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A Cold-Sensitive *Listeria monocytogenes* Mutant Has a Transposon Insertion in a Gene Encoding a Putative Membrane Protein and Shows Altered (p)ppGpp Levels†

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A cold-sensitive *Listeria monocytogenes* mutant designated cld-14 was obtained by transposon Tn*917* mutagenesis. The gene interrupted by Tn*917* in cld-14 was the *L. monocytogenes* LMOf2365_*1485* homolog, which exhibits 45.7% homology to the *Bacillus subtilis yqfF* locus. LMOf2365_*1485*, here designated *pgpH*, encodes a putative integral membrane protein with a predicted molecular mass of 81 kDa. PgpH is predicted to contain a conserved N-terminal signal peptide sequence, seven transmembrane helices, and a hydrophilic C terminus, which likely extends into the cytosol. The Tn*917* insertion in *pgpH* is predicted to result in production of a premature polypeptide truncated at the fifth transmembrane domain. The C terminus of PgpH, which is probably absent in cld-14, contains a highly conserved HD domain that belongs to a metal-dependent phosphohydrolase family. Strain cld-14 accumulated higher levels of (p)ppGpp than the wild type accumulated, indicating that the function of PgpH may be to adjust cellular (p)ppGpp levels during low-temperature growth. The cld-14*pgpH*$^{+}$ complemented strain was able to grow at a low temperature, like the parent strain, providing direct evidence that the activity of PgpH is important in low-temperature adaptation. Because of its predicted membrane location, PgpH may play a critical role in sensing the environmental temperature and altering cellular (p)ppGpp levels to allow the organism to adapt to low temperatures.

A unique feature of *Listeria monocytogenes* is this foodborne pathogen's ability to grow at refrigeration temperatures. Hence, understanding the mechanisms of *L. monocytogenes* low-temperature sensing and adaptation is essential for developing control methods that reduce public health risks and expensive product recalls.

Studies of *L. monocytogenes* exposed to cold have revealed that multiple processes are involved in low-temperature adaptation; these processes include adjustments of membrane lipid composition (1, 9), alterations in membrane transport and nutrient uptake (4, 10, 14, 24), regulation of protein synthesis (3), and reassembly of ribosomes (18). In our previous study (17) we identified 24 *L. monocytogenes* genes that were responsive to low-temperature growth. These genes were involved in regulatory responses, general stress responses, amino acid metabolism, and catabolism. Insertional inactivation of genes with Tn*917* and subsequent selection of *L. monocytogenes* mutants defective for low-temperature growth allowed us to further identify the genes and molecular mechanisms of low-temperature growth in *L. monocytogenes* (3, 25).

In this report, we describe *L. monocytogenes* strain cld-14, which carries transposon Tn*917* inserted into a gene designated *pgpH*, encoding PgpH, which contains a conserved HD domain belonging to the metal-dependent phosphohydrolase family. The levels of the highly phosphorylated guanosine nucleotide (p)ppGpp were altered in the mutant compared to the levels in the parent strain.

The accumulation of (p)ppGpp in response to amino acid starvation has been called the stringent response. In *Escherichia coli*, the *spoT* gene, encoding a protein containing the conserved HD domain, has been reported to be responsible for the hydrolysis of (p)ppGpp (22). Cellular (p)ppGpp inhibits the initiation of transcription of operons encoding rRNAs and decreases the peptide elongation rate of many transcripts at translation. Therefore, increased (p)ppGpp levels result in down-regulation of stable RNA synthesis (rRNA and tRNA), reduced synthesis of certain proteins, and up-regulation of mRNA synthesis for genes encoding enzymes involved in amino acid biosynthesis (6).

