textit{L. monocytogenes} \(^{14,17}\), thereby removing plasmid copy number as a variable in assessing the true contribution of \textit{betL} to \textit{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 (–35 promoter binding sites, it seemed plausible that the increased osmotolerance. This phenomenon and has been reported previously in the literature \(^{18}\). Promoter variants in genes encoding compatible solute uptake is not a new phenomenon and has been reported previously in the literature \(^{18}\). Promoter variants in genes encoding compatible solute uptake is not a new phenomenon and has been reported previously in the literature \(^{18}\). 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 \(^{21,23}\). Interestingly, \textit{prepL} (encoding betaine uptake in \textit{E. coli}) is controlled by changes in DNA topology \(^{21}\), while the osmoregulated promoter for \textit{opuA}, encoding a betaine uptake system in \textit{B. subtilis}, is likely subject to similar control. Indeed, both the osmoregulated \textit{prepL} and \textit{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 \(^{21,23}\),—the latter being the predicted distance between the –10 and –35 binding sites of the \textit{betL} \( ^{\star}\)–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. \(^{21}\). Promoters with sub-optimal spacer regions, like the \textit{betL} \( ^{\star}\)–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. \(^{21}\). Indeed, Alice and Sanchez-Rivas \(^{21}\) observed a direct link between osmotolerance and DNA supercoiling in
B. subtilis, while Grau et al. 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, 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 previous 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.

DNA manipulations and sequence analysis. Plasmid DNA was isolated using the Qiong QiAprep Spin Miniprep Kit (Qigene). E. coli was transformed by standard methods while electro-transformation of L. monocytogenes was achieved by the protocol outlined by Park and Stewart.35 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 (silico LifeSciences Ltd).

Generation and screening of betL*. Random mutagenesis was performed using the strategy outlined previously.36 Plasmid pRS1 harbouring the listerial betL gene was transformed into the mutator strain Epicurian coli NM3 (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 (Alco, Dept. Bioph., Agha).

Site directed mutagenesis. Single nucleotide additions and deletions within the putative betL ORF-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.37 Essentially L. monocytogenes cells were grown, at 37 °C with shaking, to mid-exponential phase in BHI. Ten milliliters of culture were centrifuged and re-suspended in 1 ml of BHI with 4% added NaCl, for
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., and cDNA was synthesized by adding 1 μl of 5× RT buffer (Roche), 2 μl of 100 mM dithiothreitol, 0.5 μl of a denucleoside 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.

Table 1. Bacterial strains and plasmids

| Strain or plasmid | Strains | Relevant genotype or characteristics* | Source or reference |
|-------------------|---------|--------------------------------------|--------------------|
| L. monocytogenes  | LO28    | Serotype 1/2c                         | P. Coissant, Institut Pasteur |
| L208::BCGΔopU    | LO28 ΔbetL, ΔopU, Δgbbu                 | Wemekamp-Kamphuis et al., ¹⁰⁴⁹ |
| E. coli          | MK8H13  | MC4100Δ(purPΔ101D)ΔproA2ΔproO1         | Haardt et al., ²⁷⁴⁹ |
| X1-Red           | endA1 gen::thi - F rust 7596 ups44 rolB1 lac mucO15 mucE mucT10 (Tet) | Stratagene |
| Plasmids         | pUC18   | ColE1 on, Apr                         | Viera and Messing²⁴⁹ |
|                  | pCPL1   | pUC18 containing 2.5 kb of L. monocytogenes genomic DNA | Slettor et al.,²⁴⁹ |
|                  | pC37f2  | E. coli/λ phage shuttle vector, 3.7 kb, Cmr  | Slettor et al.,²⁴⁹ |
|                  | pPL2    | Site-specific bacterial integrative vector, 6.1 kb, Cmr | Slettor et al.,²⁴⁹⁹ |
|                  | pRS1    | pC372-betL                           | This study |
|                  | RS1Δmm  | Randomly mutated RS1 from E. coli X1-Red | This study |
|                  | pRS2    | pPL2::betL*                          | This study |
|                  | pRS3    | pPL2::betL                           | This study |

*Ap*r ampicillin resistance, *Cmr* chloramphenicol resistance, *Tet* tetracycline resistance.

Table 2. Primer sequences used in this study

| Primer     | Sequence (5'-3') |
|------------|------------------|
| bet/LPrFl  | CAT CAG CAT TCC CTC GCC CCA TTA TTA CTA GGG CCA TAT C |
| bet/LPrFl  | CAT TCT AGA GCT CTA TTC CCA TTA CTA GGG CCA TAT C |
| XbaIKO     | TAA GGG CCA CCA TCT TAG ACC |
| EcoRIKO    | GCA CGA ATT CAC CAA GTA |
| pRS1       | TTG CTC TTC CAA TGT TAG |
| L2:        | GAG TGG TTA ATG TTT GAT |

*Nucleotides introduced to create restriction sites are underlined.

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No potential conflicts of interest were disclosed.

Megaview-III digital camera and analySIS software.

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