Role of Cold Shock Proteins in Growth of *Listeria monocytogenes* under Cold and Osmotic Stress Conditions[▽]

Barbara Schmid,¹ Jochen Klumpp,² Eveline Raimann,¹ Martin J. Loessner,²
Roger Stephan,^{1*} and Taurai Tasara¹

Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, CH-8057 Zurich, Switzerland,¹ and Institute of Food Science and Nutrition, ETH Zurich, CH-8092 Zurich, Switzerland²

Received 17 September 2008/Accepted 6 January 2009

The gram-positive bacterium *Listeria monocytogenes* is a food-borne pathogen of both public health and food safety significance. It possesses three small, highly homologous protein members of the cold shock protein (Csp) family. We used gene expression analysis and a set of mutants with single, double, and triple deletions of the *csp* genes to evaluate the roles of CspA, CspB, and CspD in the cold and osmotic (NaCl) stress adaptation responses of *L. monocytogenes*. All three Csps are dispensable for growth at optimal temperature (37°C). These proteins are, however, required for efficient cold and osmotic stress tolerance of this bacterium. The hierarchies of their functional importance differ, depending on the environmental stress conditions: CspA>CspD>CspB in response to cold stress versus CspD>CspA/CspB in response to NaCl salt osmotic stress. The fact that Csps are promoting *L. monocytogenes* adaptation against both cold and NaCl stress has significant implications in view of practical food microbial control measures. The combined or sequential exposure of *L. monocytogenes* cells to these two stresses in food environments might inadvertently induce cross-protection responses.

Listeria monocytogenes is a gram-positive food-borne pathogen of public health concern as well as a food safety challenge. This pathogen, primarily encountered through contaminated ready-to-eat foods, infects high-risk individuals, such as immunocompromised adults, pregnant women, and neonates, and leads to listeriosis, a rare but highly invasive disease with severe clinical signs and relatively high mortality (20 to 30%) (32, 35). The ubiquity of these organisms in nature and in a wide range of food-related environments means that they are a considerable microbial control challenge in food production (9). Moreover, *L. monocytogenes* organisms efficiently adapt and sometimes proliferate, despite exposure to low temperatures, low pH, and elevated salt (NaCl) concentrations, conditions used in preserving ready-to-eat food products (10, 24, 36). Improvement of food safety measures taken against this pathogen will depend on further insights gained into molecular cell response mechanisms underlying the various stress resistance phenotypes displayed by these organisms.

The cold shock protein (Csp) family consists of small, highly conserved, and structurally related nucleic acid binding proteins that presumably have important roles in regulation of various microbial physiological processes (8). These proteins are widely distributed among prokaryotes, including *L. monocytogenes*, and are often encoded through differentially regulated multiple gene families per organism (16, 29, 38). Csps are thought to serve as nucleic acid chaperones that bind RNA and DNA and thus may facilitate the control of processes such as replication, transcription, and translation within bacterial cells (8). To date, the multiple-homolog nature and functional vari-

ability of Csps are mostly derived from studies conducted with *Escherichia coli* and *Bacillus subtilis*. Nine Csps (CspA to CspI) are found in *E. coli*, and five (CspA, CspB, CspE, CspG, and CspI) have been linked to modulation of cold adaptation functions (12, 23, 26, 38). The CspC and CspE proteins have been implicated in chromosomal condensation and cell division and in the regulation of RpoS and UspA stress response proteins (2, 17, 19, 30, 43). Furthermore, the CspD protein of this organism has been suggested to regulate nutrient and stationary-phase stress adaptation responses (42). In *B. subtilis*, three Csps (CspA, CspB, and CspD) have been described and have been associated with regulation of both normal growth and cold and stationary-phase stress adaptation responses (14, 39). There is, however, limited knowledge regarding the function of Csps found in various psychrotolerant food-borne pathogens (25, 27, 40). An improved understanding of the role of these proteins in the normal growth and stress adaptation of *L. monocytogenes* is of particular interest, given the challenges that these organisms pose to food safety. Three Csp family proteins (CspA, CspB, and CspD) are found within the sequenced genomes of *L. monocytogenes*, but their functions are not yet understood (11). The present study was therefore conducted to gain further insights into Csp involvement in the normal growth and stress adaptation responses of this bacterium. To achieve this, *csp* gene expression patterns and the growth phenotypes of *csp* gene family deletion mutants were evaluated in response to optimal temperature (37°C), cold stress (4 and 10°C), and elevated NaCl salt concentration conditions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and general techniques. The wild-type and mutant bacterial strains, as well as the different plasmid vectors, used in this study are listed in Table 1. The clinical reference strain *L. monocytogenes* EGD-e (11) and its derivatives carrying various *csp* gene family deletion mutants were used. The *E. coli* host for all plasmid construction and propagation steps was the XL-1 Blue

* Corresponding author. Mailing address: Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, Winterthurerstr. 272, CH-8057 Zurich, Switzerland. Phone: 41 44 635 8657. Fax: 41 44 635 8908. E-mail: stephanr@fsafety.uzh.ch.

[▽] Published ahead of print on 16 January 2009.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Genetic deletion	Reference or source
Strains			
EGD-e	Wild type (1/2a)	None	11
$\Delta cspA$	$\Delta cspA$	153-bp in-frame <i>cspA</i> deletion	This study
$\Delta cspB$	$\Delta cspB$	147-bp in-frame <i>cspB</i> deletion	This study
$\Delta cspD$	$\Delta cspD$	147-bp in-frame <i>cspD</i> deletion	This study
$\Delta cspAB$	$\Delta cspAB$	<i>cspA</i> and <i>cspB</i> in-frame deletions	This study
$\Delta cspAD$	$\Delta cspAD$	<i>cspA</i> and <i>cspD</i> in-frame deletions	This study
$\Delta cspABD$	$\Delta cspABD$	<i>cspA</i> , <i>cspB</i> , and <i>cspD</i> in-frame deletions	This study
Plasmids			
pKSV7			33
pKSV7- $\Delta cspA$			This study
pKSV7- $\Delta cspB$			This study
pKSV7- $\Delta cspD$			This study
pPL2			21
pPL2- <i>cspA</i>			This study
pPL2- <i>cspD</i>			This study

