Transcriptional Regulation and Posttranslational Activity of the Betaine Transporter BetL in *Listeria monocytogenes* Are Controlled by Environmental Salinity

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While the genetic elements contributing to the salinity tolerance of *Listeria monocytogenes* have been well characterized, the regulatory signals and responses (genetic and/or biochemical) that govern these mechanisms have yet to be elucidated. Encoded by betL, the first genetic element to be linked to listerial osmohomeostasis, the secondary betaine uptake system BetL is a member of the betaine-carnitine-choline transporter family. Preceded by consensus σ^+^- and σ^−^-dependent promoter sites, betL is constitutively expressed and transcriptionally up-regulated in response to salt stress. The nisin-controlled expression system was used to achieve salinity-independent, controlled betL expression in *Listeria*. In the absence of NaCl-activated transcriptional control, BetL activity was found to be a function of environmental salinity, showing optimal activity in buffer supplemented with 1 to 2% NaCl (osmolality, 417 to 719 mosmol/kg). In addition, BetL was activated rapidly (half-life, 2 min) in response to an osmotic upshift imposed by adding 2% NaCl to 50 mM potassium phosphate buffer.

The ubiquitous food-borne pathogen *Listeria monocytogenes* is highly adapted to life in challenging environments (10). The ability of the organism to survive, and indeed thrive, at elevated osmolarities and reduced temperatures is attributed mainly to the accumulation of osmo- and cryoprotective compounds termed osmolytes, or compatible solutes (13, 27). Indeed, recent evidence suggests that osmolyte uptake in *L. monocytogenes* is linked not only to the ability of the organism to grow and survive in foods but also to the ability of the organism to cause infection (26, 29). The preferred compatible solute for the majority of bacteria, and the most effective osmolyte in *L. monocytogenes*, is the trimethyl ammonium compound glycine betaine. Present at relatively high concentrations in foods of plant origin (22), betaine has been shown to stimulate the growth of *L. monocytogenes* between 0.3 and 0.7 M NaCl, resulting in a 2.1-fold increase in the growth rate at 0.7 M NaCl (1) and a 1.8-fold increase at 4°C (13).

Although betaine was previously believed to be accumulated by a single transporter (20), recent genetic analysis revealed that *L. monocytogenes* takes up betaine via more than one system (28). The principal transporters include the multicomponent, ATP-dependent GbuABC system (12) and the ion-motive-force-dependent secondary transporter BetL (24). Each system exhibits distinct substrate specificities and kinetic parameters and thus is presumably optimized for maximal effects in diverse ecological niches (29).

Since the osmolyte transport systems of *L. monocytogenes* have been cataloged (28), the next major challenge is to elucidate the individual contribution of each system to the overall salt stress response. Determining how and when individual systems are activated, to what extent, and in response to which signal(s) (internal or external salinity and/or osmolality, turgor pressure, or related parameters, such as membrane tension) will ultimately provide a means of predicting when, and how quickly, the organism reacts. It is envisaged that this information will eventually facilitate the design of effective control measures for restricting the spread of the pathogen, both in foods prior to ingestion and subsequently within the animal host.

Studies of solute accumulation by other organisms have revealed that osmoprotectant uptake may be controlled at the levels of both transporter gene expression and transporter activity (32). For example, transporters BetP and EctP of *Corynebacterium glutamicum*, both of which are BetL sequence homologues, can be osmotically activated (18). Previously, we demonstrated that betL is osmoregulated at the transcriptional level (25). Using the nisin-controlled expression (NICE) system for salinity-independent gene expression (3), we now demonstrate that BetL is itself activated in response to changes in salinity. Rapid activation of preexisting 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 medium salinity.

**Materials and Methods**

**Media, chemicals, and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. *L. monocytogenes* strains were grown in brain heart infusion (BHI) broth (Oxoid, Unipath Ltd., Basingstoke, United Kingdom). Nisin (Sigma Chemical Co., St. Louis, Mo.) was prepared in 0.05% acetic acid (100 mg/ml) and diluted 1:10 in dimethyl sulfoxide. 1-[^14]C- radiolabeled N,N,N-trimethylglycine (55 mCi/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, Mo.). Antibiotics were prepared as concentrated stocks as described by Maniatis et al. (15) and added to media at the required levels. When necessary, the medium salinity was adjusted by adding NaCl and osmolality was measured with a vapor pressure osmometer (Wescor, Logan, Utah).

