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Two-Component-System Histidine Kinases Involved in Growth of *Listeria monocytogenes* EGD-e at Low Temperatures

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Two-component systems (TCSs) aid bacteria in adapting to a wide variety of stress conditions. While the role of TCS response regulators in the cold tolerance of the psychrotrophic foodborne pathogen *Listeria monocytogenes* has been demonstrated previously, no comprehensive studies showing the role of TCS histidine kinases of *L. monocytogenes* at low temperature have been performed. We compared the expression levels of each histidine kinase-encoding gene of *L. monocytogenes* EGD-e in logarithmic growth phase at 3°C and 37°C, as well as the expression levels 30 min, 3 h, and 7 h after cold shock at 5°C and preceding cold shock (at 37°C). We constructed a deletion mutation in each TCS histidine kinase gene, monitored the growth of the EGD-e wild-type and mutant strains at 3°C and 37°C, and measured the minimum growth temperature of each strain. Two genes, *yycG* and *lisK*, proved significant in regard to induced relative expression levels under cold conditions and cold-sensitive mutant phenotypes. Moreover, the Δ*resE* mutant showed a lower growth rate than that of wild-type EGD-e at 3°C. Eleven other genes showed upregulated gene expression but revealed no cold-sensitive phenotypes. The results show that the histidine kinases encoded by *yycG* and *lisK* are important for the growth and adaptation of *L. monocytogenes* EGD-e at low temperature.

Listeria monocytogenes is a low-G+C, Gram-positive, nonspore-forming coccobacillus ubiquitously found in the environment (1, 2). It has been isolated from foods and food-processing premises, where it has been confirmed to be able to persist for several years (3–7). *L. monocytogenes* is the causative agent of listeriosis, with a reported annual incidence of approximately 0.3 case per 100,000 population in the United States and 0.2 to 0.8 case per 100,000 population in Europe (8–10). Although relatively rare, listeriosis is nevertheless a severe foodborne disease with a mortality of up to 20 to 30% (1, 8, 11, 12). *L. monocytogenes* surmounts a wide range of stress conditions and is able to grow at temperatures as low as −0.4°C (13). This sets critical challenges for the modern food industry, since *L. monocytogenes* is consequently able to grow in ready-to-eat foods with a long shelf-life and in products stored at refrigeration temperatures. Thus, it is crucial to study and elucidate the factors enhancing the cold tolerance of *L. monocytogenes*.

Two-component regulatory signaling systems (TCSs) are among the major systems that aid bacteria in adapting to many arduous stress factors encountered in nature and during food processing. A typical TCS is constituted of a transmembrane sensor histidine kinase (HK) and a cognate cytoplasmic response regulator (RR) (14–17). The signaling pathway of the TCS is based on protein phosphorylation. In the simplest form of signal transduction, the sensor domain of the HK detects a specific stimulus or stress factor in cells or in their environment. This leads to the autophosphorylation of a specific His residue in the HK dimerization domain. The cognate RR then catalyzes the transfer of the phosphoryl group to its own Asp residue in the regulatory domain. Finally, phosphorylation leads to the activation of the effector domain, which usually functions as a transcription factor, and, eventually, to an appropriate response to the particular stimulus encountered (14–17).

Apart from *L. monocytogenes*, the importance of TCSs has been clarified for the cold tolerance of many foodborne and other pathogenic bacteria. One of the most thoroughly studied TCSs responding to cold stress is *Bacillus subtilis* DesK/DesR. Following cold shock, it induces the transcription of the membrane phospholipid desaturase-encoding *des* gene, optimizing cell membrane fluidity and thus improving the survival of the bacterium (18, 19). Despite the relatedness between *L. monocytogenes* and *B. subtilis*, no DesK/DesR homologue has been found in *L. monocytogenes* (20). Concerning *Clostridium botulinum*, Lindström et al. reported that the functional TCS CBO0366/CBO0365 is needed for growth of *C. botulinum* ATCC 3502 at 15°C (21). However, when Palonen et al. found the TCS CheA/CheY to be important for the growth of *Yersinia pseudotuberculosis* IP32953 at 3°C, an individual mutation in the HK-encoding *cheA* gene resulted in impaired growth, whereas an equivalent mutation in the cognate RR-encoding *cheY* gene did not affect the growth at 3°C (22). Palonen et al. also found four HKs and two RRs of *Y. pseudotuberculosis* IP32953 to be upregulated at 3°C, while the expression levels of the cognate RRs and HKs, respectively, remained unaltered (22). This demonstrates that certain HKs or RRs may have individual roles in the stress tolerance of bacteria. Thus, by concentrating solely on one component of a TCS in stress experiments, relevant information may be unheeded.

