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Identification and Disruption of BetL, a Secondary Glycine Betaine Transport System Linked to the Salt Tolerance of *Listeria monocytogenes* LO28

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The trimethylammonium compound glycine betaine (*N*,*N*,*N*-trimethylglycine) can be accumulated to high intracellular concentrations, conferring enhanced osmo- and cryotolerance upon *Listeria monocytogenes*. We report 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.

In the early 1980s a number of major outbreaks of human listeriosis established *Listeria monocytogenes* as an important foodborne pathogen (13). Even allowing for improvements in diagnostic techniques and greater awareness, the incidence of listeriosis appears to be increasing (26). This is extremely significant given that mortality rates of 23% have been reported for the organism (36). *L. monocytogenes* can survive a variety of environmental stresses, growth having been reported at NaCl concentrations as high as 10% (30) and at temperatures as low as −0.1°C (39). The ability of the organism to withstand hostile environments is illustrated by an outbreak of listeric septicemia which was linked to consumption of salted mushrooms (7.5% NaCl) stored at low temperatures (17). 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 trimethylamino 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 (2), resulting in a 2.1-fold increase in the growth rate at 0.7 M NaCl (3) and a 1.8-fold increase at 4°C (20). Patchett et al. (32) described glycine betaine uptake in *L. monocytogenes* as a highly specific, constitutive, energy-dependent system which was subsequently shown to be Δψ-driven via cotransport with Na^+ (11) and regulated at the protein level by a novel osmolyte-sensing mechanism (37). 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 (4).

While much information regarding the physiological characterization 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* (18) and two binding-protein-dependent transport systems, *opuA* (19) and *opuC* (*proU*) (25). The secondary transport system *betP*, isolated by Peter et al. (33), is involved in glycine betaine accumulation in *Corynebacterium glutamicum*.

In this communication, we describe the isolation, characterization, 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

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 (29). *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-sterilized 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. (29) 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 (16). Plasmid DNA was isolated with the Qiagen QIAprep spin miniprep kit (Qiagen, Hilden, FRG). *E. coli* was transformed by standard methods (29), while electrotransformation of *L. monocytogenes* was achieved by the protocol outlined by Park and Stewart (31). Restriction fragments were isolated with the Qiaex II gel extraction kit (Qiagen). PCR reagents (*Taq* polymerase and deoxynucleoside triphosphates dNTPs) were purchased from Boe-

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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*lacU169*(ϕ80*lacZ*ΔM15)R17 *recA1 endA1 gyrA96 thi-1 relA1*	12
MKH13	MC4100Δ(*putPA*)101Δ(*proP*)2Δ(*proU*)	19
Plasmids		
pUC18	Ap^r ColE1 *ori*	38
pCPL1	pUC18 containing 2.5 kb of *L. monocytogenes* genomic DNA	This study
pVE6007	Cm^r Ts derivative of pWV01	28
pORI19	Em^r Ori$^+$ RepA$^-$ *lacZ′*	24
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.

hrnger 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 synthesizer (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 analyses were done by using Lasergene (DNASTAR Ltd., London, United Kingdom). Homology searches were performed with the BLAST program (1).

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 site 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 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 the failure of the truncated plasmid to complement MKH13.

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. (24). 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 551-bp fragment (nt 703 to 1253 [Fig. 1]) from the center of the *betL* gene was generated by PCR with primers *Xba*IKO (5′ TAAGCGCCACTCTAGACC 3′) (nt 703 to 720 [Fig. 1]) and *Eco*RIKO (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*RI and ligated into similarly digested pORI19 to give pCPL2 (Fig. 1), which was then transformed into *L. monocytogenes* LO28G (LO28 harboring pVE6007). A temperature upshift 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 402 to 423 [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 (10) 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 [^{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 energize the cells, and where indicated, 3% NaCl was added to subject the cells to osmotic upshock. After 20 min of incubation at 30°C, assays were initiated by the addition of [^{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 paper have been submitted to GenBank and assigned accession no. 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 synthesize 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 characterization. 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 1729. 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 (34). 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 (4). Upstream of the putative promoter site is a short inverted repeat with a ΔG of −13 kcal 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