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DNA manipulations and sequence analysis. Restriction enzymes, RNase, and T4 DNA ligase were obtained from Boehringer GmbH (Mannheim, Germany). Plasmid DNA was isolated with the QIAprep spin miniprep kit (QIAGEN, Hilden, Germany). Escherichia coli was transformed by standard methods (15), while electrodtransformation of L. monocytogenes was achieved by the protocol outlined by Park and Stewart (19). PCR reagents (Taq DNA polymerase and deoxynucleoside triphosphates) were purchased from Boehringer and used according to the manufacturer’s instructions with a PCR express system (Hybaid, Middlesex, United Kingdom). Unless otherwise stated, PCR was carried out following lysis of cells with Igepal CA-630 (Sigma). Oligonucleotide primers used for PCR and sequence purposes were synthesized on an Oligo 1000M DNA synthesizer (Beckman Instruments, Inc., Fullerton, Calif.). Nucleotide sequence determination was performed with an ABI 373A automated sequencer by using a dye terminator sequencing kit (Applied Biosystems, Warrington, United Kingdom).

Incorporating betL into the NICE expression system. PCR primers betL. (5’ GCTACCATATGGAAATACATACAGAGA 3’), betL. (5’ CCGCAAGCTTTTCCTTTGTGAAAAATAATCTTCAAAC 3’), and incorporated NcoI and HindIII cut sites (underlined) were used to amplify a promoterless copy of the betL. gene (47 bp upstream of the TTG initiation codon and 126 bp downstream of the TAA termination codon; encompassing the coding region and native ribosomal binding site but not the upstream promoter regions) from the chromosome of L. monocytogenes LO28. The resultant PCR product was digested with NcoI and HindIII and subsequently cloned into similarly digested pNZ8048, creating a transcriptional fusion between the nisin-inducible nisA. promoter on pNZ8048 and the promoterless betL. gene. The resultant plasmid construct, designated pCPL17 and confirmed by sequence analysis, was then cloned into LO28ΔδBG. Harboring the facilitator plasmid pNZ9530, this strain lacks the principal betaine uptake systems BetL and Gbu and thus exhibits no detectable betaine uptake. Control strains included LO28ΔδBG (pNZ8048), which contains both pNZ9530 and pNZ8048 and is devoid of betaine uptake, and LO28ΔδG, which possesses a chromosomal copy of betL. that is transcribed from its own native promoters.

Transcriptional analysis. RNA isolation and reverse transcription-PCR (RT-PCR) were carried out as previously described (25). RNA was isolated from overnight cultures following nisin induction or imposition of a salt stress. For studies of the transcriptional response to added NaCl, overnight cultures of L. monocytogenes grown at 37°C in BHI were used to inoculate fresh media at a level of 1%. When the optical density at 600 nm (OD600) of the culture reached 0.5, salt stress (4% NaCl) was applied for 30 min. For induction with nisin, cultures were grown to an OD600 of 0.2 and either induced with a 0.1% concentration of the supernatant from an overnight culture of the nisin-producing strain Lactococcus lactis NZ9700 or preinduced with 4.5 μg of nisin powder/ml for 1 h and then induced with 45 μg of nisin powder/ml (a concentration high enough to induce transcription, yet low enough to ensure no difference in the nisin sensitivities of the two strains [data not shown]) for 30 min before RNA was isolated. Following RT, primers XhrIKO and EcoRIKO, described previously (25), were used to amplify the resulting cDNA. In all cases, control PCR primers were used to confirm the complete removal of DNA from non-reverse-transcribed RNA preparations and subsequently following the RT reaction to ensure that levels of cDNA for samples that were to be compared were equal.

Transport assays. Radiolabeled betaine uptake studies were carried out as described by Cullham et al. (5), with some minor modifications. Essentially, log-phase cells grown in BHI were harvested by centrifugation, washed twice, and resuspended in 50 mM potassium phosphate buffer (pH 6.8) to an OD600 of 1.0. Glucose was added to a final concentration of 5 mM to energize the cells, and where indicated below, NaCl was added to subject the cells to salt shock. After 3 min of incubation at 25°C, assays were initiated by the addition of [14C]glycine (at a final concentration of 40 μM and a specific radioactivity of 5 Ci/mol). Cells were collected on 0.45-μm pore-size cellulose nitrate filters (Schleicher & Schuell, GmbH, Dassel, Germany, and Millipore Canada Ltd.) under vacuum. Filters were then washed with 5 ml of buffer (of the same osmolality as the assay buffer), and the radioactivity trapped in the cells was measured by liquid scintillation counting.

To determine the kinetics of activation of BetL, bacteria were prepared for transport as described above, uptake was initiated with betaine after preincubation in the standard assay mixture supplemented with 2% NaCl (incubations ranged from 10 s to 20 min), and initial uptake rates were determined. Nonlinear regression was used to fit the resulting data to the following relationship: vt = vt0 1 - e-αt + vt0e-αt, where vt is the initial rate of betaine uptake at time t, vt0 is the initial rate of betaine uptake at an infinite time after activation, and α is the activation rate constant. t1/2 for transporter activation is equal to ln 2/α.