In the sequenced genome of *L. monocytogenes* wild-type EGD-e, genes for 16 TCSs have been identified, among which 15

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristic	Reference or source[a]
Strains		
L. monocytogenes strains		
EGD-e	Wild-type strain; serotype 1/2a	23
Δ*lmo0050*	In-frame deletion of the *lmo0050* gene (1,296 bp) with 1,101 nucleotides	This study
Δ*lmo0288* (Δ*yycG*)	Out-of-frame deletion of the *yycG* gene (1,833 bp) with 1,682 nucleotides	This study
Δ*lmo0692* (Δ*cheA*)	In-frame deletion of the *cheA* gene (1,857 bp) with 1,752 nucleotides	This study
Δ*lmo1021*	In-frame deletion of the *lmo1021* gene (1,059 bp) with 930 nucleotides	This study
Δ*lmo1061*	Out-of-frame deletion of the *lmo1061* gene (1,446 bp) with 1,249 nucleotides	This study
Δ*lmo1173*	Out-of-frame deletion of the *lmo1173* gene (1,458 bp) with 1,078 nucleotides	This study
Δ*lmo1378* (Δ*lisK*)	In-frame deletion of the *lisK* gene (1,452 bp) with 1,116 nucleotides	This study
Δ*lmo1508*	In-frame deletion of the *lmo1508* gene (1,440 bp) with 1,230 nucleotides	This study
Δ*lmo1741*	In-frame deletion of the *lmo1741* gene (1,041 bp) with 786 nucleotides	This study
Δ*lmo1947* (Δ*resE*)	In-frame deletion of the *resE* gene (1,791 bp) with 1,542 nucleotides	This study
Δ*lmo2011*	In-frame deletion of the *lmo2011* gene (1,740 bp) with 1,437 nucleotides	This study
Δ*lmo2421* (Δ*cesK*)	In-frame deletion of the *cesK* gene (1,143 bp) with 1,020 nucleotides	This study
Δ*lmo2500* (Δ*phoR*)	In-frame deletion of the *phoR* gene (1,776 bp) with 1,641 nucleotides	This study
Δ*lmo2582*	Out-of-frame deletion of the *lmo2582* gene (1,380 bp) with 1,339 nucleotides	This study
Δ*lmo2679* (Δ*kdpD*)	In-frame deletion of the *kdpD* gene (2,691 bp) with 2,652 nucleotides	This study
Δ*yycG*c	Δ*yycG* $tRNA^{Arg}$::p*yycG*c; complemented strain	This study
Δ*lisK*c	Δ*lisK* $tRNA^{Arg}$::p*lisK*c; complemented strain	This study
EGD-epPL2	EGD-e $tRNA^{Arg}$::pPL2	30
Δ*yycG*pPL2	Δ*yycG* $tRNA^{Arg}$::pPL2	This study
Δ*lisK*pPL2	Δ*lisK* $tRNA^{Arg}$::pPL2	This study
E. coli strains		
DH5α/pMAD	DH5α containing the shuttle vector plasmid pMAD	31
TOP10	Electrocompetent strain	Invitrogen
NEB5α	Electrocompetent strain	New England Biolabs
HB101	Conjugation donor containing the helper plasmid pRK24	CRBIP
Plasmids		
pMAD	Cloning shuttle integration vector plasmid	34
pMAD-Δ*yycG*	pMAD containing homologous region up- and downstream of EGD-e *yycG*	This study
pMAD-Δ*lisK*	pMAD containing homologous region up- and downstream of EGD-e *lisK*	This study
pPL2	Site-specific integration vector	37
p*yycG*c	pPL2 containing 613 bp of upstream nucleotides and coding sequence of *yycG*	This study
p*lisK*c	pPL2 containing 1,443 bp of upstream nucleotides, coding sequence, and downstream terminator sequence of *lisK*	This study

[a] CRBIP, Biological Resource Centre of the Institut Pasteur.