strain (3). The plasmids pKSV7 (32) and pPL2 (21) were employed for genetic disruption and complementation, respectively. Bacterial genomic DNA was isolated with the DNeasy blood and tissue kit (Qiagen). Plasmids were prepared using the plasmid midikit (Qiagen). PCR amplification was performed using the FastStart high-fidelity PCR system (Roche Molecular Diagnostics GmbH, Penzberg, Germany). Restriction enzymes, T4 DNA ligase, and *Taq* DNA polymerase were used in accordance with the protocols of the supplier (Roche). PCR products and DNA restriction fragments were cut and purified from agarose gels using the mini elute gel extraction kit (Qiagen). Oligonucleotides were all ordered from Microsynth AG (Balgach, Switzerland). DNA sequencing was also performed at Microsynth AG. Bacterial strains were grown using brain heart infusion (BHI) (Oxoid) or Luria-Bertani (LB) (Difco Laboratories) broth or chemically defined minimal media (DM) (31). Osmolarity in salt-supplemented DM (DMS; 2.2% NaCl) and BHI plus 3% NaCl media was adjusted by addition of NaCl to the desired levels.

Genetic deletion and complementation. A set of *csp* gene deletion mutants was created in the EGD-e strain background. The splicing-by-overlap extension (SOE) PCR protocol was used for nonpolar deletions of the *csp* gene family (18). The $\Delta cspA$, $\Delta cspB$, and $\Delta cspD$ constructs were created by PCR amplification of EGD-e DNA templates by using the various SOE primers listed in Table 2. The SOE-PCR products were, thereafter, cloned into the pKSV7 plasmid by using the incorporated restriction enzyme sites. The pKSV7- $\Delta cspA$, pKSV7- $\Delta cspB$, and pKSV7- $\Delta cspD$ plasmid constructs were confirmed by DNA sequencing. The deletion of the targeted genes through homologous recombination and allelic exchange was facilitated by following previously described protocols with some modifications (4). Briefly, *L. monocytogenes* cells were transformed with the plasmid DNA constructs as previously described (28). Transformants were selected on LB plates in the presence of chloramphenicol (10 μ g/ml) after 48 to 72 h of incubation at 30°C. Integration mutants were enriched for 3 to 6 generations at 42°C in 10 ml BHI broth plus chloramphenicol (10 μ g/ml). The integrated strains were grown on BHI agar plates in the presence of chloramphenicol (10 μ g/ml) for 24 to 48 h at 42°C. Colonies of integrants were confirmed after colony PCR analysis with a primer combination that bound to defined regions in the pKSV7 plasmid and EGD-e genomic DNA. Plasmid excision was facilitated through growth of the integrants in BHI broth for 3 to 50 generations. During this period, samples were drawn at every third generation and plated onto BHI agar plates. The colonies were thereafter plated onto BHI plates with and without antibiotic to identify chloramphenicol-sensitive colonies in which the plasmid had been excised. PCR analysis and subsequent DNA sequencing of the targeted regions confirmed deletion mutants. The $\Delta cspA$, $\Delta cspB$, and $\Delta cspD$ single-deletion mutant strains were generated initially. These strains were sub-

TABLE 2. Oligonucleotide primers used in this study^f

Primer	Sequence ^g
SOE-P1-CspA-A	GGAATTCCGGAAAACAACAAGTGA CGCT ^a
SOE-P1-CspA-B	TGCTTGAGGTCCGCGACCTTGTTCCT TGTTTCATGTTCTT
SOE-P1-CspA-C	AGGAACATGGAACAAGGTCGCGGACC TCAAGCAGCT ^c
SOE-P1-CspA-D	ACGCGTCGACACCACATCACCGATTAT CGGAATA ^b
SOE-P2-CspB-A	TCCCCCGGGGCGTCCGGCAAGTGTG AATTGGTTAGTGAGCGAG ^c
SOE-P2-CspB-B	TGCTTGTGGGCCACGAACGTGTACCT GTTTGCATATTTCACT
SOE-P2-CspB-C	AACAGGTACAGTTCGTGGCCCAAG CAGAAAAAG ^c
SOE-P2-CspB-D	TCCCCCGGG CGCACTGGAATACAAG CTGGGACTATGCCCGCTTT ^c
SOE-P3-CspD-A	TCCCCCGGGGCCACCATTTTGTTCATA GAGGAAGGA ^c
SOE-P3-CspD-B	CAATGCAAAAT GGAAAGTACGCGG CGCTCAAG CAG
SOE-P3-CspD-C	CTGCTTGAGCGCGCGTACTTTCCCAT TTTGCAATTGAAATAAATCC ^c
SOE-P3-CspD-D	TCCCCCGGGCGCACTGGAATACAAG CTGGGACTATGCCCGCTTT ^c
CspA-comp-P1	AAAACATGCAG CTGATTTAATCGCAC TTAGAGAAAATTAATCA ^d
CspA-comp-P2	TCCCCCGGG TTACGCTTTTGAACGT TAGCTGCTT ^c
CspA-fw	AACATGGAACAAGGTACAG*
CspA-rv	GTTGGCCTTCTTCAACG*
CspB-fw	CAAACAGGTACAGTTAAATGGTTTA*
CspB-rv	ACGATTTCAAATTCACGCTTTGA*
CspD-fw	TACGGTTTTATCGAATCAGAC*
CspD-rv	ACGTTAGCTGCTTGTAG*
16S rRNA-fw	CTTCCGCAATGGACGAAAGT*
16S rRNA-rv	CTCATCGTTTACGGCGTG*

^a The EcoRI restriction site incorporated in this primer to facilitate cloning is underlined.

^b The SalI restriction site incorporated in this primer to facilitate cloning is underlined.

^c The SmaI restriction site incorporated in this primer to facilitate cloning is underlined.

^d The PstI restriction site incorporated in this primer to facilitate cloning is underlined.

^e The regions complementary to SOE-P1-CspA-B, SOE-P1-CspB-B, and SOE-P1-CspD-B primers are in italics.

^f Oligonucleotides were synthesized at Microsynth AG (Balgach, Switzerland). Primers were designed using the LightCycler probe and primer design software (Roche Molecular Diagnostics GmbH, Penzberg, Germany).