In gram-negative bacteria, two genes that encode (p)ppGpp synthetase/hydrolase activities have been described. The *relA* gene encodes (p)ppGpp synthetase, and *spoT* encodes a (p)ppGpp 3′ pyrophosphohydrolase that also has (p)ppGpp synthetase activity (6). However, several studies and database entries suggest that in gram-positive bacteria there is only one *relA*/*spoT* homologue, which encodes a protein capable of both (p)ppGpp synthesis and degradation. The *relA* gene has been reported to be responsible for (p)ppGpp synthesis after amino acid starvation in *L. monocytogenes* (23). No *spoT* homolog was found in the *L. monocytogenes* genome.

In this paper we describe a *Listeria* cold-sensitive mutant, cld-14, which has an inactivated *pgpH* gene, which codes for a metal-dependent phosphohydrolase. The loss of the phosphohydrolase function might directly correlate with the altered

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† This article is dedicated to the memory of Tricia Mason.

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TABLE 1. Oligonucleotides used for PCRs in this study

Oligonucleotide	Sequence[a]
F	GGAGCATATCACTTTTCTTGGAGAG
L1	ACGGTTGAAAACTGTACC
L2	GATACAAATTCCTCGTAG
cld-14-0	CGGAGCCAATAGTTTAC
cld-14-1	CATGATATAGGAAAAACA
cld-14-2	GCAGTTCGTTCTTCCAC
lq1	TGA**TCTAGA**CGATGTAGTCAGACACCCGCT
lq2	CAA**CTGCAG**ATTCGCCCCAATCAATCT
lq3	GAT**GGATCC**GTGAAACTAGCCAA
lq4	GCT**GGATCC**TTCGAAACGATTTTTGG

[a] Boldface type indicates restriction enzyme sites introduced into the primers.

(p)ppGpp levels in the mutant compared to the levels in the parent strain. We found that higher-than-wild-type levels of (p)ppGpp accumulated in the cld-14 mutant upon amino acid starvation, which has implications for the mechanisms used by *Listeria* for temperature sensing and adaptation to low temperatures.

MATERIALS AND METHODS

Bacterial strains and mutagenesis. *L. monocytogenes* strain 10403S was grown at 37°C in brain heart infusion (BHI) broth (BD Diagnostic Systems, Sparks, MD) as previously described (17). Mutagenesis of strain 10403S with pLTV3 carrying Tn*917* was performed as described by Camilli et al. (5). Following transformation of 10403S with pLTV3, the resulting Erm^r Lin^r Tet^r strain (DP-L910) was inoculated and grown to the stationary phase at 30°C, and bacteria having transposon insertions were selected based on their ability to grow at 41°C in the presence of antibiotic selection. This temperature switch treatment resulted in a library of bacteria with the transposon integrated into chromosomal DNA at random locations, which yielded Erm^r Lin^r Tet^s colonies. A library consisting of approximately 10,000 Tn*917* mutants was scored for impaired growth on BHI agar at 5°C, and master replica plates were incubated at 30°C. Twenty mutants showing various degrees of sensitivity to low-temperature growth conditions were isolated from the master plates, and in the present work we focused on one of these mutants, designated cld-14. The cld-14 cells were grown in BHI medium containing erythromycin (10 μg ml^{-1}) and lincomycin (25 μg ml^{-1}) at 37°C with shaking at 240 rpm to the mid-exponential phase or the stationary phase. *E. coli* strains DH5α and BL21(DE3)(pLysS) were grown at 37°C in Luria broth (LB) supplemented with 100 μg ml^{-1} ampicillin when necessary. *E. coli* strains CF1943 (*E. coli* parental strain W3110), CF1944 [*E. coli* CF1943 (*relA*::*kan*)], and CF1946 [*E. coli* CF1943 (*relA*::*kan spoT*::*cm*)] were kindly provided by M. Cashel (Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD) and were grown in LB with appropriate antibiotics (6).