are complete signaling systems and 1, encoded by *lmo2512* (*degU*), is an orphan RR (23, 24). Previously, the contribution of RRs to the cold stress tolerance of *L. monocytogenes* was studied extensively (25, 26). Chan et al. demonstrated by whole-genome transcriptional analysis of *L. monocytogenes* wild-type strain 10403S that the RR-encoding *lmo0287* gene (*yycF*) was induced at 4°C in relation to that in cells grown at 37°C (25). Furthermore, by performing mutational analysis, Chan et al. found three RRs, encoded by *lmo1060*, *lmo1172*, and *lmo1377* (*lisR*), to be required for the cold adaptation of the same strain at 4°C (26). However, HKs and their role in the survival of *L. monocytogenes* at low temperature have been less studied. Liu et al. discovered that the mRNA level of the HK-encoding gene *lmo1508* of *L. monocytogenes* strain 10403S was increased at 10°C compared to 37°C by using a method for selective capture of transcribed sequences (27). This suggests that *lmo1508* has a role in the cold response of *L. monocytogenes*. Nevertheless, to our knowledge, no comprehensive studies on the utility of HKs in the growth of *L. monocytogenes* at refrigeration temperatures have been performed to date.

To examine the role of HKs in the growth of *L. monocytogenes* at refrigeration temperatures, we detected the expression levels of the 15 known HKs at 3°C and after cold shock at 5°C by using reverse transcription-quantitative PCR (RT-qPCR), and we examined the phenotype of each individual HK knockout deletion mutant at 3°C. Of all the known HKs of *L. monocytogenes*, we identified two, encoded by *lmo0288* (*yycG*) and *lmo1378* (*lisK*), as important for the cold tolerance of this notable foodborne bacterium, as well as several other HKs with putative roles.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *L. monocytogenes* wild-type strain EGD-e (23), derived mutant strains with one of the known 15 TCS HK genes deleted, the Δ*yycG*c and Δ*lisK*c complemented strains, the vector controls EGD-epPL2, Δ*yycG*pPL2, and Δ*lisK*pPL2, and the plasmids and *Escherichia coli* strains used for genetic manipulation are presented in Table 1. All *L. monocytogenes* strains were grown at 37°C on blood or brain heart infusion (BHI) agar (Bacto, Becton Dickinson, Sparks, MD) or in BHI broth, and *E. coli* strains were grown at 30°C on Luria-Bertani (LB) agar (Difco, Becton Dickinson, Sparks, MD) or in LB

TABLE 2 Primers for mutant strain construction and complementation[a]