^g *, quantitative real-time RT-PCR primer.

sequently used in a second round of mutagenesis to target a second *csp* gene to create the $\Delta cspAB$, $\Delta cspAD$, and $\Delta cspBD$ double-deletion mutants. Finally, the *cspA* gene was targeted in the $\Delta cspBD$ strain, generating the $\Delta cspABD$ triple-deletion mutant strain. Defective phenotypes of $\Delta cspA$ and $\Delta cspD$ strains were complemented using a pPL2 plasmid-based system as described previously (21). DNA fragments consisting of the complete *cspA* and *cspD* genes, as well as their entire upstream sequence between them and the preceding gene, were amplified from the EGD-e DNA templates and subsequently cloned between the PstI and SmaI restriction sites of the pPL2 vector to create the pPL2-*cspA* and pPL2-*cspD* complementation plasmids. Upstream sequences included the predicted promoter elements and ribosome binding sites preceding these two genes. These constructs were confirmed by DNA sequencing and were subsequently used to transform the $\Delta cspA$ and $\Delta cspD$ strains, respectively. Colonies of transformants obtained on LB agar plates in the presence of chloramphenicol (10 μ g/ml) were analyzed for proper integration of pPL2-*cspA* and pPL2-*cspD* constructs into the PSA prophage integration site by PCR analysis using the previously described NC16/PL95 primer set (21).

Growth conditions. Stationary phase inocula (10^9 CFU/ml) of wild-type and mutant strains were prepared by growth of single colonies in 10 ml BHI overnight (16 to 18 h) in a shaking incubator (220 rpm) at 37°C. The stationary-phase inocula were serially diluted 10-fold to 10^5 CFU/ml in 0.85% NaCl-peptone. Thereafter, aliquots of 100 μ l were used for inoculation of 10 ml BHI, DM, or DMS, resulting in 10^3 CFU/ml starter cultures. Growth evaluation in BHI and DM at different temperatures involved preparation of three duplicate sets from each strain, which were subsequently incubated at 37, 10, and 4°C, without shaking. For growth experiments in DMS (DM plus 2.2% NaCl), 10-ml duplicate cultures were inoculated and similarly incubated at 37°C. The growth kinetics in each sample was monitored by standard colony counting at defined time intervals. The results presented in this study depict means and standard deviations of three independent experimental runs.

Cold and NaCl stress adaptation experiments. For cold stress exposure, 10-ml BHI stationary-phase cultures of the wild-type EGD-e strain were prepared by overnight growth at 37°C. The stationary-phase cultures were centrifuged ($4,000 \times g$ for 5 min), and the pellets were resuspended in 10 ml fresh BHI and divided into two 5-ml aliquots, which were habituated to 4°C and 37°C by 2 h of incubation. Thereafter, 1.5-ml samples were drawn, and total RNA templates were isolated as outlined below. For NaCl stress exposure, stationary-phase inocula, prepared as described above, were used to inoculate 10 ml of BHI or BHI plus 3% NaCl at 10^3 CFU/ml. These cultures were then incubated without shaking at 37°C for 10 (BHI) and 15 (BHI-NaCl) h to reach the late log phase, which was confirmed by colony counting in each case. At this point, 1.5-ml aliquots were drawn and directly processed for total RNA as outlined below or frozen at -20°C until processed at a later date.

Total RNA extraction. The RNA extraction procedure that was employed combined rapid mechanical lysis and column-based RNA purification. The 1.5-ml aliquots from each culture were centrifuged at 4°C (cold-adapted cells) or room temperature (non-cold-adapted cells) for 5 min at $4,000 \times g$. The supernatants were discarded, and pellets were resuspended in 0.5 ml lysis buffer provided in the RNeasy plus minikit (Qiagen). The mixtures were transferred onto the lysing bead matrix in MagNA lyser tubes (Roche Molecular Diagnostics GmbH, Penzberg, Germany). Bacterial cells were mechanically disrupted using the MagNA lyser instrument (6,500 rpm for 60 s). Total RNA was purified from the lysates on RNA binding columns according to the RNeasy plus minikit protocol and included two DNA removal steps. The cell lysates were passed through a genomic DNA removal column, and an on-column DNase I digestion step was performed. Total RNA was eluted using 30 μ l of RNase-free water, and the yield was determined by measuring absorption at 260 nm using the Nanodrop ND1000 (Nanodrop Instruments, DE). RNA template integrity was further analyzed under UV light after denaturing agarose gel electrophoresis and ethidium bromide staining.

Reverse transcription. Reverse transcription (RT) was performed using the Quantitect RT kit (Qiagen). Three hundred nanograms of total RNA from each sample were converted into cDNA in 20- μ l reaction mixtures. Similar amounts of total RNA from each sample were also subjected to the cDNA synthesis reaction without the inclusion of the reverse transcriptase enzyme as controls. These were subsequently used as "minus RT" controls to assess potential residual DNA contamination in total RNA templates.

Real-time PCR and quantification of gene expression. Primers used in this study are listed in Table 2. The real-time PCRs were performed in the LightCycler 480 instrument (Roche Molecular Diagnostics, Rotkreuz, Switzerland). The reactions were performed in a final reaction volume of 10 μ l: 2.5 μ l (3.75 ng) cDNA (1:10 dilution of cDNA generated as described above); 2.5 μ l (0.4 μ M) of gene-specific forward and reverse primer mix; 5 μ l of 2 $\times$ LightCycler 480 SYBR green I master mix. The real-time PCR run protocol was comprised of preincubation (4 min at 95°C), 40 amplification cycles (10 s at 95°C; 20 s at 56°C; 20 s at 72°C; 5 s at 80°C with a single fluorescent measurement); and melting curve (65 to 97°C at 2.2°C/s plus continuous fluorescent measurement). Primers and reaction conditions were optimized for specificity and target amplification, with efficiencies of 90 to 100%. Transcript levels were determined using the LightCycler 480 relative quantification software (Roche Molecular Diagnostics). Initially a reference gene validation under the different experimental conditions was performed as previously described (37), and the 16S rRNA transcripts were established as the most suitable internal control reference gene for relative mRNA quantification in our study. Based on this, we quantified *csp* gene transcripts relative to the 16S rRNA transcript level of the same sample.