DNA sequences adjacent to the 5′ end of Tn*917*. Molecular biology techniques were generally performed as described by Sambrook et al. (21). Chromosomal DNA of wild-type strain 10403S and cld-14 were prepared using a Wizard Genomic DNA purification kit (Promega, Madison, WI) as described previously (17). About 100 ng of cld-14 genomic DNA was digested with XbaI, purified by phenol-chloroform extraction, and self-ligated prior to transformation into DH5α cells. Colonies that appeared on LB agar plates containing kanamycin (50 μg ml^{-1}) carried a recombinant plasmid, designated pLTV3cld-14. DNA sequencing of pLTV3cld-14 was performed using primers L1 and L2 (Table 1) derived from Tn*917* sequences. Additional DNA sequences were obtained by primer walking using primers cld-14-1 and cld-14-2. DNA sequences were obtained by using an ABI Prism 310 and an ABI Prism dye terminator cycle sequencing Ready Reaction kit (Perkin-Elmer, Foster City, CA). Sequence analyses were performed using an NCBI BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/).

DNA sequences adjacent to the 3′ end of Tn*917*. Inverse PCR, as described by Cotter et al. (8), was used to obtain sequences near the 3′ end of the Tn*917* insertion. Briefly, about 1 μg of cld-14 genomic DNA was digested with EcoRI, purified, and ligated using T4 DNA ligase. The ligated genomic DNA was used as a template in an inverse PCR performed with primer F and primer cld-14-0 (Table 1). The PCR products were cloned into the TA vector (Invitrogen, Carlsbad, CA).

Complementation of cold-sensitive mutant cld-14. The full-length *pgpH* gene of strain 10403S (about 2.5 kb) was amplified by PCR using primers lq1 and lq2 (Table 1) and was cloned into the pTZ18 R vector at XbaI and PstI sites (introduced into primers). The 2.5-kb XbaI-PstI fragment containing a 160-bp promoter region, the full-length open reading frame, and a 280-bp 3′ terminator sequence was cloned into the XbaI and PstI sites of the pKSV7 shuttle vector (obtained from D. Portnoy, Department of Molecular and Cell Biology, University of California, Berkeley). Strain cld-14 cells were transformed with pKSV7pgpH by electroporation as described previously (20), and the resulting Amp^r and Chl^r colonies on BHI agar plates were designated the cld-14*pgpH*$^+$ strain. Analyses of growth at 10°C of cld-14*pgpH*$^+$, as well as cld-14 and 10403S, in BHI broth were performed by monitoring the optical density at 600 nm at intervals. The data represent averages from duplicate experiments.

Expression of the *pgpH* gene in *E. coli*. Using primers lq3 and lq4 (Table 1), a 1.0-kb *pgpH* fragment was amplified and subcloned, in frame with His tag coding sequences, into the pRSETa vector (Invitrogen) to overexpress the HD domain of the *pgpH* gene in BL21(DE3)(pLysS) cells. Protein was expressed according to the manufacturer's instructions. About 10 μg protein was used for electrophoresis on a 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel.

Assay of (p)ppGpp levels after amino acid starvation. Overnight cultures of *L. monocytogenes* cells were diluted and resuspended to an optical density at 600 nm of 0.5 in 100 μl of unlabeled morpholinepropanesulfonic acid (MOPS) starvation medium lacking phosphate and amino acids (7, 23). The suspensions were centrifuged, and the cells were labeled with $H_3{}^{32}PO_4$ (5 μCi ml^{-1}) in MOPS starvation medium for 30 min in the presence or absence of serine hydroxamate (1.5 mg ml^{-1}) and L-valine (0.5 mg ml^{-1}). The cell pellets were resuspended in 50 μl of MOPS medium, and then an equal volume of 13 M formic acid was added and the samples were subjected to three freeze-thaw cycles. The acid extracts were centrifuged, and 5 μl of supernatant was spotted onto polyethyleneimine cellulose (Sigma, St. Louis, MO) for thin-layer chromatography in 1.5 M KH_2PO_4. Nucleotides were visualized by autoradiography.

