APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 2002, p. 1541–1547 Vol. 68, No. 4
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.4.1541–1547.2002

Cloning of *rel* from *Listeria monocytogenes* as an Osmotolerance Involvement Gene

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Received 1 October 2001/Accepted 31 January 2002

Transposon insertional mutants of *Listeria monocytogenes* were constructed to identify genes involved in osmotolerance, and one mutant that showed reduced growth under high osmotic pressure was obtained. The cloned gene from the transposon insertion site of the mutant, named *rel*, was 2,214 bp in length and had very high homology to *relA* of *Bacillus subtilis*, which encodes guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) [collectively designated (p)ppGpp] synthetase during stringent response. The mutant showed a deficiency in (p)ppGpp accumulation. In the parental strain, the amount of intracellular (p)ppGpp was not increased after an osmotic upshift but was slightly decreased compared with the level before the upward shift. The reduced osmotolerance of the mutant was restored to a level almost equal to that of the parent strain when the chromosomal region that included *rel* of *L. monocytogenes* was introduced into the mutant. After exposure to methyl glucoside, the *rel* mutant accumulated (p)ppGpp at a higher level than the basal level and partially restored the ability to grow in NaCl-supplemented brain heart infusion broth. The mutant was found to grow in chemically defined minimal medium supplemented with glycine betaine or carnitine, so-called compatible solutes, and 4% NaCl. Our results suggest that the appropriate intracellular concentration of (p)ppGpp is essential for full osmotolerance in *L. monocytogenes* and that its mechanism is different from that for the accumulation of compatible solutes.

Listeria monocytogenes is the causative agent of listeriosis and is transmitted mainly by food to humans (6). Its characteristics, such as ubiquitous distribution in the environment (33) and strong tolerances to NaCl and refrigeration temperatures (26), make it difficult to prevent food contamination with *L. monocytogenes*. Recent studies have shown that *Listeria* accumulates so-called compatible solutes, such as glycine betaine (betaine), carnitine, and proline, under hyperosmotic stress to counteract the outward flow of water (2, 12). Such accumulations have also been observed in a wide range of other organisms, including *Bacillus subtilis* (3) and *Escherichia coli* (4). In *L. monocytogenes*, the *betL* gene and *gbu* operon have been identified as betaine transporter-encoding genes (27, 13), and *opuC* has been identified as a carnitine transporter-encoding operon (7). Disruption of these genes reduces the osmotolerance of *L. monocytogenes* (7, 13, 27). Both *betL* (7) and *opuC* (27) have σ^B-dependent promoters, and the *sigB* (the σ^B-encoding gene) mutant strain also shows a reduced ability to accumulate betaine, resulting in lower osmotolerance than that in the parent strain (1). On the other hand, there are some reports about the influence of disruption of osmotic stress-related genes to virulence of *L. monocytogenes*; *sigB* (32) and *proBA* (proline transporter-encoding gene) (28) mutants showed no differences from the parental strain in rates of recovery from organs of mice. But strains with mutations in *opuC* (29) and *clpC* (22), which encodes ClpC ATPase, showed reduced virulence compared to the parental strain. To further analyze the mechanism of osmotolerance, we made transposon insertional mutants of *L. monocytogenes* to obtain mutants with decreased NaCl resistance and succeeded in obtaining one clone that showed less resistance than the parental strain. We report the identification of the *rel* gene in *L. monocytogenes* derived from the mutant, its analysis, and the involvement of (p)ppGpp, which is synthesized by the *rel* gene product on growth in NaCl-supplemented broth. We also examined whether the *rel* mutant can use extracellular compatible solutes as osmoprotectants. Finally, we examined the recovery of *rel* mutants from mice to determine the influence of *rel* gene disruption on the virulence of *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this study are shown in Table 1. *L. monocytogenes* strains were grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.), and *E. coli* was cultured in L broth (24). Plasmid pUC18 (Toyobo, Osaka, Japan) was used to clone fragments for sequencing. pMK4 (provided by A. Bubert) (30) is an *E. coli-B. subtilis* shuttle vector. pMK4Em was a derivative of pMK4 created by the insertion of an approximately 1.8-kb *Bam*HI fragment that contained the erythromycin (EM) resistance genes from pBR322::MudEm at the same site. Where appropriate, antibiotics were used at the following concentrations: ampicillin (AP), 150 μg/ml for *E. coli*; chloramphenicol (CM), 5 μg/ml for *L. monocytogenes* and 10 μg/ml for *E. coli*; EM, 5 μg/ml for *L. monocytogenes* and 150 μg/ml for *E. coli*. To examine the efficacy of compatible solutes in *L. monocytogenes* strains, chemically defined minimal medium (CDM medium) (2) supplemented with 4% NaCl and with 1 mM betaine (Sigma, St. Louis, Mo.), 1 mM DL-carnitine (Sigma), or 10 mM L-proline (Sigma) was used. For in vivo (p)ppGpp measurements, CDM medium was modified as follows: KH_2PO_4 and Na_2HPO_4 concentrations