Statistical analysis. Statistical analysis was performed using the Stat View 4.02 (Abacus Concepts Inc., Berkeley, CA) program. The bacterial colony counts were converted into log CFU/ml followed by calculation of the means and standard deviations. The statistical significances of differences in the bacterial colony counts between the wild type and the *csp* mutants were evaluated using

TABLE 3. Amino acid sequence comparison matrix of *L. monocytogenes* CspS, *E. coli* CspA, and *B. subtilis* CspB^a

Csp	Sequence identity (%)				
	CspA _{Lm}	CspB _{Lm}	CspD _{Lm}	CspA _{Ec}	CspB _{Bs}
CspA _{Lm}	100				
CspB _{Lm}	73	100			
CspD _{Lm}	73	67	100		
CspA _{Ec}	62	59	58	100	
CspB _{Bs}	74	80	72	61	100

^a Lm, *L. monocytogenes*; Ec, *E. coli*; Bs, *B. subtilis*.

one-way analysis of variance at a significance level (α) of 0.05. The comparative data derived from quantitative gene expression analysis were analyzed using Student's *t* test, and differences with *P* values of <0.05 were considered to be statistically significant.

Bioinformatics. The Vector NTI Advance 10.3 program (Invitrogen) was used for DNA and protein sequence manipulations. Protein motif search and hairpin predictions were performed using the DNASIS MaxVersion 2.6 (Miraibio, San Francisco, CA). Sequence homologies and protein sequence alignments were investigated using the BLASTn and BLASTp (NCBI) programs and multiple sequence alignment with hierarchical clustering (7).

RESULTS

Comparative nucleotide and amino acid sequence analysis of *L. monocytogenes* CspS. Nucleotide sequence comparison of CspS in the EGD-e strain revealed high levels of sequence similarity, with identities ranging from 77 to 84% (data not shown). All the *csp* open reading frames in this organism are preceded by relatively long stretches of 5' untranslated leader regions (5' UTR) ranging from 198 bp (*cspA*) to 363 bp (*cspD*), a situation which is similar to what has previously been described for some *csp* genes of other bacterial species (1, 34). These long 5' UTR regions were predicted to be rich in RNA hairpin loop secondary structures (DNASIS MaxVersion 2.6 program; Miraibio, San Francisco, CA), which might have implications for the regulation of *csp* gene expression in this organism. The amino acid sequence comparison matrix of *L. monocytogenes* EGD-e CspS, and the homologs of *E. coli* (CspA_{Ec}) and *B. subtilis* (CspB_{Bs}) are presented in Table 3. As shown, *L. monocytogenes* CspA, CspB, and CspD also share high levels of amino acid sequence identity (67 to 73%). These proteins are also closely related to the *B. subtilis* (72 to 80%) and *E. coli* (58 to 62%) homologs. In fact, *L. monocytogenes* CspA and CspB are more closely related to *B. subtilis* CspB_{Bs} (74 to 80%) than to the other CspS of this organism. An amino acid sequence alignment further highlights general sequence conservation and the canonical nucleic acid binding motifs, RNP-1 and RNP-2 (Fig. 1). Although some amino acid substitutions are found within the RNP-1 and RNP-2 motifs, these are conserved. As shown, a tyrosine-to-phenylalanine substitution occurs in *L. monocytogenes* CspB RNP-1 and an isoleucine-to-valine substitution in *L. monocytogenes* CspB and CspD RNP-2 motifs, compared to the motifs of *E. coli* and *B. subtilis* homologs.

Cold stress-associated induction of *csp* gene expression. The effect of *L. monocytogenes* cold adaptation on the expression pattern of its *csp* gene family was examined. To achieve this, stationary-phase *L. monocytogenes* EGD-e cells originally grown at 37°C were adapted to cold stress conditions by incu-

RNP1
RNP2

LmCspA -MNMEQGTVKWFNAEKGFGFIERE-NGDDVVFVHFSATIQGDGFKSLDEGQAVTFDVEEGQRGPQAANVQKA-
 LmCspB ----QN.K.....N...Y...SD-G.E.I...T.....Y...E.....E.V..N..A.....E...
 LmCspD ---.QT.....S.....V.-G...I.....E.E...T.....S.E.EIV.....EK.T.L.
 BsCspB ----LE.K.....S.....V.-GQ.....E...T.E.....S.EIV..N.....T.EA
 EcCspA MSGKMT.I.....D.....TPDDGSK.....N..Y.....K.S.TI.S.AK..A.G..TSL-

FIG. 1. Amino acid sequence alignment of *L. monocytogenes* CspA (CspA_{Lm}), CspB (CspB_{Lm}), and CspD (CspD_{Lm}) and the *E. coli* (CspA_{Ec}) and *B. subtilis* (CspB_{Bs}) homologs. The sequences were aligned using the program for multiple sequence alignment with hierarchical clustering (6). Dots represent identical amino acids in all five Csp homologs, and dashes represent the gaps placed in the sequences by the alignment program to optimize the alignment. The highly conserved nucleic acid binding motifs (RNP-1 and RNP-2) of cold shock domain proteins are highlighted in bold and underlined.

bation for 2 h at 4°C. As a control, an equal aliquot of the same EGD-e culture was also adapted to optimal temperature conditions by similar incubation for 2 h at 37°C. Total RNA isolated from these samples was used to determine the level of *csp* gene family (*cspA*, *cspB*, and *cspD*) transcripts in quantitative RT-PCR assays. A summary of the relative *csp* gene expression ratios at 4 and 37°C, as well as cold stress-dependent induction, are presented in Table 4. As shown, there is low constitutive expression of *cspA*, *cspB*, and *cspD* transcripts in EGD-e cells held at 37°C, but all *csp* gene transcripts are significantly induced ($P < 0.05$) in cold-adapted EGD-e cells at 4°C. Under these conditions, mean cold stress-dependent inductions of 23-fold, 4.5-fold, and 7.4-fold were observed for *cspA*, *cspB*, and *cspD* transcripts, respectively.