RESULTS AND DISCUSSION

Controlled expression of betL. The NICE system (3) was used to overcome salt-induced transcriptional control of the betL gene and to determine whether BetL is regulated at the biochemical level in response to increasing salinity. Originally developed in lactic acid bacteria (11) and subsequently demonstrated to function in a variety of gram-positive bacteria (7), the system consists of two compatible replications: the regulatory plasmid pNZ9530, carrying the nisRK regulatory genes, and the expression vector pNZ8048, harboring the nisA promoter (PnisA). In the presence of nisin and, to a lesser extent, galactose and lactose (4), transcription is induced from PnisA in a dose-dependent manner. In the case of nisin, induction is via
We suggest that this consequence of nisin-independent induction of PnisA encoding by pCPL17 was comparable to that of the wild type added nisin, the transcript level for iments. Interestingly, it was observed that in the absence of BG for use as a negative control in subsequent exper-

ments in BHI under nonstress conditions (Fig. 1). To avoid changing the initiation codon from TTG to ATG and to directly determine the effects of increasing salinity on BetL activation, independently of the regulation of betL transcription.

The host strains used in this study were L. monocytogenes LO28ΔG, in which the chromosomally encoded wild-type BetL (regulated by salinity at the transcriptional level) is the only remaining functional betaine transporter, and LO28ΔBG, which is completely devoid of betaine uptake (31). To remove the betL gene from salt-induced transcriptional control, a promoterless copy of betL was cloned downstream of the Pnis promoter between the NcoI and HindIII cut sites on pNZ8048. To avoid changing the initiation codon from TTG to ATG and thus possibly affecting the translational control of the resulting gene construct (28), the betL Fnis primer was designed to incorporate the native betL ribosomome-binding site and a termination codon to prevent read-through from the ATG start codon at the NcoI site. The resulting construct, designated pCPL17, was subsequently transformed into LO28nΔBG (harboring pNZ9530) to form LO28nΔBG(pCPL17). Plasmid pNZ8048 lacking an insert was also introduced into LO28nΔBG for use as a negative control in subsequent experiments. Interestingly, it was observed that in the absence of added nisin, the transcript level for betL nis (the betL construct encoded by pCPL17) was comparable to that of the wild type following growth in BHI under nonstress conditions (Fig. 1).

We suggest that this “leaky” transcription is most likely a consequence of nisin-independent induction of PnisA by lactose and galactose, which are present in the growth medium (4). Proof that the NICE system is indeed functional and that native-salt-induced transcriptional control has been replaced by salinity-insensitive PnisA induction was obtained by RT-PCR transcriptional analysis of LO28ΔG and LO28nΔBG (pCPL17) (Fig. 1). Exposure of LO28ΔG to salt stress resulted in an increase in transcript levels similar to that observed previously (25). However, no transcriptional up-regulation was observed for LO28nΔBG(pCPL17), proving that salt-induced transcriptional up-regulation had been removed. In contrast, while the addition of nisin had no effect on betL transcription in LO28ΔG, transcription was significantly induced in LO28nΔBG(pCPL17). Densitometric analysis revealed a ca. eightfold increase in the betL transcript level following exposure to 45 μg of nisin/ml. This increase compares with the 10- to 11-fold induction observed previously for Bacillus subtilis (7).

BetL activity is determined by environmental salinity. As expected, the transporter-deficient strain LO28ΔBG had no detectable betaine uptake in potassium phosphate buffer (50 mM) in the presence or absence of added NaCl (3%) (Table 2). Thus, at least under the conditions tested (NaCl added at 0 to 6%) (Fig. 2), this strain is devoid of betaine uptake activity and provides an ideal background for the study of BetL activity in vivo. Strain LO28ΔG (betL<sup>−</sup>) did not take up betaine in the absence of a salt stress, but the addition of 3% NaCl resulted in a significant increase in the betaine uptake rate. Since a

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Glycine betaine uptake rate (nmol/min/mg of protein)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>LO28ΔG</td>
<td>Δgbu, wild-type betL</td>
<td>ND 2.8</td>
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<tr>
<td>LO28ΔBG</td>
<td>ΔbetL Δgbu</td>
<td>ND ND</td>
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<tr>
<td>LO28nΔBG(pCPL17)</td>
<td>ΔbetL Δgbu, betL under PnisA control</td>
<td>ND 2.5</td>
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<sup>a</sup> ND, no detectable betaine uptake.