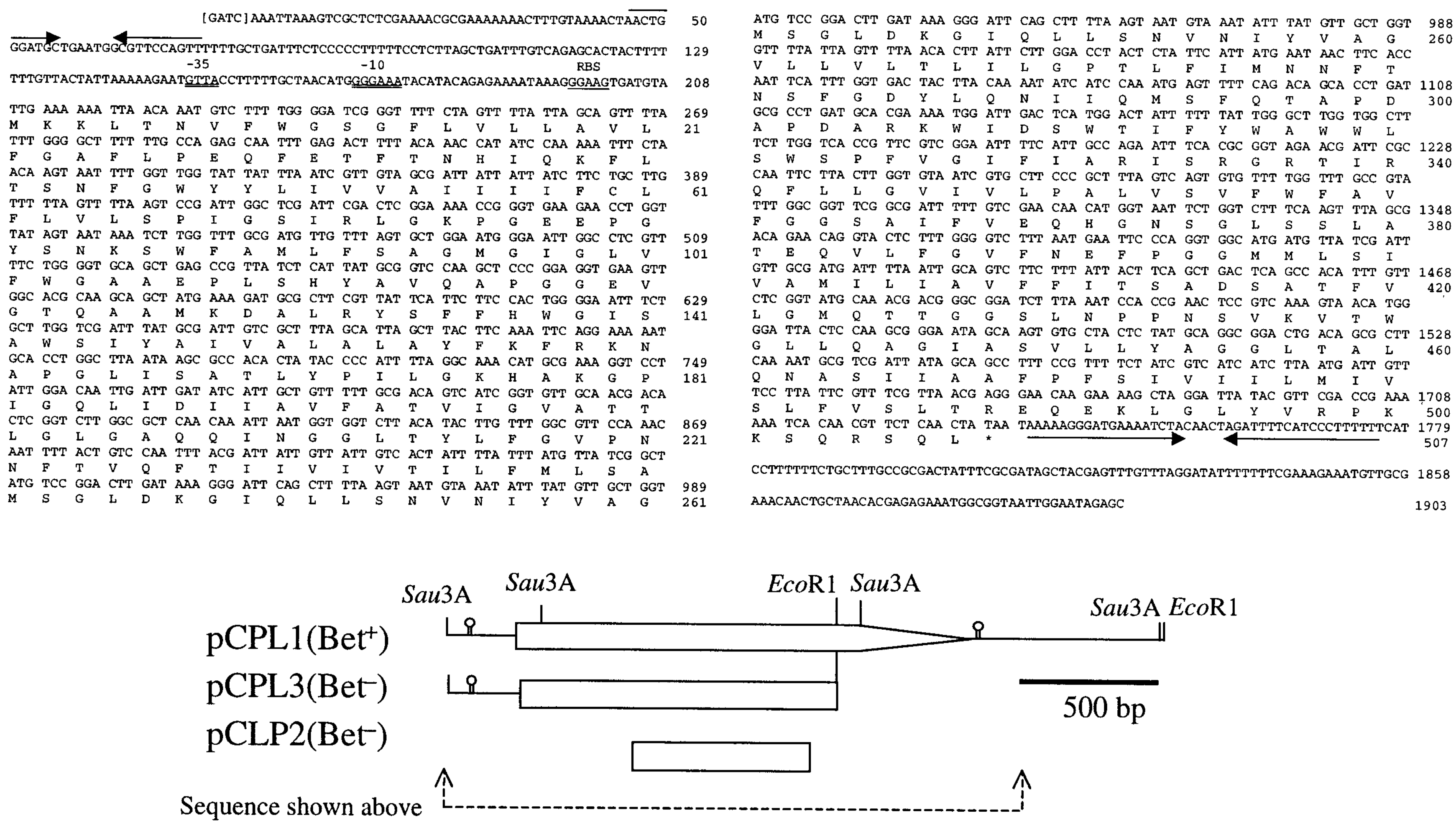

FIG. 1. DNA sequence of the *betL* gene and deduced amino acid sequence of the BetL protein. The likely ribosome-binding site (RBS) and the putative σ^B-dependent −10 and −35 sites are underlined. Inverted repeats are indicated by pairs of arrows. A graphic illustration of the cloned fragment of LO28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