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source
Strains		
L. monocytogenes EGD		Laboratory stock
L. monocytogenes ED1 (*rel*::Tn*917 lx*)		This study
L. monocytogenes ED1/pMK4Em		This study
L. monocytogenes ED1/pMK4Em*rel*		This study
E. coli DH5α		Toyobo
E. coli MC1061		Laboratory stock
Plasmids		
pDlux917	Ap^r Cm^r Em^r in *E. coli* and Cm^r Em^r in gram-positive bacteria	Shih-Tung Liu
pMK4	Ap^r Cm^r in *E. coli* and Cm^r in gram-positive bacteria	A. Bubert (28)
pUC19	Ap^r	Takara
pMK4Em	Ap^r Cm^r Em^r in *E. coli* and Cm^r Em^r in gram-positive bacteria; constructed with pMK4 *Bam*HI digest and Em^r fragment from pBR322::MudEm *Bam*HI digest	This study

were reduced to 1/200 of those in the original medium composition (low-phosphate CDM medium).

Transposon mutagenesis. Transposon Tn*917 lx*, a derivative of Tn*917ac1* (5) which contains *luxA-luxB* genes for generating transcriptional fusion and ColE1*ori* for gene cloning, was used for transposon mutagenesis. For transposon mutagenesis, the temperature-sensitive delivery vector pDlux917, provided by Shih-Tung Liu (Chang-Gung Medical College, Kwei-Shan, Taoyuan, Taiwan), which carried Tn*917 lx* was introduced into *L. monocytogenes* strain EDG by electroporation with a Gene Pulser (Bio-Rad Laboratories, Hercules, Calif.), according to the method described by Park and Stewart (19). A single colony of a strain harboring the donor plasmid pDlux917 was used to inoculate medium containing CM (5 μg/ml) and a reduced concentration (0.15 μg/ml) of EM to keep the thermosensitive plasmid, and the inoculated bacterium was incubated at 25°C. For the induction of transposition, cells were cultured at 42°C overnight in 5 ml of BHI medium containing 0.15 μg of EM per ml. To cure the episomal form of pDlux917, cells were subsequently incubated at 42°C overnight. Cultures were then plated on BHI agar containing CM at 37°C overnight. CM-resistant (Cm^r) and EM-sensitive (Em^s) colonies were isolated from each colony and purified once on the same selective plates. These colonies were streaked on BHI agar plates supplemented with 6% NaCl. Mutants whose growth was markedly reduced, as assessed by colony diameter, only on the NaCl-supplemented plates but not on normal BHI agar plates were selected. Mutants that had the single transposon in their chromosome were selected by Southern hybridization with pDlux917 as the probe and a DNA labeling kit and a digoxigenin luminescent detection kit (Boehringer Mannheim, Mannheim, Germany).

Cloning of Tn*917 lx*-flanking regions from Tn*917 lx* mutants. Tn*917 lx* has ColE1*ori*, the replication origin in *E. coli*, and an AP resistance gene. There are no *Eco*RI, *Bam*HI, or *Pvu*II sites in the region of ColE1*ori* and the AP resistance gene in Tn*917 lx*. Thus, chromosomal DNAs from NaCl-sensitive mutants were digested with either *Eco*RI, *Bam*HI, or *Pvu*II and self-ligated. The resulted plasmids were transformed into *E. coli* strain DH5α (Toyobo) and selected with AP resistance. After purification of the plasmids, the flanking region was subcloned into plasmid pUC18 and then sequenced.

DNA sequence and database screen. Purified plasmid templates created using a Qiagen plasmid mini-kit (Qiagen, Valencia, Calif.) were used in cycle-sequencing reactions with a Thermo Sequenase fluorescent labeling kit (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom) and fluorescein isothiocyanate-labeled M13 forward and reverse primers (Shimadzu, Kyoto, Japan) according to the manufacturer's instructions. All sequence samples were run on a DSQ-2000L sequencer (Shimadzu). Open reading frames (ORFs) were initially identified with DNASIS software (Hitachi, Tokyo, Japan). For subsequent analysis, each ORF was compared to the current nonredundant protein database of the GenomeNet Database Service (http://www.genome.ad.jp.) by using BLAST and FASTA software through the Internet.