Application	Gene[b]	Primer name	Primer sequence (5′ → 3′)[c]
Mutant strain construction	*lmo0050*	0050-1	NNNNNNGGATCCTTATGGCTCAGACGGTATTCT
	lmo0050	0050-2	*TCATCCCCATTCTTCTACAT*GGCACTTACAAAAACAATCA
	lmo0050	0050-3	*TGATTGTTTTTGTAAGTGCC*ATGTAGAAGAATGGGGATGAA
	lmo0050	0050-4	NNNNNNACGCGTCCACACCTTTTGTCGTATCT
	lmo0288 (*yycG*)	0288-1 MluI	NNNNNNACGCGTCTTGTAGTAGATGACGAAAAACC
	lmo0288 (*yycG*)	0288-2	*CTAACCCTGTTCCTCCCATT*GTAAGCGAAAATACCCAAAA
	lmo0288 (*yycG*)	0288-3	*TTTTGGGTATTTTCGCTTAC*AATGGGAGGAACAGGGTTAGG
	lmo0288 (*yycG*)	0288-4	NNNNNNACGCGTGCTTAGGAAAAGATTGCGTTTG
	lmo0692 (*cheA*)	0692-1	NNNNNNGGATCCCGTGAACAATCAATCCATCG
	lmo0692 (*cheA*)	0692-2	*CTGAAAAAGCCGCCAAGTGT*CATGCTATCCACCTCCATTTCT
	lmo0692 (*cheA*)	0692-3	*AAATGGAGGTGGATAGCATG*ACACTTGGCGGCTTTTTCAG
	lmo0692 (*cheA*)	0692-4	NNNNNNACGCGTGCCTGTTTCCCTTTTTCCTC
	lmo1021	1021-1 MluI	NNNNNNACGCGTCGTAAGGAGGTGTGTCAGC
	lmo1021	1021-2	*TTATTTTGATGGTGCCGG*AGCAAACAGCCATCATCAG
	lmo1021	1021-3	*CTGATGATGGCTGTTTGCTCC*GGCACCATCAAAATAA
	lmo1021	1021-4	NNNNNNACGCGTCACCAAATCGCCCAAGAC
	lmo1061	1061-1 MluI	NNNNNNACGCGTAAAAAGAACAGTAGGAGGCAAA
	lmo1061	1061-2	*ATAATAAACAACCCTAAACC*AGAGCCGTAAAAAGTAAAATGA
	lmo1061	1061-3	*ATTTTACTTTTTACGGCTCT*GGTTTAGGGTTGTTTATTATTGC
	lmo1061	1061-4	NNNNNNACGCGTCGCTGTATTTTCTTTGTCATCTG
	lmo1173	1173-1 MluI	NNNNNNACGCGTCGTGACAGGAATGAATGGAAG
	lmo1173	1173-2	*ATGACCGTTGTCCGTTACTT*TTTGGTTGTTTTTGGTGGTG
	lmo1173	1173-3	*CACCACCAAAAACAACCAAAA*AAGTAACGGACAACGGTCAT
	lmo1173	1173-4	NNNNNNACGCGTATCAAACAGGGATAAGTTCG
	lmo1378 (*lisK*)	1378-1 MluI	NNNNNNACGCGTAGCGGTTGCTAATGATGGAC
	lmo1378 (*lisK*)	1378-2	*TCTGGCTTTATCTACACGGT*GCAGTTCTTTTACTTCTGGTTCTTCT
	lmo1378 (*lisK*)	1378-3	*ACCAGAAGTAAAAGAACTGC*ACCGTGTAGATAAAGCCAGAAGC
	lmo1378 (*lisK*)	1378-4	NNNNNNACGCGTCGAAAGAAGAACAAGCAACTATTC
	lmo1508	1508-1 MluI	NNNNNNACGCGTAATGTAAGAGGAGTGAGAACAGAA
	lmo1508	1508-2	*CCAATACCAGATTCGCCA*CAAGCAATGAGCAAAATGATAAA
	lmo1508	1508-3	*ATCATTTTGCTCATTGCTTG*TGGCGAATCTGGTATTGG
	lmo1508	1508-4	NNNNNNACGCGTACAGGGCGTCGTAAAGAAGT
	lmo1741	1741-1 MluI	NNNNNNACGCGTGGTCAAAAACAACCCCGATA
	lmo1741	1741-2	*CTCGTGCTGGGCTATTTCT*GTGGCGTAGGAACAGAAGTGA
	lmo1741	1741-3	*CACTTCTGTTCCTACGCCA*CAGAAATAGCCCAGCACGAGA
	lmo1741	1741-4	NNNNNNACGCGTAAGGCGGTAAGTTTGTTCCA
	lmo1947 (*resE*)	1947-1 MluI	NNNNNNACGCGTGCCCCGAAGATGAACGAATA
	lmo1947 (*resE*)	1947-2	*CAAAACAGCCAGTATCATC*ATGGAAGCACACAATGGAAAA
	lmo1947 (*resE*)	1947-3	*TTTTCCATTGTGTGCTTCC*ATGATGATACTGGCTGTTTTGTCT
	lmo1947 (*resE*)	1947-4	NNNNNNACGCGTTTTGTCGTGAACTGAGGGAGT
	lmo2011	2011-1 MluI	NNNNNNACGCGTGAAGTTTGTTCGGTTATTCCAA
	lmo2011	2011-2	*CAATCTCCAAAGAGCGGAA*GATACGAGCCACATCGGTCT
	lmo2011	2011-3	*AGACCGATGTGGCTCGTATC*TTCCGCTCTTTGGAGATTG
	lmo2011	2011-4	NNNNNNACGCGTGTGGGATTGTGTTTGGCTTT
	lmo2421 (*cesK*)	2421-1	NNNNNNGGATCCGAAAATCTCGCCTTGTTCA
	lmo2421 (*cesK*)	2421-2	*GTTTCTTGGGTTTTGGCGT*TTATCGTCAAACTGCCACTAAT
	lmo2421 (*cesK*)	2421-3	*TAGTGGCAGTTTGACGATAA*ACGCCAAAACCCAAGAAAC
	lmo2421 (*cesK*)	2421-4	NNNNNNGAATTCGACTGGAAAGGACAATGAGTATGA
	lmo2500 (*phoR*)	2500-1 MluI	NNNNNNACGCGTCATTCCGACAACCGCTACTC
	lmo2500 (*phoR*)	2500-2	*TATGGCTGAAAATCGGGTT*TTCCATCGTCAAACATCTCG
	lmo2500 (*phoR*)	2500-3	*CGAGATGTTTGACGATGGAA*AACCCGATTTTCAGCCATA
	lmo2500 (*phoR*)	2500-4	NNNNNNACGCGTGCTTCTATTGTTACCTTGCTACA
	lmo2582	2582-1	NNNNNNACGCGTTCCCAGCCAACGAAAATAAC
	lmo2582	2582-2	*AGTTGTCACGATGCT*ATCCGATTCACATTCCGTTT
	lmo2582	2582-3	*AAACGGAATGTGAATCG*ACAAGCATCGTGACAACT
	lmo2582	2582-4	NNNNNNACGCGTGAGGCAAGTGATGGGGTAGA
	lmo2679 (*kdpD*)	2679-1	NNNNNNACGCGTTTCTTCTGCCATCCGGTATC
	lmo2679 (*kdpD*)	2679-2	*ATGGAAACGAATCGTCCAAGC*GGAGGAGATGGAAAATGAA
	lmo2679 (*kdpD*)	2679-3	*TTCATTTTCCATCTCCTCCG*CTTGGACGATTCGTTTCCAT
	lmo2679 (*kdpD*)	2679-4	NNNNNNACGCGTGCGTGCTTTATCCAGGTGTT