RESULTS AND DISCUSSION

Identification of the transposon insertion site in cld-14. Southern blot analysis revealed that there was only a single insertion of Tn*917* in strain cld-14 (data not shown). The DNA flanking the transposon in cld-14 was sequenced, and BLAST searches indicated that Tn*917* was inserted into a gene corresponding to LMOf2365_*1485* in the fully sequenced strain *L. monocytogenes* F2365 (19). Similar homologs are also present in all *L. monocytogenes* strains sequenced to date (at the loci LMOf2365_*1485*, LMOf6854_*1509*, LMOh7858_*1562*, and, in EGD-e, lmo1466) (11, 19). The gene was designated *pgpH*, and the most similar nonlisterial homolog was the *yqfF* sequence of *Bacillus subtilis* (15). Wild-type *pgpH* encodes a 718-amino-acid putative integral membrane protein with a molecular mass of 81 kDa, which has an N-terminal signal peptide sequence, seven transmembrane helices, and a C-terminal sequence predicted to extend into the cytosol (16). The C-terminal region contains an HD domain, described by Aravind and Koonin (2) as a conserved doublet of the predicted catalytic residues histidine (H) and aspartic acid (D) in the metal-dependent phosphohydrolase family. Based on its location, the Tn*917* (5.63-kb) insertion in *pgpH* should lead to a premature polypeptide consisting of 447 amino acids that is truncated at the fifth transmembrane domain. This suggests that the C-terminal HD domain is not produced in *pgpH* strain cld-14. A comparison of the growth of strain 10403S with that of cld-14 at 10°C revealed that cld-14 had a longer lag phase, a lower growth rate, and a lower final culture turbidity (Fig. 1). Complementation of cld-14 with *pgpH*$^+$ (strain cld-14*pgpH*$^+$) resulted in growth similar to that of strain 10403S at a low temperature.

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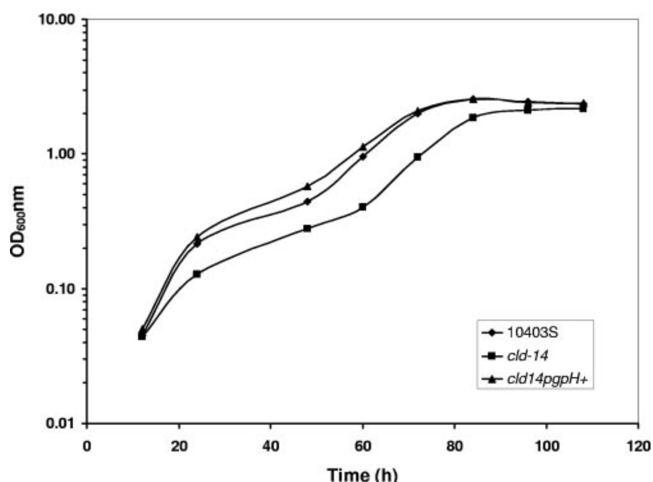

FIG. 1. Growth of *L. monocytogenes* strains 10403S, cld-14, and cld-14*pgpH*$^+$ (cld-14 complemented with *pgpH*) at 10°C. OD_{600}nm, optical density at 600 nm.

Expression of *pgpH* in *E. coli* and assay for dGTPase activities. Since the predicted HD domain of PgpH may have either dGTPase (Mg^{2+}-dependent hydrolysis of dGTP to dG and PPP_i) or (p)ppGpp synthetase/hydrolase activities (2), we attempted to express *pgpH* in *E. coli* with the goal of assaying dGTPase activity. Attempts to express the intact *pgpH* gene in *E. coli* were unsuccessful, possibly due to the transmembrane domains of the protein that prevented it from folding into a soluble polypeptide. Instead, a partial *pgpH* sequence that included the HD domain near the C terminus was expressed in *E. coli* BL21(DE3)(pLysS). Isopropyl-β-D-thiogalactopyranoside (ITPG) induced expression of an ~29-kDa protein, which indicated that a partial PgpH molecule with the HD domain was produced and was soluble (Fig. 2). dGTPase assays using the purified partial PgpH molecule containing the HD domain and dGTP, GTP, and deoxynucleoside triphosphate substrates did not show that there was P_i production based on a colorimetric inorganic phosphate assay conducted after acid hydrolysis of the reaction mixture to convert any PPP_i to P_i. Similarly, no dGTPase activity was detected with a more sensitive assay using radiolabeled [α-^{32}P]dGTP as the substrate.