PCR and cloning of the *rel* region from *L. monocytogenes*. To amplify the intact gene of *rel*, we designed a pair of primers, rel-A1 (5′-ATGGAGATAAGAGGGTGAA-3′) and rel-B1 (5′-AATCAGTAACAGACGGCCT-3′), based on the sequencing results. For PCR, TaKaRa Ex Taq DNA polymerase (Takara Shuzo, Otsu, Japan) was used. The PCR product from the chromosomal region of *L. monocytogenes* including *rel* and ORF1 was cloned into *Sma*I-digested pMK4Em shuttle vector in *E. coli* strain MC1061 and introduced into the *rel* mutant, named ED1, by electroporation. Transformants were selected for EM resistance, and their genotype was confirmed by PCR with primers rel-A1 and rel-B2 (5′-TCTCAACTAAATCTCGGCC-3′) and TaKaRa LA Taq DNA polymerase (Takara Shuzo). ED1/pMK4Em was also constructed.

Detection of in vivo (p)ppGpp synthesis in *L. monocytogenes*. The (p)ppGpp accumulation patterns in *L. monocytogenes* were assessed by the modified method of stringent response in *B. subtilis* (31). Overnight cultures in BHI were washed twice with an equal volume of low-phosphate CDM medium and resuspended in the same medium, following incubation for 1 h at 37°C with vigorous agitation. $H_3{}^{32}PO_4$ (150 mCi/ml; Amersham Pharmacia Biotech) was added to the bacterial suspension, which was adjusted at a fixed optical density (OD) and incubated at 37°C for 2 h. After labeling with isotope, an equal volume (100 μl) of bacterial suspension was used for the following experiment. For the stringent response, serine hydroxamate (Sigma), a serine metabolic inhibitor, was added at a 1.5-mg/ml final concentration. For osmotic upshift and carbon source starvation, NaCl and α-methyl-D-(+)-glucoside (Wako, Osaka, Japan), glucose metabolic inhibitor, was added at 8 and 1% (wt/vol) final concentrations, respectively. After incubation at 37°C for 30 min, stressed cells were washed twice with low-phosphate CDM medium and resuspended in 50 μl of the same medium. Labeled bacterial cell suspensions were then mixed with an equal volume of 13 M formic acid. Samples were subjected to three cycles of freezing and thawing and centrifuged at 7,500 × *g* for 5 min. Supernatants (10 μl) were spotted on polyethyleneimine-cellulose plates F (Merck, Darmstadt, Germany) and air dried. Thin-layer chromatography was performed in 1.5 M non-pH-adjusted KH_2PO_4. Relative amounts of (p)ppGpp were calculated with a GT8700 scanner (Epson, Suwa, Japan) and NIH image software (http://rsb.info.nih.gov/nih-image/).

Growth in BHI under high osmotic pressure. To assess the osmotic sensitivity of the wild-type and mutant strains of *L. monocytogenes*, aliquots of overnight cultures (100 μl) were added to 10 ml of BHI containing 7.5% NaCl or 10 ml of BHI without NaCl. Cultures were incubated at 37°C with agitation, and growth was monitored by measurement of OD at 660 nm (OD_{660}) with a mini-photo OD meter (Taitec, Koshigaya, Japan) for the intervals indicated in the figure legends. Similar assays were performed in BHI supplemented with 8% KCl or 42% sucrose. To investigate the effect of accumulated (p)ppGpp on the growth in BHI supplemented with NaCl, the *rel* mutant was grown in BHI with 3.5% NaCl after exposure to methyl glucoside. After washing and resuspension in CDM medium, bacterial cells were incubated with 1% (wt/vol) methyl glucoside for 30 min and inoculated in BHI with and without 3.5% NaCl at 37°C, followed by measurement of OD_{660} every 1 h.

Growth in CDM medium supplemented with 4% NaCl and compatible solutes. To examine the efficacy of compatible solutes in *L. monocytogenes* strains growing under hyperosmotic conditions, the OD_{660} values of the parental strain and the *rel* mutant cultured in CDM medium were measured. Bacteria were grown in BHI for 24 h at 37°C with agitation, washed twice with CDM medium to remove BHI broth, and then inoculated in CDM medium, CDM medium supplemented with 4% NaCl, or CDM medium with 4% NaCl that was supplemented either with 1 mM glycine betaine (Sigma) or 1 mM DL-carnitine (Sigma). The cultures were incubated with shaking at 37°C, and growth was monitored by measurement of OD_{660} as described above. The concentration of NaCl was reduced to 4% in this medium, while the BHI medium was supplemented with 7.5% NaCl, since the growth of *Listeria* in CDM medium supplemented with NaCl at concentrations above 5% is extremely limited even when compatible solutes are added (2).