NaCl stress-dependent induction of *cspA* and *cspD* gene transcripts. Next, the influence of EGD-e strain growth under NaCl stress on *csp* gene family expression was investigated. For this purpose, late log phase EGD-e cells grown in BHI plus 3% NaCl and controls that had been similarly grown in regular BHI were compared. The *csp* gene expression results from these studies are presented in Table 5. As shown, both *cspA* and *cspD* transcripts were also significantly enhanced ($P < 0.05$) in NaCl stress-adapted EGD-e cells compared to the control of regular BHI-grown EGD-e cells. In contrast, there was no statistically significant increase associated with *cspB* transcripts ($P > 0.05$) in NaCl stress-adapted EGD-e cells (Table 5). The mean NaCl stress-dependent inductions achieved were 2.8-fold and 4.4-fold for *cspA* and *cspD* transcripts, respectively.

Creation of *cspA* deletion mutants in *L. monocytogenes* EGD-e. To gain more insights into the phenotypic contributions of CspA to *L. monocytogenes* growth under different conditions, various deletion mutants were created in the EGD-e strain (Table 1). The deletions of the targeted CspA were all performed in frame and verified through PCR and DNA sequence analysis of the targeted DNA regions (data not shown).

TABLE 4. Relative *csp* gene expression induction of cold stress-adapted stationary-phase EGD-e cells

Gene	Relative gene expression ratio at ^a :		Fold induction at 4°C vs 37°C ^b
	37°C	4°C	
<i>cspA</i>	0.02 ± 0.01	0.46 ± 0.20	23
<i>cspB</i>	0.37 ± 0.20	1.48 ± 0.30	4
<i>cspD</i>	0.05 ± 0.04	0.37 ± 0.10	7.4

^a The levels of *csp* gene transcripts are expressed relative to the levels of 16S rRNA reference gene transcripts.

^b The fold inductions in *csp* gene expression ratios of cold-adapted cells at 4°C are relative to those of control cells adapted to the optimal temperature (37°C).

Furthermore, quantitative RT-PCR assays with gene-specific primer pairs confirmed that transcripts corresponding to targeted *cspA* genes were also absent in the relevant mutant strains (data not shown). The phenotypic restoration in Δ *cspA* and Δ *cspD* strains was accomplished using pPL2-*cspA* and pPL2-*cspD* plasmid constructs. This process restored deleted *cspA* and *cspD* genes by integration into the PSA prophage *attB* site of the *L. monocytogenes* EGD-e chromosome in respective mutant strains.

CspA are required for efficient *L. monocytogenes* growth under cold stress. The growth kinetics of the wild type and the various *cspA* deletion mutant strains are summarized in Table 6. Representative growth curves depicting the growth of the wild type and selected *cspA* deletion mutants are presented in Fig. 2. As shown, an individual *cspA*, *cspB* or *cspD* gene deletion resulted in no detectable growth phenotype differences from the parent wild-type strain at 37°C in rich (BHI) and minimal (DM) nutrient conditions (Fig. 2A and B). Similarly, double deletions as well as triple deletions of all known *csp* genes produced no detectable growth phenotype defects at 37°C, as summarized by Table 6. Meanwhile, the growth analysis at 4°C showed that *cspB* deletion also had no influence, while deletion of *cspA* or *cspD* impairs *L. monocytogenes* growth under cold stress (Fig. 2C and D). In fact, the Δ *cspA* strain completely failed to grow at 4°C and 10°C in BHI and DM. In support of a causal link, the cold growth phenotype of the Δ *cspA* strain was successfully restored by a pPL2-*cspA* complementation (Fig. 2E). The cold growth phenotype in the Δ *cspD* strain was only significantly poor ($P < 0.05$) compared to that of the wild type at 4°C, but not at 10°C (Fig. 2C and D and Table 6). The cold-sensitive phenotype of this mutant was primarily observed with the defined minimal nutrient conditions of DM. But it was less pronounced within the rich complex nutrient background in BHI (Fig. 2C and D). Similarly,

TABLE 5. Relative *csp* gene expression induction of BHI-NaCl stress-adapted log phase EGD-e cells

Gene	Relative gene expression ratio in ^a :		Fold induction in BHI-NaCl vs BHI ^b
	BHI	BHI-3% NaCl	
<i>cspA</i>	0.06 ± 0.01	0.17 ± 0.02	2.8
<i>cspB</i>	0.56 ± 0.31	0.83 ± 0.35	NS ^c
<i>cspD</i>	0.15 ± 0.01	0.66 ± 0.32	4.4

^a The levels of *csp* gene transcripts are expressed relative to the levels of 16S rRNA reference gene transcripts.

^b The fold inductions in the *csp* gene expression ratios of BHI-NaCl-grown cells are relative to those of regular BHI-grown controls.

^c The gene expression induction differences between stress- and non-stress-exposed control cells were not statistically significant (NS) ($P > 0.05$).

TABLE 6. Overview of *csp* gene deletion mutant growth phenotypes at 37, 10, and 4°C

Strain	Growth phenotype at indicated temperature in ^a :					
	BHI			DM		
	37°C	10°C	4°C	37°C	10°C	4°C
Wild type	++++	++++	++++	++++	++++	++++
$\Delta cspA$	++++	—	—	++++	—	—
$\Delta cspB$	++++	++++	++++	++++	++++	++++
$\Delta cspD$	++++	++++	+++	++++	++++	++
$\Delta cspAB$	++++	—	—	++++	—	—
$\Delta cspAD$	++++	—	—	++++	—	—
$\Delta cspBD$	++++	++++	+++	++++	++++	+
$\Delta cspABD$	++++	—	—	++++	—	—

^a The growth phenotype of each mutant strain is expressed relative to the wild-type strain. +++++ to +, most to least growth, respectively; —, no growth.

growth analysis of double ($\Delta cspAB$, $\Delta cspAD$, and $\Delta cspBD$) and triple ($\Delta cspABD$) Csp deletion strains further confirmed that a *cspA* or *cspD* gene deletion compromises the cold growth phenotypes of *L. monocytogenes* EGD-e (Table 6), while *cspB* gene functions seemed dispensable as long as the *cspA* and *cspD* genes were retained in this bacterium. This hypothesis was confirmed by cold growth analysis of a $\Delta cspDB$ mutant strain in DM at 4°C (Fig. 2F). As shown, growth in this mutant strain is further compromised by *cspB* deletion, compared to growth of the $\Delta cspD$ or $\Delta cspB$ strain (Fig. 2F). Thus, loss of *cspB* gene functions, combined with a *cspD* deletion, also leads to impaired cold growth of EGD-e cells, suggesting some minimal CspB protein contribution to cold adaptation functions of this organism.