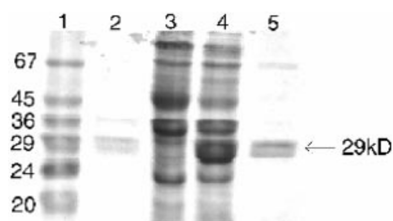

FIG. 2. Expression of the truncated PgpH protein containing the HD domain in *E. coli* BL21(DE3)(pLysS) cells. Lane 1, protein molecular weight marker; lane 2, fraction 3 of Ni column-purified fusion protein containing a His tag and the HD domain; lane 3, protein crude extract from *E. coli* BL21(DE3)(pLysS) carrying pRSETa*pgpH* without IPTG induction; lane 4, protein crude extract from *E. coli* BL21(DE3)(pLysS) carrying pRSETa*pgpH* with IPTG induction; lane 5, fraction 4 of Ni column-purified fusion protein containing a His tag and the HD domain.

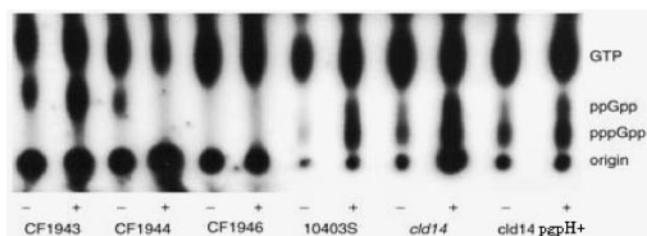

FIG. 3. Accumulation of (p)ppGpp after amino acid starvation. Bacterial cells were labeled with $H_3{}^{32}PO_4$ in MOPS starvation medium without phosphate and amino acids and incubated for 30 min with serine hydroxamate and L-valine stimulation (+) or without stimulation (−). Acid extracts were spotted onto polyethleneimine cellulose for thin-layer chromatography in 1.5 M KH_2PO_4 (pH 3.4). CF1943, *E. coli* W3110 parental strain; CF1944, *E. coli* CF1943 (*relA*::*kan*); CF1946, *E. coli* CF1943 (*relA*::*kan spoT*::*cm*); 10403S, *L. monocytogenes* 10403S; cld14, cld-14; cld14 pgpH+, cld-14*pgpH*$^+$.