(Continued on following page)

TABLE 2 (Continued)

Application	Gene[b]	Primer name	Primer sequence (5′ → 3′)[c]
Complementation	Operon 051	Komp 0288F1	NNNNGGATCCGGTGCGGATGACTATGTAACG
	Operon 051	Komp oper 0288R	NNNNTCTAGACCATTTTCTTTCCACTCAAACC
	Operon 220	Komp oper 1378F2	NNNNGGATCCTTTGCTCGTTACATTTCTGC
	Operon 220	Komp oper 1378R2	NNNNTCTAGATTATTCGTTTCCTTCACAGC

[a] All primers were developed for this study.
[b] Putative operon 051 includes the *lmo0288* (*yycG*), *lmo0289*, and *lmo0290* genes, and putative operon 220 includes the *lmo1377* (*lisR*) and *lmo1378* (*lisK*) genes (36).
[c] Restriction sites are underlined. Overlapping areas are marked with italics. N, any of the bases, i.e., adenine (A), cytosine (C), guanine (G), or thymine (T).

broth. Appropriate antibiotics (Sigma-Aldrich, St. Louis, MO) were added to the agars and broths when needed. Primers for the construction of the deletion mutant and complemented strains (Table 2), for sequencing of the target gene deletion areas of the mutants (Table 3), and for RT-qPCR (Table 4) were designed using Primer3 (v. 4.0.0) (28, 29; http://primer3.ut.ee/ [accessed 27 January 2015]).

RT-qPCR. Growth experiments for RT-qPCR analysis of HK gene expression were carried out according to the method of Markkula et al. (30). To study the relative expression levels of 15 HK-encoding genes at 3°C and 37°C (experiment I), the wild-type strain EGD-e was grown to logarithmic growth phase (optical density at 600 nm [OD_{600}], 0.7) at each temperature, after which samples were taken and total RNA extracted. To evaluate the effect of cold shock at 5°C on the expression levels of the HK genes in relation to their expression before cold shock (t_0; 37°C) (experiment II), wild-type cells grown to the logarithmic growth phase (OD_{600}, 0.7) at 37°C were abruptly cooled to 5°C. Samples were taken and total RNA extracted 30 min, 3 h, and 7 h after cold shock. Both experiments were repeated three times. RNA extraction, quality control, and duplicate reverse transcription reactions from 800 ng of total RNA were carried out according to the methods of Mattila et al. (31). For real-time qPCR, each cDNA sample was diluted 1:1,000. Reactions were performed in duplicate by using a DyNAmo Flash SYBR green qPCR kit (Thermo Fischer Scientific, Inc., Waltham, MD) according to the supplier's instructions, as well as a RotorGene qPCR 3000 device (Corbett Research, Sydney, Australia). The expression levels of the target genes at the logarithmic growth phase at 3°C (experiment I) or at three time points after cold shock at 5°C (experiment II) were calibrated to the expression levels at logarithmic growth phase at 37°C or at t_0 preshock, respectively. The method of Pfaffl was used, which considers the amplification efficiencies of primers (32). The 16S rRNA (*rrn*) gene, which has been shown to be the most suitable reference gene for *L. monocytogenes* under cold stress (33), was used as an internal normalization reference. Differences in the expression levels of the target genes between 3°C or post-cold shock and 37°C or t_0, respectively, were tested by the paired *t* test (Microsoft Excel 2010; Microsoft Redmond Campus, Redmond, WA).