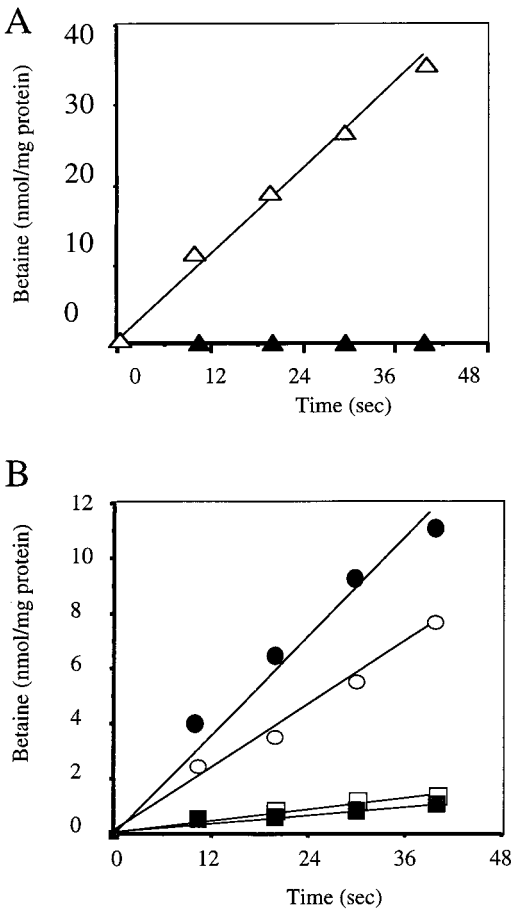

FIG. 2. (A) BetL-mediated glycine betaine uptake in *E. coli* MKH13. Uptake of [1-^{14}C]glycine betaine was assayed in low-osmolarity cultures at a final substrate concentration of 10 μM. *E. coli* MKH13(pCPL1) (BetL$^+$) was grown in M9 medium to mid-log phase and assayed for glycine betaine uptake (△). 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 energized 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, [^{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.

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 (22) from *E. coli* (38% identity) and two gram-positive secondary transporters, OpuD from *B. subtilis* (57% identity) and BetP from *C. glutamicum* (41% identity). Both OpuD (18) and BetP (33) 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 (21), 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 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* (18), its actual function is as yet unknown.

Analysis of BetL kinetics in *E. coli* MKH13. Uptake studies using [^{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^{-1} mg of protein^{-1} 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 (37). Since no measurable uptake of [^{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, we assume that either the σ^B-dependent *Listeria* promoter is recognized 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 LO28B were grown to stationary phase in BHI broth, serially diluted in Ringers, and plated on BHI agar containing an additional 4% NaCl. While LO28 gave large colonies within 48 h at 37°C, LO28B was able only 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 (6, 40). Osmolytes (often referred to as compatible

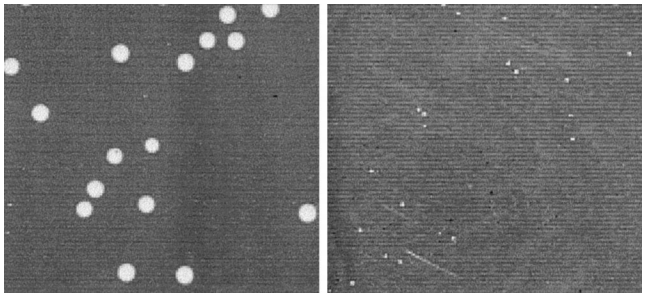

FIG. 3. Growth of *L. monocytogenes* LO28 (left) and the BetL$^-$ mutant LO28B (right) on BHI agar containing an additional 4% NaCl after 48 h at 37°C.

solutes because they can be accumulated to high intracellular concentrations without adversely affecting cellular processes) can be either taken up from the environment or synthesized de novo, and they act by counterbalancing external osmotic strength, thus preventing water loss from the cell and plasmolysis. Synthesized in relatively large quantities by plants (14), glycine betaine is the preferred compatible solute for the majority of bacteria (8, 9). 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 (5, 23), *L. monocytogenes* cannot synthesize glycine betaine from these molecules; thus, accumulation must occur via a transport system (3).

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) (7), while *B. subtilis* has three glycine betaine transport systems, OpuD, OpuA, and OpuC (18, 19, 25). 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 eucaryotic ATP-binding cassette transporters or traffic ATPases (15). Members of this family, including OpuA (19) and OpuC (25) of *B. subtilis* and ProU of *E. coli* (27), 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 (35). 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 characterized σ^B-dependent promoters (4). This is significant given that Becker et al. (4) 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. monocytogenes*. While it has been proposed that glycine betaine uptake in *L. monocytogenes* is controlled by activation of a constitutive enzyme (20) regulated by a novel osmolyte-sensing mechanism (37), 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 preexisting BetL (18).

The K_m value of 7.9 μM for BetL synthesized in *E. coli* MKH13 is similar to the value of 10 μM observed in *L. monocytogenes* (37) 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* (20). This is further evidenced by the dramatic decrease in the rate of glycine betaine uptake observed following disruption of *betL*. While nonspecific 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 characterized 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* (11, 20, 37), 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 synthesized BetL proteins.

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