Mouse virulence assay. Seven-week-old female BALB/c mice (Charles River, Kanagawa, Japan) were used for all infection experiments and housed at the National Institute of Public Health according to Institutional Animal Committee

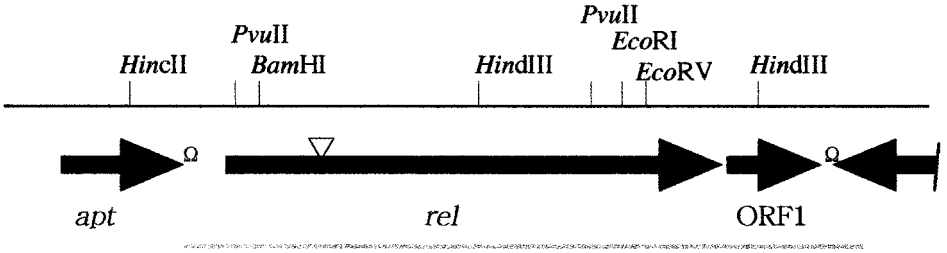

FIG. 1. Organization of the *rel* region in *L. monocytogenes* EGD. Ω, potential transcriptional terminator; ▽, transposon insertional site; solid line, chromosome region sequenced in this study and its restriction sites; black arrows, size and orientation of putative genes; gray line (bottom), chromosomal region amplified and used for complementation.

guidelines. The experiments were reviewed and approved by the Institutional Animal Committee. Approximately 2×10^8 *Listeria* cells were suspended in 0.1 ml of saline and intragastrically inoculated into five mice. The animals were sacrificed at day 3 after infection, and numbers of CFU in the spleen and liver were determined on Luria-Bertani agar plates (24). The mean values and standard deviations were calculated with StatView software (Abacus Concepts, Berkeley, Calif.). *P* values of <0.05 were considered to indicate statistical significance.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper are registered in the DDBJ/EMBL/GenBank nucleotide sequence database under accession no. AB051847.

RESULTS

Isolation of the osmotolerance-deficient mutant of *L. monocytogenes* by random Tn*917 lx* insertion into the chromosome. A total of 2,000 transposon-insertional mutants were streaked on BHI agar with and without 6% NaCl, resulting in 13 mutants on the BHI agar plates with 6% NaCl that showed reduced growth. However, Southern hybridization using *Eco*RI or *Bam*HI-digested chromosomal DNA with a digoxigenin-labeled DNA probe specific for pDlux917 suggested that 12 of these mutants had the transposon in the same fragment of the chromosome (data not shown). The remaining mutant possessed two copies of the transposon in its chromosome. Thus, only one of these 12 mutants, strain ED1, was chosen for further detailed characterization.

Cloning and sequencing analysis of the *rel* region of *L. monocytogenes*. To investigate the cause of reduced osmotolerance, the Tn*917 lx*-flanking DNA sequence was determined and the insertion site for the mutant was assigned as described in Materials and Methods. A homology search in the DNA database revealed that the mutant possessed Tn*917 lx* within a gene encoding a protein homologous to the RelA, a ppGpp synthetase, of *B. subtilis* (31) (GenBank accession no. U68377). To analyze this region in *L. monocytogenes*, an approximately 4-kb DNA fragment inserted by Tn*917 lx* from mutant strain ED1 was sequenced, and three ORFs, including a *relA* homolog and a partial ORF, were identified. The 519-bp coding region of a putative protein sequence 205 bp upstream of the *relA* homolog and transcribed in the same orientation showed 73.8% identity with adenosine phosphoribosyltransferase from *B. subtilis* encoded by *apt* (accession no. U68377). A third ORF, located 15 bp downstream of the *relA* homolog and oriented similarly, encoded a 150-amino-acid putative protein with 54.7% identity to YrvI from *B. subtilis*, the function of which is unknown (accession no. U68377). A fourth partial ORF, located downstream of the YrvI homolog and transcribed divergently, encoded a putative protein with 41.4% identity to the C-terminal half of the YrvJ protein (similar to *N*-acetylmuramoyl-L-alanine amidase) from *B. subtilis* (accession no. U68377). Based on the significant similarity between these ORFs in *L. monocytogenes* and *B. subtilis* genes, and on their conserved order on the chromosome, we designated these ORFs *apt*, *rel*, and ORF1 (Fig. 1). To clone the intact *L. monocytogenes rel* gene, chromosomal DNA from the EGD strain was used as a template for PCR with primers rel-A1 and rel-B1. Nucleotide sequencing of the cloned *rel* region revealed that a putative ribosome-binding site, GAGGG, was present 9 bp upstream of the *rel* initiation codon, and the *rel* ORF of 2,217 bp was predicted to encode a protein of 738 amino acids with a calculated M_r of 84,731. The insertion of the transposon Tn*917 lx* in ED1 was found to have occurred between A484 and G485 of the *rel* structural gene, resulting in deletion of the C-terminal 567 amino acid residues of the *rel* gene product. The deduced amino acid sequence of Rel had a very high degree of similarity to the equivalent gene products of *B. subtilis* (79.6% identity), *Streptococcus equisimilis* (17) (58.7% identity; GenBank accession number X72832), and *Staphylococcus aureus* (8) (63.3% identity; DDBJ accession number D76414) (Fig. 2).