***L. monocytogenes* CspS contribute to efficient cellular growth under NaCl stress.** The growth responses of the wild type and various *cspA* deletion mutants were also investigated in DM plus 2.2% NaCl (DMS). The loss of *cspD* gene function significantly compromises the NaCl stress tolerance capacity of EGD-e cells (Fig. 3A). The growth of the $\Delta cspD$ in DMS was significantly slower than that of the wild-type strain. This was despite the fact that both strains grew similarly in regular DM (Fig. 2B). Phenotypic defects of $\Delta cspD$ growth in DMS were partly restored through pPL2-*cspD*-based genetic complementation (Fig. 3A). On the other hand, individual *cspA* and *cspB* gene deletions did not influence EGD-e growth phenotypes in DMS (Fig. 3B and Table 7). A double deletion of these two genes, however, significantly reduced growth in DMS (Fig. 3B). The loss of *cspD* gene function in *cspA/D* ($\Delta cspAD$) and *cspB/D* ($\Delta cspBD$) double-deletion mutants was also confirmed to impair growth phenotypes of EGD-e under NaCl stress conditions of DMS (Table 7). A *cspA/B/D* triple-deletion mutant also leads to complete growth failure of EGD-e cells in DMS, indicating that at least one Csp must be retained for *L. monocytogenes* growth at high NaCl salt concentrations.

DISCUSSION

Csp family proteins have previously been linked to regulation of both normal growth and stress adaptation processes against cold and nutrient starvation as well as to promotion of survival stationary-phase cells in some organisms (13–15, 41, 42). In cold-adapted *L. monocytogenes* cells, the induction of Csp-like proteins, as well as *cspA* gene transcripts, was previ-

FIG. 2. Growth of wild-type EGD-e and various *csp* gene family deletion mutants of this strain. (A to D) Growth kinetics of the wild-type, $\Delta cspA$, $\Delta cspB$, and $\Delta cspD$ strains in BHI and DM at optimal (37°C) and cold stress (4°C) temperatures. (E) Growth of wild-type, $\Delta cspA$, and pPL2-*cspA*-complemented $\Delta cspA$ strains at 10°C in BHI. (F) Growth kinetics of $\Delta cspB$, $\Delta cspD$, and $\Delta cspBD$ at 4°C in DM. The results represent the means ($\pm$ standard deviations) from duplicates of three independent experimental runs.

FIG. 3. Growth of the wild-type EGD-e and various *csp* gene family deletion mutants under NaCl stress in DMS. Growth kinetics in DMS of wild-type, $\Delta cspD$, and pPL2-*cspD*-complemented $\Delta cspD$ strains (A) and wild-type, $\Delta cspA$, $\Delta cspB$, and $\Delta cspAB$ strains (B). The results are means ($\pm$ standard deviations) from duplicates of three independent experimental runs.

ously documented (5, 40). The current study was conducted in order to expand this knowledge and to explore the Csp's contribution in other stress responses of this organism. In contrast to *B. subtilis*, in which at least one *csp* gene is essential for viability (14), none of the three *csp* genes is essential for *L. monocytogenes* viability. In fact, a mutant carrying deletions of all three *csp* genes could be generated without any discernable defects in the growth phenotype at 37°C, even under the defined minimal nutrient conditions of DM. It thus appears that functions encoded by the *L. monocytogenes* Csp genes are not critical requirements for viability or efficient growth under optimal temperature conditions. To the contrary, a hierarchy (*cspA* > *cspD* > *cspB*) was established in *csp* gene importance for cold adaptation and *L. monocytogenes* growth at low temperatures (4 and 10°C). The deletion of the *cspA* gene completely abolishes cold growth, and *cspD* deletion leads to significantly reduced cold (4°C) growth efficiency of this organism. The functions of the *cspB* gene on the other hand are largely dispensable. There were no cold growth defects associated with the deletion of this gene, as long as the *cspA* or *cspD* gene was retained.

The potential role of Csp's in osmotic stress adaptation so far stems from the fact that expression of the *cspC* gene in *Bordetella bronchiseptica* is significantly induced following exposure of this organism to 2 M NaCl (34). We show here that direct growth of *L. monocytogenes* EGD-e under NaCl stress in BHI-NaCl leads to significant induction of *cspA* and *cspD* gene expression, although the overall NaCl stress-associated *csp* inductions appeared lower compared to those in cold-adapted

cells. The induction trend observed of *cspD* > *cspA* was consistent with the subsequent trend observed in the stress sensitivity ($\Delta cspD$ > $\Delta cspA$ or $\Delta cspB$) of the *csp* gene deletion mutants. In fact, a single deletion of *cspA* or *cspB* gave no discernable NaCl stress growth phenotypes in DMS cultures. The $\Delta cspAB$ double-deletion mutant was, however, also significantly impaired during growth under similar NaCl stress conditions. It also seems that at least one *csp* gene is required for *L. monocytogenes* growth at higher NaCl concentrations. A $\Delta cspABD$ triple-deletion mutant completely failed to grow under NaCl stress conditions in DMS cultures.

In this study, we have presented both gene expression and stress growth phenotypic evaluation data suggesting that *L. monocytogenes* Csp's are functionally required for efficient cold and osmotic stress adaptation responses in this bacterium, although the precise nature of Csp involvement in such stress adaptation mechanisms still remains to be investigated. Key cold stress challenges include negatively supercoiled DNA and stabilized RNA secondary structures. These impair cellular replication, transcription, and translation processes. Therefore, increased Csp synthesis and activity at low temperatures provides DNA and RNA chaperone functions (8, 29), which are needed in cold-exposed *L. monocytogenes* cells to help resolve these nucleic acid structural hurdles. The Csp functional contributions to NaCl osmotic stress adaptation in *L. monocytogenes* are not yet clear. One possibility is that Csp chaperones might also promote the increased production of sodium ion extrusion transporter proteins through their effects in facilitating transcription and translation processes. This might enhance protection of *L. monocytogenes* cells from NaCl toxicity through increased extrusion of intracellular sodium ions. Alternatively, as observed in eukaryotic cells, high cellular NaCl concentration might lead to increased levels of cellular DNA damage in *L. monocytogenes* cells (20). It is thus plausible that Csp's through their DNA chaperone activity facilitate repair of NaCl stress-damaged DNA and promote *L. monocytogenes* under NaCl-associated osmotic stress.