Construction of *L. monocytogenes* histidine kinase deletion mutants. Each of the 15 known *L. monocytogenes* TCS HK genes was individually deleted by allelic replacement, without an associated antibiotic resistance gene, using a pMAD plasmid shuttle vector (30, 34). *E. coli* strain DH5α was used for pMAD cloning (31). An insert constituting up- and downstream regions (700 to 800 bp) of the target gene was constructed by splicing by overlap extension PCR (35). In order to build the vector pMADΔHK, the inserts and pMAD were digested using MluI (10 U/μl) and BamHI (20 U/μl) or EcoRI (20 U/μl) (New England BioLabs, Ipswich, MA) and ligated into pMAD by using T4 ligase (Thermo Fischer Scientific, Inc.). pMADΔHK was propagated in *E. coli* TOP10 electrocompetent cells (Invitrogen, Life Technologies, Carlsbad, CA). *L. monocytogenes* wild-type strain EGD-e was transformed with pMADΔHK by electroporation (25 μF, 200 Ω, 2.3 kV). Single-crossover mutants were selected at 39°C with erythromycin (5 μg/ml) and double-crossover mutants at 39°C without antibiotic. The deletions were confirmed by PCR and by sequencing of the target regions at the Institute of Biotechnology (University of Helsinki, Finland).

Complementation. To verify the cold-sensitive mutant phenotypes of the Δ*yycG* and Δ*lisK* strains, inserts including the coding sequences of the wild-type copy of the target gene and related genes in putative operons (36), accompanied by the related upstream region, including the putative promoter, were transformed into the respective mutant strains according to the methods of Lauer et al. and Markkula et al. (30, 37), using the site-specific phage integration vector pPL2, provided by Martin Loessner (Swiss Federal Institute of Technology, Zürich, Switzerland). In brief, the insert was restriction digested with XbaI and BamHI, making it compatible with the SpeI and BamHI restriction sites of pPL2, and ligated into the multiple-cloning site of pPL2 to make p*yycG*c and p*lisK*c. The plasmids p*yycG*c and p*lisK*c were cloned into chemically competent *E. coli* NEB5α cells (New England BioLabs) and transformed into the conjugation donor *E. coli* HB101 (Biological Resource Centre of the Institut Pasteur, Paris, France), which contains the helper plasmid pRK24. Transformants were then conjugated into recipient *L. monocytogenes* strains according to the method of Ma et al. (38), and the strains carrying the pPL2 constructs were selected by chloramphenicol (25 μg/ml) on ALOA agar (Lab M, Ltd., Lancashire, United Kingdom) at 37°C. Integration of pPL2 into each recipient *L. monocytogenes* strain was confirmed by PCR using primers NC16 and PL95 (37), and the presence of the insert was confirmed using gene-specific primers.

Growth curve analyses. Growth curve analyses of the mutants and the wild-type strain EGD-e were carried out according to the method of Markkula et al. (30). Five colonies of each strain were individually inoculated into 10 ml of BHI broth and incubated overnight at 37°C. The cultures were diluted in fresh BHI broth (1:100), and 350 μl of each suspension was transferred to a separate well of a 100-well honeycomb plate. The strains were grown in a Bioscreen C microbiology reader (Growth Curves, Helsinki, Finland) at 3°C for 23 days and at 37°C for 24 h, and the OD_{600} was measured at 1-h and 15-min intervals, respectively. The mean maximum growth rate and maximum optical density of each strain were obtained using DMFit, Web edition, software (Computational Microbiology Research Group, Institute of Food Research, Colney, Norwich, United Kingdom [http://browser.combase.cc/DMFit.aspx; accessed 27 January 2015]), using the model of Baranyi and Roberts (39). The statistical significances of differences between the growth rates and maximum optical densities of the mutant strains and those of the wild type were tested using two-tailed Student's *t* test (Microsoft Excel 2010). Correspondence between the OD_{600} values and viable cell numbers for the wild-type strain EGD-e and each deletion mutant strain was confirmed by plate counts in the early and late logarithmic and early stationary growth phases. Growth curve analyses of the Δ*yycG*c and Δ*lisK*c complemented mutant strains and the vector controls EGD-epPL2, Δ*yycG*pPL2, and Δ*lisK*pPL2 were performed as described above, with a growth period of 40 days.