The *rel* mutant showed reduced (p)ppGpp synthetic activity. To determine whether the *rel* homologue was functional in *L. monocytogenes*, the (p)ppGpp-synthetic activities of the wild-type strain and the Tn*917 lx* mutant ED1 were measured. The wild type was found to accumulate large amounts of (p)ppGpp in response to amino acid deprivation by treatment with serine hydroxamate (Fig. 3, lane 3). A similar but weaker pattern was observed when *L. monocytogenes* cells were transferred to medium depleted of glucose as a carbon source by addition of methyl glucoside (Fig. 3, lane 5). In contrast, the mutant strain ED1 could synthesize smaller amounts of (p)ppGpp than the wild-type strain under the conditions of amino acid depletion (Fig. 3, lane 4) and carbon starvation (lane 6), even though the amount of (p)ppGpp was increased in the mutant grown under these starvation conditions.

Effect of *rel* mutation on osmotolerance in *L. monocytogenes*. The osmotolerance of the *rel* mutant cultured in BHI broth was compared with that of the parental strain. The *rel* mutant and its vector-transformed derivative had reduced growth rates at the exponential phase of growth in BHI with and without 3.5% NaCl (final concentration, 4%), but cell density of the stationary phase of the mutant culture reached a level similar to that of the parental strain and *rel*-complemented mutant after 24 h (Fig. 4b). But in BHI supplemented with 7.5% NaCl (final

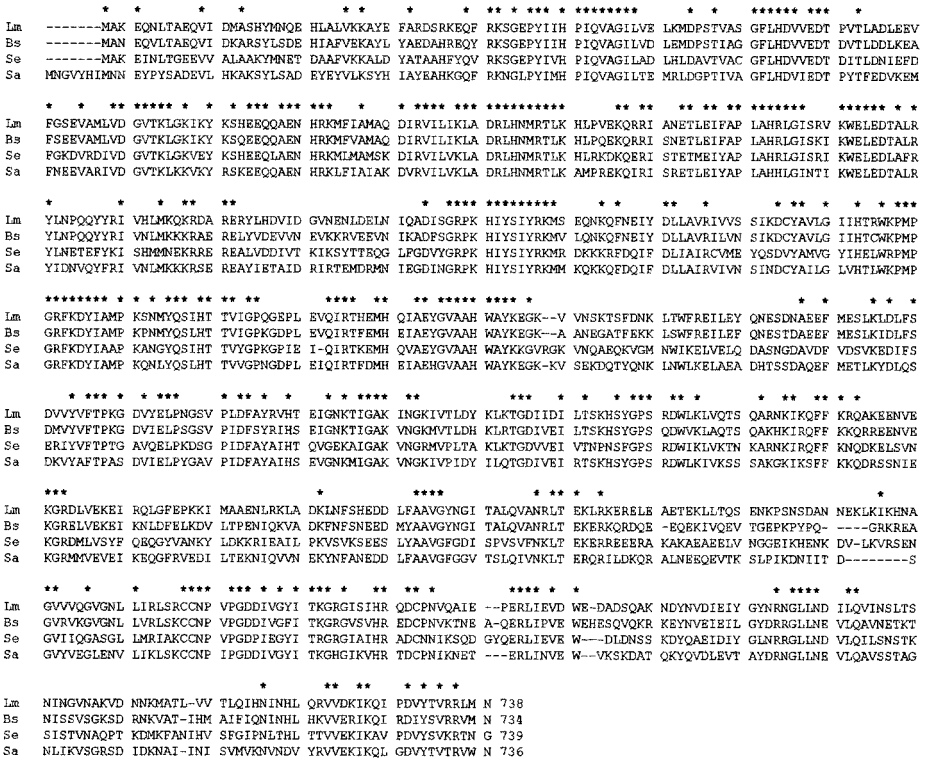

FIG. 2. Alignment of the predicted amino acid sequence of *L. monocytogenes* Rel with RelA of *B. subtilis*, Rel of *S. equisimilis*, and RelA of *S. aureus*. Asterisks indicate amino acids present in all sequences.