The fact that Csp's seem to promote *L. monocytogenes* adaptation against both cold and NaCl stress also has significant implications in view of practical food microbial control measures. The combined or sequential exposure of *L. monocytogenes* cells to these two stresses in food environments might inadvertently induce cross-protection responses. Cold

TABLE 7. Overview of Csp deletion mutant growth phenotypes in DM and DMS¹

Strain	Growth phenotype in ^a :	
	DM	DMS
Wild type	++++	++++
$\Delta cspA$	++++	++++
$\Delta cspB$	++++	++++
$\Delta cspD$	++++	++
$\Delta cspAB$	++++	++
$\Delta cspAD$	++++	++
$\Delta cspBD$	++++	++
$\Delta cspABD$	++++	—

^a The growth phenotype of each mutant strain is expressed relative to the wild-type strain. +++++ to ++, most to least growth, respectively; —, no growth.

stress induced by low temperature may inadvertently cross-protect cells against NaCl stress due to induction of *cspA*, *cspD*, and *cspB* gene expression. Conversely, exposure to higher NaCl salt concentrations in some foods before cooling can also adapt cells to grow at low temperatures due to induction of *cspA* and *cspD* gene functions in response to NaCl stress. A similar phenomenon was recently described for the spoilage bacterium *Shewanella putrefaciens* (22) and for an lmo1078 gene transposon deletion mutant of *L. monocytogenes* (6). A mutant in this gene, which encodes a putative UDP-glucose phosphorylase enzyme, also exhibited cold and NaCl salt stress sensitivity.