Influence of *pgpH* on (p)ppGpp levels. *L. monocytogenes* and *E. coli* were subjected to amino acid starvation in the presence or absence of serine hydroxamate (1.5 mg ml^{-1}) and L-valine (0.5 mg ml^{-1}). The (p)ppGpp accumulation due to amino acid starvation is shown in Fig. 3. For the controls, the *E. coli* wild-type strain CF1943 accumulated ppGpp, the *relA* mutant strain CF1944 accumulated less ppGpp, and the *relA spoT* double mutant CF1946 did not accumulate a detectable amount of (p)ppGpp (Fig. 3). For *L. monocytogenes*, strain 10403S accumulated both pppGpp and ppGpp in response to amino acid starvation, strain cld-14 accumulated substantially more pppGpp and ppGpp than the parent strain accumulated, and strain cld-14*pgpH*$^+$, the complemented mutant, accumulated (p)ppGpp at levels that were similar to the levels accumulated by 10403S under amino acid starvation conditions but were noticeably higher than the levels accumulated by 10403S without stimulation (Fig. 3). The *pgpH* mutation resulted in a general accumulation of (p)ppGpp. The small difference between the behavior of the parent strain and the behavior of complemented strain cld-14*pgpH*$^+$ without stimulation may have been due to differences in location and copy number of the functional *pgpH* gene. In wild-type strain 10403, *pgpH* is part of the chromosome, and in complemented strain cld-14*pgpH*$^+$, *pgpH* is located in a plasmid vector. The regulation of *pgpH* may be influenced functionally by the chromosomal position of *pgpH*, which is not duplicated in strain cld-14*pgpH*$^+$. Under conditions in which there was sufficient amino acid, the levels of (p)ppGpp were similar in cld-14*pgpH*$^+$ and in the cld-14 mutant (Fig. 3), but they were more than the levels in the wild type, which may have been due to reduced expression of the plasmid-encoded enzyme. In contrast, under amino acid starvation conditions, it appeared that the *pgpH* gene in cld-14*pgpH*$^+$ became quite active, resulting in a decreased level of (p)ppGpp compared to the level in the cld-14 mutant. The PgpH activity stimulated by amino acid starvation in cld-14*pgpH*$^+$ appeared to be similar to the activity in the wild type since the (p)ppGpp level was similar to that in the wild type. These findings indicate that strain cld-14 is not able to degrade accumulated (p)ppGpp, suggesting that the (p)ppGpp hydrolase activity in this strain is defective. Since the C-terminal sequence of PgpH is not translated in cld-14, it appears that the relevant (p)ppGpp hydrolase activity resides

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in the C-terminal region of PgpH. However, it remains to be determined whether changing the Mg^{2+} concentration would directly affect the (p)ppGpp level since Mg^{2+} might play a key role in phosphohydrolase enzymatic activity. Although informatics evidence supports the hypothesis that PgpH has a phosphohydrolase function, additional studies are needed to rule out the possibility that the increased level of (p)ppGpp might be a more general effect triggered by the *pgpH* mutation rather than directly due to loss of PgpH activity, because other factors could contribute to pppGpp production and metabolism.

(p)ppGpp accumulation and low-temperature growth. Under low-temperature growth conditions, cld-14 grew poorly compared to the parent strain. Since the Tn*917* insertion in cld-14 correlated with higher-than-normal (p)ppGpp accumulation in amino acid-starved cells, higher-than-normal (p)ppGpp accumulation might also occur in cld-14 at a low temperature, thereby delaying and slowing the growth of cld-14 in the cold. The wild-type strain's lower cellular (p)ppGpp levels may be due to functional PgpH that allows the organism to adapt to low-temperature growth. It has been reported that in *E. coli* the(p)ppGpp level decreases following a temperature shift from 37°C to 10°C and that this change plays a physiological role in the regulation of gene expression and adaptation for growth at low temperatures (13).

Role of *pgpH*. The (p)ppGpp hydrolase activity of PgpH, predicted to be present at the protein C terminus in the cytosol, may provide either a primary system or a secondary system for modulating the cellular levels of (p)ppGpp in response to low temperatures. ppGpp has been shown to accumulate in *B. subtilis* in response to low temperatures (12), and PgpH may provide a mechanism for restoring normal (p)ppGpp levels following the pseudostarvation conditions (nutritional downshift) induced at low temperatures (13).

Bacterial thermosensing functions might reside in the membrane, which is compatible with the proposed membrane location of PgpH. In support of the hypothesis that PgpH is a potential mediator of cold signaling, a proposed function for the HD domain of metal-dependent hydrolases is in signal transduction (2). The PgpH N terminus, which likely extends outside the membrane, might serve as a cold sensor, while the C-terminal (p)ppGpp hydrolase inside the cell may modulate (p)ppGpp levels, which are part of the cold shock and cold acclimation responses (6).

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