concentration, 8%), the *rel* mutant and its vector-transformed derivative showed limited growth, while the growth of the complemented mutant was partially restored (Fig. 4c). The growth rate of the mutant was much lower than that of the parental strain, and the cell density of the mutant culture did not reach the level of the parental strain even after 72 h. The growth of all strains was maximum at 72 h, and the OD_{660} of each strains was decreased when the incubation was extended for 96 h (data not shown). Similar results were obtained when these strains grew in BHI supplemented with KCl or sucrose (data not shown). In BHI supplemented with 12% NaCl (final concentration, 12.5%), the OD_{660} of the *rel* mutant was about one-seventh of that of the parental strain even at 72 h (data not shown). The *rel* mutant and its derivatives were grown normally in BHI broth without additional NaCl (Fig. 4a). These results showed that inactivation of *rel* caused reduction of osmotolerance in this organism. Since the *rel* mutation was suggested to affect synthesis of (p)ppGpp, we examined the effect of high concentrations of NaCl on intracellular amount of (p)ppGpp and compared the amount of (p)ppGpp in the mutant with that in the parental strain. Both the parental and mutant strains showed slightly reduced or nearly identical lev-

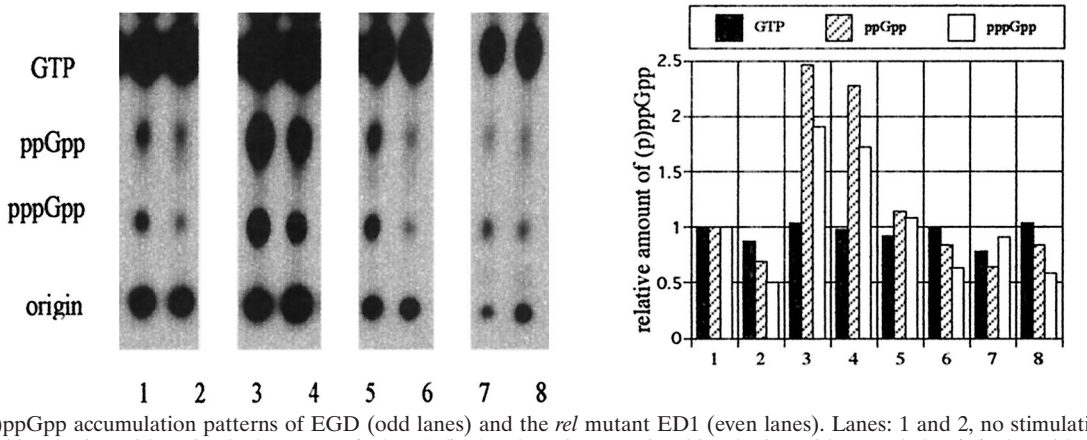

FIG. 3. (p)ppGpp accumulation patterns of EGD (odd lanes) and the *rel* mutant ED1 (even lanes). Lanes: 1 and 2, no stimulation; 3 and 4, after 30 min of incubation with serine hydroxamate (1.5 mg/ml); 5 and 6, after 30 min of incubation with α-methyl-D-(+)-glucoside (1% wt/vol); 7 and 8, after 30 min of incubation with NaCl (final concentration, 8%). The experiments were performed three times with similar results.

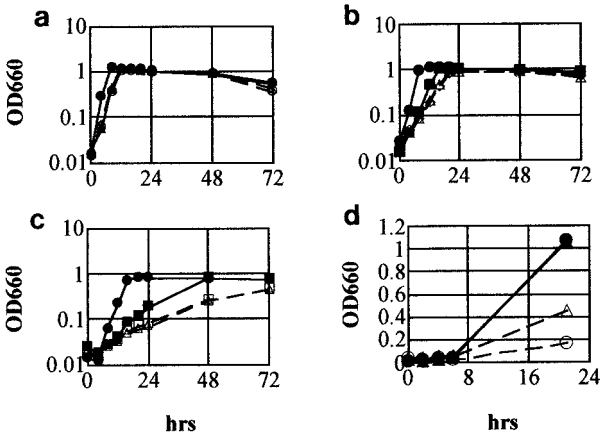

FIG. 4. (a to c) Growth of EGD (●), *rel* mutant ED1 (○), ED1/pMKEm *rel* (▲), and ED1/pMKEm (△). Cultures were grown in BHI (a), BHI with 3.5% NaCl (final concentration, 4%) (b), and BHI with 7.5% NaCl (final concentration, 8%) (c) at 37°C. (d) Growth of the *rel* mutant in BHI after 60 min of incubation in CDM medium (●) or CDM medium with methyl glucoside (1%, wt/vol) (▲) and BHI with 3.5% NaCl after 60 min of incubation in CDM medium (○) or in CDM medium with methyl glucoside (1% wt/vol) (△). The experiments were performed five times with similar results.