ACKNOWLEDGMENT

B. Schmid was funded by a research grant from the University of Zurich.

REFERENCES

- Bae, W., P. G. Jones, and M. Inouye. 1997. CspA, the major cold shock protein of *Escherichia coli*, negatively regulates its own gene expression. *J. Bacteriol.* **179**:7081–7088.
- Bae, W., S. Phadtare, K. Severinov, and M. Inouye. 1999. Characterization of *Escherichia coli* *cspE*, whose product negatively regulates transcription of *cspA*, the gene for the major cold shock protein. *Mol. Microbiol.* **31**:1429–1441.
- Bullock, W. O., J. M. Fernandez, and J. M. Short. 1987. XL1-blue: a high efficiency plasmid transforming *recA* *Escherichia coli* strain with β -galactosidase selection. *BioTechniques* **5**:376–379.
- Camilli, A., L. G. Tilney, and D. A. Portnoy. 1993. Dual roles of *plcA* in *Listeria monocytogenes* pathogenesis. *Mol. Microbiol.* **8**:143–157.
- Chan, Y. C., S. Raengpradub, K. J. Boor, and M. Wiedmann. 2007. Microarray-based characterization of the *Listeria monocytogenes* cold regulon in log- and stationary-phase cells. *Appl. Environ. Microbiol.* **73**:6484–6498.
- Chassaing, D., and F. Auvray. 2007. The *lmo1078* gene encoding a putative UDP-glucose pyrophosphorylase is involved in growth of *Listeria monocytogenes* at low temperature. *FEMS Microbiol. Lett.* **275**:31–37.
- Corpet, F. 1988. Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res.* **16**:10881–10890.
- Ermolenko, D. N., and G. I. Makhatadze. 2002. Bacterial cold-shock proteins. *Cell. Mol. Life Sci.* **59**:1902–1913.
- Farber, J. M., and P. I. Peterkin. 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Rev.* **55**:476–511.
- Gandhi, M., and M. L. Chikindas. 2007. *Listeria*: a foodborne pathogen that knows how to survive. *Int. J. Food Microbiol.* **113**:1–15.
- Glaser, P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend, F. Baquero, P. Berche, H. Bloeker, P. Brandt, T. Chakraborty, A. Charbit, F. Chetouani, E. Couve, A. de Daruvar, P. Dehoux, E. Domann, G. Dominguez-Bernal, E. Duchaud, L. Durant, O. Dussurget, K. D. Entian, H. Fsihi, F. Garcia-del Portillo, P. Garrido, L. Gautier, W. Goebel, N. Gomez-Lopez, T. Hain, J. Hauf, D. Jackson, L. M. Jones, U. Kaerst, J. Kreft, M. Kuhn, F. Kunst, G. Kurapat, E. Madueno, A. Maitournam, J. M. Vicente, E. Ng, H. Nedjari, G. Nordstiek, S. Novella, B. de Pablos, J. C. Perez-Diaz, R. Purcell, B. Rammel, M. Rose, T. Schluter, N. Simoes, A. Tierrez, J. A. Vazquez-Boland, H. Voss, J. Wehland, and P. Cossart. 2001. Comparative genomics of *Listeria* species. *Science* **294**:849–852.
- Goldstein, J., N. S. Pollitt, and M. Inouye. 1990. Major cold shock protein of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **87**:283–287.
- Graumann, P., K. Schroder, R. Schmid, and M. A. Marahiel. 1996. Cold shock stress-induced proteins in *Bacillus subtilis*. *J. Bacteriol.* **178**:4611–4619.
- Graumann, P., T. M. Wendrich, M. H. Weber, K. Schroder, and M. A. Marahiel. 1997. A family of cold shock proteins in *Bacillus subtilis* is essential for cellular growth and for efficient protein synthesis at optimal and low temperatures. *Mol. Microbiol.* **25**:741–756.
- Graumann, P. L., and M. A. Marahiel. 1999. Cold shock proteins CspB and CspC are major stationary-phase-induced proteins in *Bacillus subtilis*. *Arch. Microbiol.* **171**:135–138.
- Graumann, P. L., and M. A. Marahiel. 1998. A superfamily of proteins that contain the cold-shock domain. *Trends Biochem. Sci.* **23**:286–290.
- Harrington, E. W., and N. J. Trun. 1997. Unfolding of the bacterial nucleoid both in vivo and in vitro as a result of exposure to camphor. *J. Bacteriol.* **179**:2435–2439.
- Horton, R. M., Z. L. Cai, S. N. Ho, and L. R. Pease. 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *BioTechniques* **8**:528–535.
- Hu, K. H., E. Liu, K. Dean, M. Gingras, W. DeGraff, and N. J. Trun. 1996. Overproduction of three genes leads to camphor resistance and chromosome condensation in *Escherichia coli*. *Genetics* **143**:1521–1532.
- Kültz, D., and D. Chakravarty. 2001. Hyperosmolality in the form of elevated NaCl but not urea causes DNA damage in murine kidney cells. *Proc. Natl. Acad. Sci. USA* **98**:1999–2004.
- Lauer, P., M. Y. Chow, M. J. Loessner, D. A. Portnoy, and R. Calendar. 2002. Construction, characterization, and use of two *Listeria monocytogenes* site-specific phage integration vectors. *J. Bacteriol.* **184**:4177–4186.
- Leblanc, L., C. Leboeuf, F. Leroi, A. Hartke, and Y. Auffray. 2003. Comparison between NaCl tolerance response and acclimation to cold temperature in *Shewanella putrefaciens*. *Curr. Microbiol.* **46**:157–162.
- Lee, S. J., A. Xie, W. Jiang, J. P. Etchegaray, P. G. Jones, and M. Inouye. 1994. Family of the major cold-shock protein, CspA (CS7.4), of *Escherichia coli*, whose members show a high sequence similarity with the eukaryotic Y-box binding proteins. *Mol. Microbiol.* **11**:833–839.
- Lianou, A., and J. N. Sofos. 2007. A review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. *J. Food Prot.* **70**:2172–2198.
- Mayr, B., T. Kaplan, S. Lechner, and S. Scherer. 1996. Identification and purification of a family of dimeric major cold shock protein homologs from the psychrotrophic *Bacillus cereus* WSBC 10201. *J. Bacteriol.* **178**:2916–2925.
- Nakashima, K., K. Kanamaru, T. Mizuno, and K. Horikoshi. 1996. A novel member of the *cspA* family of genes that is induced by cold shock in *Escherichia coli*. *J. Bacteriol.* **178**:2994–2997.
- Neuhaus, K., K. P. Francis, S. Rapposch, A. Gorg, and S. Scherer. 1999. Pathogenic *Yersinia* species carry a novel, cold-inducible major cold shock protein tandem gene duplication producing both bicistronic and monocistronic mRNA. *J. Bacteriol.* **181**:6449–6455.
- Park, S. F., and G. S. Stewart. 1990. High-efficiency transformation of *Listeria monocytogenes* by electroporation of penicillin-treated cells. *Gene* **94**:129–132.
- Phadtare, S. 2004. Recent developments in bacterial cold-shock response. *Curr. Issues Mol. Biol.* **6**:125–136.
- Phadtare, S., and M. Inouye. 2001. Role of CspC and CspE in regulation of expression of RpoS and UspA, the stress response proteins in *Escherichia coli*. *J. Bacteriol.* **183**:1205–1214.
- Premaratne, R. J., W. J. Lin, and E. A. Johnson. 1991. Development of an improved chemically defined minimal medium for *Listeria monocytogenes*. *Appl. Environ. Microbiol.* **57**:3046–3048.
- Ramaswamy, V., V. M. Cresence, J. S. Rejitha, M. U. Lekshmi, K. S. Dharasana, S. P. Prasad, and H. M. Vijila. 2007. *Listeria*—review of epidemiology and pathogenesis. *J. Microbiol. Immunol. Infect.* **40**:4–13.
- Smith, K., and P. Youngman. 1992. Use of a new integrational vector to investigate compartment-specific expression of the *Bacillus subtilis* spoIIIM gene. *Biochimie* **74**:705–711.
- Stubs, D., T. M. Fuchs, B. Schneider, A. Bosserhoff, and R. Gross. 2005. Identification and regulation of cold-inducible factors of *Bordetella bronchiseptica*. *Microbiology* **151**:1895–1909.
- Swaminathan, B., and P. Gerner-Smidt. 2007. The epidemiology of human listeriosis. *Microbes Infect.* **9**:1236–1243.
- Tasara, T., and R. Stephan. 2006. Cold stress tolerance of *Listeria monocytogenes*: a review of molecular adaptive mechanisms and food safety implications. *J. Food Prot.* **69**:1473–1484.
- Tasara, T., and R. Stephan. 2007. Evaluation of housekeeping genes in *Listeria monocytogenes* as potential internal control references for normalizing mRNA expression levels in stress adaptation models using real-time PCR. *FEMS Microbiol. Lett.* **269**:265–272.
- Wang, N., K. Yamanaka, and M. Inouye. 1999. CspI, the ninth member of the CspA family of *Escherichia coli*, is induced upon cold shock. *J. Bacteriol.* **181**:1603–1609.
- Weber, M. H., and M. A. Marahiel. 2002. Coping with the cold: the cold shock response in the Gram-positive soil bacterium *Bacillus subtilis*. *Philos. Trans. R. Soc. Lond. B* **357**:895–907.
- Wemkamp-Kamphuis, H. H., A. K. Karatzas, J. A. Wouters, and T. Abee. 2002. Enhanced levels of cold shock proteins in *Listeria monocytogenes* LO28 upon exposure to low temperature and high hydrostatic pressure. *Appl. Environ. Microbiol.* **68**:456–463.
- Yamanaka, K., L. Fang, and M. Inouye. 1998. The CspA family in *Escherichia coli*: multiple gene duplication for stress adaptation. *Mol. Microbiol.* **27**:247–255.
- Yamanaka, K., and M. Inouye. 1997. Growth-phase-dependent expression of *cspD*, encoding a member of the CspA family in *Escherichia coli*. *J. Bacteriol.* **179**:5126–5130.
- Yamanaka, K., T. Mitani, T. Ogura, H. Niki, and S. Hiraga. 1994. Cloning, sequencing, and characterization of multicopy suppressors of a *mukB* mutation in *Escherichia coli*. *Mol. Microbiol.* **13**:301–312.