**FIG. 2.** Impact of NaCl on glycine betaine uptake via BetL. Betaine uptake rates were measured as a function of NaCl concentration for L. monocytogenes LO28nΔBG(pCPL17) (●) and the control strain, LO28nΔBG(pNZ8048) (○). Each point represents the mean ± standard error from four independent experiments.
comparable result was obtained with strain LO28nΔBG (pCPL17), in which betL expression is salinity independent. BetL is itself activated by salt stress (Table 2). Previously, Gerhardt et al. (9), employing an in vitro-based approach, also identified a betaine-specific permease that is activated by salt and can function in membrane vesicles. We suggest that this transporter (later named betaine porter I) and BetL are in fact one and the same. In further support of this proposal is the observation that no porter I activity can be detected against a betL null background (2, 31).

To determine the salt concentration at which BetL activity is maximal, the initial rate of betaine uptake was determined over a range of NaCl concentrations (Fig. 2). The uptake rate was optimal between 1 and 2% NaCl. The coupling ion for betaine uptake via BetL is likely to be Na⁺, as it is for other members of the betaine-carnitine-choline transporter family, including the BetL functional homologue BetP of C. glutamicum (21, 23). The Kₚ of BetP for Na⁺ is 4 mM (21). The increasing betaine uptake activities via BetL observed as the NaCl concentration rose to 1% (0.17 M) may in part reflect limiting concentrations of Na⁺ as the counterion for betaine symport, but they are also likely to indicate osmotic activation of BetL in that range. The decreased activity of BetL at NaCl concentrations greater than 2% (0.33 M) may result from the impact of high salinity on the maintenance of the sodium motive force generated through respiration. In any case, the salt concentration for which BetL is maximally active (1 to 2%) is significantly lower than 4%, the salt concentration at which maximum betaine uptake was previously reported for Listeria (13). This observation is strengthened by the fact that the salinity and osmolality of the base medium used in the present study (50 mM potassium phosphate buffer) were significantly lower than those of the modified Pine’s medium used by Ko et al. (13) (70 mmol/kg as opposed to 210 mmol/kg). Taken together with the finding of Mendum and Smith (16) that inactivating Gbu significantly affects betaine uptake only at NaCl concentrations greater than 4%, this result suggests that while the multicomponent Gbu system most likely plays the dominant role at high NaCl concentrations, it is the BetL system which appears to be most important at lower salinities.

Kinetics of activation for BetL. Having determined the salt concentration at which BetL activity was optimal, our next step was to establish the rate of activation, i.e., how quickly the protein reacted to an imposed increase in salinity (Fig. 3). The kinetics of BetL activation could be described by the formula vₚ = v₀(1 - e⁻Kt) + v₀e⁻Kt. BetL activity increased from 1.5 nmol/min/mg of protein to approximately 3 nmol/min/mg of protein with a t½ of 2 min. The t½ for the activation of ProP in E. coli following a hyperosmotic shift imposed with NaCl is similar (17). This rapid activation of BetL is consistent with the finding of Mendum and Smith (16) that even in a gbu mutant, the rate of betaine uptake immediately following an increase in salinity was indistinguishable from that in the wild type. Taken together, these results suggest that activation of preexisting BetL protein represents the most immediate response to increased salinity. A recent report by Fraser et al. (8) showed that following exposure to 3% (0.5 M) NaCl, betL and gbu were induced to approximately the same extent. Thus, it is unlikely that the dominance of BetL activity immediately following an increase in salinity arose from differences in expression levels.

Conclusions. The immediate response of preexisting BetL protein to relatively low salt concentrations (1 to 2% NaCl) suggests that this secondary betaine uptake system represents one of the primary respondents to rapid changes in medium salinity, whereas during prolonged exposure, it is the Gbu system that is most important. Thus, in addition to being tailored for optimal effects in diverse environmental niches (29), each system appears to prevail in a distinct time frame and salinity range within the overall salt stress response.

Since we have demonstrated that BetL is activated at the biochemical level independently of transcriptional control, the next step is to determine whether BetL functions as a sensor as well as a modulator of osmotic activity and indeed whether the protein reacts to other osmotic stressors, such as glucose and sucrose, in a similar manner. Given that the NICE system is controllable in a dynamic range of >1,000-fold, with induced protein levels reaching 60% of the total intracellular protein (11), an obvious advantage of the system is that it provides a convenient method for overproducing BetL. The isolated protein can subsequently be purified, reconstituted in proteoliposomes, and used for future in vitro analysis. It is envisaged that the data obtained from this in vitro approach will ultimately reveal whether BetL functions as an osmosensor and, if so, what osmotic signal(s) is sensed.

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REFERENCES