Differences in minimum growth temperatures. The minimum growth temperatures of all strains were examined using a Gradiplate W10 incubator (BCDE Group, Helsinki, Finland) according to the method of Korkeala et al. (40), with the following modifications. Overnight cultures were grown in BHI broth to logarithmic growth phase and diluted 1:1,000 in BHI broth. The dilutions were plated by the stamping technique as parallel lines on tryptic soy agar (TSA; Oxoid, Hampshire, England) con-

TABLE 3 Primers for sequencing of the deletion area[a]

Gene	Primer name	Primer sequence (5′ → 3′)
lmo0050	Seq 0050 1F	ATGACAAGAATCGGACATTT
	Seq 0050 1R	TTTTTACATACCTTTTGCCTGA
	Seq 0050 2F	CGGAAAACACTAAAAAGAAAAGC
	Seq 0050 2R	CCGTTCTTCTGCTTGCTTC
lmo0288 (*yycG*)	Seq 0288 1F	TTATGAAGGCAGGCCAGAAT
	Seq 0288 1R	GGAATAGTGCGGACGTGAAT
	Seq 0288 2F	CAGCCGAGGAAGAAGAAAAC
	Seq 0288 2R	GTGAAATAAACCGAATGTAGCC
lmo0692 (*cheA*)	Seq 0692 1F	TTATCCGTTGCTGATGCTGA
	Seq 0692 1R	TGCGTTTTCCTCCCTTTTAC
	Seq 0692 2F	CGAGGGGCTTTTCTTTTGAT
	Seq 0692 2R	GACCTTTTCCCGTTTGGATAG
lmo1021	Seq 1021 1F	GGAAGAAACATCCGACTAAAG
	Seq 1021 1R	CGCATTTTCATCCGTTG
	Seq 1021 2F	AAAGTTGGGCTTGTTGTTGC
	Seq 1021 2R	TTTTTCTGGTTTAGCGGTTAGC
lmo1061	Seq 1061 1F	CGTTCGCTACTTGATGATG
	Seq 1061 1R	CCAGGGAGCATCATTTG
	Seq 1061 2F	ATGCCGAGTATGGACGGGTA
	Seq 1061 2R	ACCTTTTGATGCGGAACCTT
lmo1173	Seq 1173 1F	GCTCTTTTACTCTTTCGCTCAA
	Seq 1173 1R	TGATTTTTCTTGTTTCACGGTCTAC
	Seq 1173 2F	ACACGGAGAAAGCAAAAGGA
	Seq 1173 2R	ACAACAAAATCCCCAGCAAA
lmo1378 (*lisK*)	Seq 1378 1F	TAACGAGTGGGTGCCTTTTT
	Seq 1378 1R	TACAGATGGGTTTTTCAGCA
	Seq 1378 2F	GCTAAACAAACAACGCTACAG
	Seq 1378 2R	CGCCCGAACAAAAGAAA
lmo1508	Seq 1508 1F	TGGGGAGTTATGAAGAAAGTATGAA
	Seq 1508 1R	TCCAAGTTTTAGCACCAATGT
	Seq 1508 2F	TCCGTGAAGTAAGCGATGTG
	Seq 1508 2R	TGTGATTATGCCGATTGTCC
lmo1741	Seq 1741 1F	AAGCAATAAACACGGCATCC
	Seq 1741 1R	TTGAAAAATGGAGAAGTAGTCGT
	Seq 1741 2F	AGTGTTGTTTTTGCCCCATC
	Seq 1741 2R	TTGTTGCTGTTGGCGTTAGT
lmo1947 (*resE*)	Seq 1947 1F	AACGAAAAGGTCTGCCAACA
	Seq 1947 1R	TGATTTGATGATGCCTGGTAA
	Seq 1947 2F	TTTACCATCCGCACCCAAT
	Seq 1947 2R	AGAATCAGCAGGAGGCACAC
lmo2011	Seq 2011 1F	CGTTTTTGGTAGGCGATTAGAC
	Seq 2011 1R	CTTAGTATGGCTTTTCCTCGTATT
	Seq 2011 2F	GACGCTCTTCCCATTCTTCTT
	Seq 2011 2R	ACGGTGTTTGTCCACTTCCA
lmo2421 (*cesK*)	Seq 2421 1F	ACAGTGCGGGACCATAATAAA
	Seq 2421 1R	GATGACGATTGAAAGTGCTCA
	Seq 2421 2F	GCATTTCCCTCCACCACTCT
	Seq 2421 2R	CAAGGCTGAAGACAACGACA
lmo2500 (*phoR*)	Seq 2500 1F	TTTTCGTCTTTACCGCCAAC
	Seq 2500 1R	AACAGCACTTTATGAGAATGAATC
	Seq 2500 2F	TTTGTGCTTTAGGGTTAGTGTTTG
	Seq 2500 2R	CCAAGAGAAGTAGTGGCGAGA
lmo2582	Seq 2582 1F	CATCCGAAACAGACAGCGTA
	Seq 2582 1R	TACGCTGTCTGTTTCGGATG
	Seq 2582 2F	AAAATTGGCAACCAAGAACG
	Seq 2582 2R	TCATCTGTGCCCACTTCAAA
lmo2679 (*kdpD*)	Seq 2679 1F	ATCAAGCGCTTCGAGTCAAT
	Seq 2679 1R	ACACGAATATCCCGAAGCAC
	Seq 2679 2F	TGCTGCGGCATATCAAGTAG
	Seq 2679 2R	AAACGTTTGGCCAATTTCAG