els of (p)ppGpp after osmotic upshift (Fig. 3, lanes 7 and 8). Next, we examined the effect of elevated intracellular (p)ppGpp on the growth of the *rel* mutant in NaCl-supplemented BHI. When the *rel* mutant was exposed to methyl glucoside for 30 min, it accumulated a small amount of (p)ppGpp; as a result, the intracellular concentration of (p)ppGpp of the mutant came close to the basal level of the parental strain (Fig. 3, lane 6). In BHI supplemented with 3.5% NaCl, the (p)ppGpp-elevated mutant grew faster than the nonstimulated mutant, whereas no differences in growth were observed between the simulated and nonstimulated mutant in BHI (Fig. 4d).

The *rel* mutant can use compatible solutes as osmoprotectants in CDM medium under 4% NaCl. Since *L. monocytogenes* can use compatible solutes, such as betaine and carnitine, as osmoprotectants (2), we further tested the ability of strain ED1 to grow in NaCl-supplemented CDM medium in the presence of betaine (Fig. 5). Inoculation of overnight cultures of both the mutant and wild-type strains inoculated into fresh CDM medium with a low NaCl concentration showed that bacterial cells were capable of growing and reaching maximum growth after 24 (wild type) or 48 (mutant) h of incubation at 37°C. However, the addition of NaCl to CDM medium at a final concentration of 4% resulted in limited growth of both strains. When the bacterial cells were inoculated into the same medium supplemented with 1 mM betaine, the growth capability of the wild-type strain was restored, although a prolonged growth lag was observed. Similarly, ED1 was able to grow to wild-type levels after 72 h of incubation in the presence of betaine. The osmotolerance of both strains was also enhanced by another osmoprotectant, carnitine (data not shown).

The *rel* mutant has a level of virulence similar to that of the parental strain. The virulence phenotype in the *rel* mutant strain was assessed by means of a mouse infection model. At 3 days postinoculation, the $\log_{10}$ CFU of strains EGD and ED1 recovered from livers were 2.989 ± 0.419 and 3.005 ± 0.496 (means ± standard deviations), respectively, and the $\log_{10}$ CFU recovered from spleens were 3.781 ± 0.470 and 2.795 ± 0.883, respectively. There was no significant difference in the accumulation of bacterial cells in the spleen or liver between the parental strain and *rel* mutant under the conditions used in this study, showing that mutation of the *rel* gene does not affect virulence in mouse infection. The experiments were performed twice, with similar results.

DISCUSSION

In this study, we identified *rel* of *L. monocytogenes* EGD as a gene involved in osmotolerance. *rel* shared very high homology to its homologues in *B. subtilis*, *S. equisimilis*, and *S. aureus* (Fig. 2). In *E. coli*, *relA* encodes the (p)ppGpp synthetase (18) and *spoT* encodes (p)ppGpp-synthetic and (p)ppGpp-degradative enzymes (25). But in *Streptomyces coelicolor*, RelA has bifunctional [(p)ppGpp-synthetic and -degradative] activities (16). Also, in gram-positive organisms, *spoT* is not identified, and Rel is thought to be such a bifunctional enzyme. Wendrich and Marahiel (31) found highly conserved residues, RKSG EPYI, common to the N-terminal region in RelA in *B. subtilis*, SpoT in *E. coli*, and Rel in *S. equisimilis* but not in RelA in *E. coli*, meaning that this residue is a putative motif responsible for the (p)ppGpp-degradative activity. We did not examine the (p)ppGpp-degradative activity of Rel in *L. monocytogenes*, but we also found the same motif at its N terminus (Fig. 2). Transposon inserted between 162 to 163 amino acids, and the mutant showed reduction of (p)ppGpp accumulation upon amino acid or carbon source starvation or osmotic shift up, but did not have a (p)ppGpp-zero phenotype (Fig. 3). This result suggests that the 162 amino acids from the N-terminal end of Rel in *L. monocytogenes* have weak (p)ppGpp synthetic activity.

In many organisms, (p)ppGpp is known as a nutritional alarmone and is accumulated in bacterial cells under nutrient-limited conditions, such as depletion of amino acid or carbon source or aminoacylated tRNA. (p)ppGpp is thought to be a stress response-related factor, because its accumulation induces inhibition of stable RNA synthesis, and the so-called

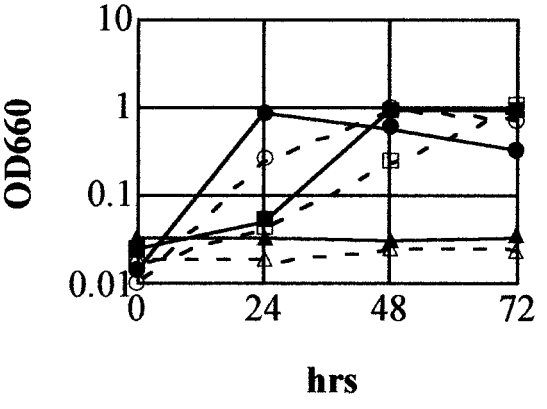

FIG. 5. Growth of EGD in CDM medium (●), CDM medium with 4% NaCl (▲), or CDM medium with 4% NaCl and 1 mM betaine (■) and ED1 in CDM medium (○), CDM medium with 4% NaCl (△), or CDM medium with 4% NaCl and 1 mM betaine (□) at 37°C. The experiments were performed three times with similar results.

stringent response occurs (4). For example, (p)ppGpp is the positive regulator of *rpoS*, which encodes the stress-responsive RNA polymerase subunit σ^S in *E. coli* (9, 15), and both induces the synthesis of proteins necessary for near-UV (300 to 400 nm) resistance in *Salmonella enterica* serovar Typhimurium (14) and increases acid tolerance in *Lactococcus lactis* (21). In addition, ppGpp accumulates in *B. subtilis* after an osmotic upshift (11), in contrast with our results in *L. monocytogenes*. In *E. coli*, mutation of *relA* affects the salt-induced acid sensitivity (23) and the suppression of temperature sensitivity by salt in the *ftsZ* (an essential gene for cell division) mutant (20).

In this study, we detected the intracellular (p)ppGpp of the *L. monocytogenes rel* mutant and its parent during stringent response and obtained the following results. (i) Like other organisms, *L. monocytogenes* accumulates (p)ppGpp under amino acid depletion induced by addition of the serine metabolic inhibitor serine hydroxamate and under carbon source depletion induced by addition of the glucose metabolic inhibitor methyl glucoside (Fig. 3, lanes 3 and 5). (ii) The amount of intracellular (p)ppGpp was reduced after an osmotic upshift (Fig. 3, lane 7). (iii) In the *rel* mutant, production of (p)ppGpp was reduced to less than that of the parental strain and became still lower after an osmotic downshift (Fig. 3, lanes 5 and 6). (iv) The growth of the *rel* mutant in the presence of a high concentration of NaCl became faster when the amount of intracellular (p)ppGpp was brought near the basal level of the parental strain by the addition of methyl glucoside (Fig. 4d). (v) The mutant showed partial restoration of osmotolerance by reinduction of the chromosomal region including *rel* (Fig. 4). From these results, we concluded that (p)ppGpp is involved in the growth of *L. monocytogenes* under high osmotic pressure and that the appropriate intracellular concentration of (p)ppGpp might also be essential to respond to osmotic stress.

The accumulation of compatible solutes to maintain cell turgor is well characterized as a bacterial osmoadaptation system (4). Disruption of the genes which encode compatible solute transporters and σ^B, which is related to promoters of these transporter genes, causes decrease of growth under high osmotic pressure (1, 7, 13). We showed that the *rel* mutant could grow at a reduced rate, but nonetheless, it reached a level nearly identical to that of the parental strain in CDM medium supplemented with NaCl and compatible solutes (Fig. 5), meaning that the *rel* mutant could use extracellular compatible solutes in 4% NaCl and that the intracellular accumulation of (p)ppGpp might be controlled by mechanisms distinct from compatible solute accumulation.

In this study, the *rel* mutant of *L. monocytogenes* was pathogenic to mice at the same level as its parent (see Results), demonstrating that the amount of (p)ppGpp accumulated via the *rel* gene is not involved in virulence, although the intracellular accumulation of ppGpp initiates the conversion from replicative to virulent forms of *Legionella pneumophila* (10).

ACKNOWLEDGMENT

This study was supported in part by grants from the Ministry of Health and Welfare of Japan.

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