[a] All primers were developed for this study.

taining 25 g agar/liter and were incubated in the Gradiplate incubator for 21 days, with a temperature gradient of 1 to 7°C. The experiment was repeated twice. The growth boundaries were observed with the naked eye and confirmed using a stereomicroscope. The minimum growth temperature was determined as the margin where dense bacterial growth discontinued. The significances of the differences between the minimum growth temperatures were determined by two-tailed Student's *t* test (Microsoft Excel 2010).

RESULTS

Relative expression levels of HK-encoding genes at low temperature. The relative expression levels of HK-encoding genes are presented in Fig. 1 and 2A to C. At 3°C (experiment I), *lmo0692* (*cheA*) was upregulated most notably (236-fold; $P < 0.001$) compared to the expression level at 37°C (Fig. 1). The HK-encoding genes *lmo1021*, *lmo1173*, *lmo1378* (*lisK*), *lmo1508*, *lmo1741*, *lmo1947* (*resE*), and *lmo2421* (*cesK*) showed 1.6- to 3-fold ($P < 0.05$) upregulation at 3°C relative to 37°C (Fig. 1). After cold shock from 37°C to 5°C (experiment II), the expression levels of *lmo0050*, *lmo0288* (*yycG*), *lmo1061*, *lmo1741*, and *lmo2679* (*kdpD*) were increased 1.9- to 3.4-fold ($P < 0.05$) at each time point measured in relation to pre-cold shock (Fig. 2A to C). The HK-encoding genes *lmo1021* and *lmo2500* (*phoR*) were upregulated 1.5- to 2.1-fold ($P < 0.05$) 30 min and 3 h after cold shock relative to t_0 (Fig. 2A and B). The cold shock induced the expression of *cheA* at 7 h (7.8-fold; $P < 0.01$) (Fig. 2C) and that of *lmo2011* at 30 min (1.6-fold; $P < 0.05$) (Fig. 2A) post-cold shock. The relative expression levels of the other HK-encoding genes were not statistically significantly upregulated either at 3°C or post-cold shock (Fig. 1 and 2A to C).

Phenotypes of *L. monocytogenes* HK deletion mutants and complemented strains. The mean maximum growth rates and maximum optical densities at 3°C and 37°C are presented in Table 5, and growth curves for the mutants and the complemented strains are shown in Fig. 3 and 4, respectively. At 3°C, the growth of the Δ*lisK* deletion mutant strain was almost completely restricted (Fig. 3A). The mean maximum growth rate of the Δ*lisK* mutant was 86% ($P < 0.001$) lower and the mean maximum optical density 73% ($P < 0.001$) lower than those of wild-type EGD-e (Table 5). At the same temperature, the Δ*yycG* strain showed a later onset of growth (by approximately 10 days), a 31% ($P < 0.001$) lower growth rate, and an 8% ($P < 0.01$) lower maximum

TABLE 4 Primers for RT-qPCR

Gene	Primer name	Primer sequence (5′ → 3′)	Reference
lmo0050	0050 F	AATCGCAGGTTTTGATGGA	This study
	0050 R	AGTTCGTTGGTTGCCGTATT	This study
lmo0288 (*yycG*)	0288 F	AGCCCAAGCAATGACTGAAG	This study
	0288 R	CGTGTCCCCGATGTCTAAAA	This study
lmo0692 (*cheA*)	0692 F	CGTCGTGAAAAATGCCAAG	This study
	0692 R	CTAAAATCGTTGCCCCAGAA	This study
lmo1021	1021 F	ATGGCTGTTTGCTCAGGTTTA	This study
	1021 R	GAAATGGCAGTAAGCGGAAC	This study
lmo1061	1061 F3	TCGTAAAAGCAGGCGAAGC	This study
	1061 R3	TCGTGCCGTCTACAACAGTC	This study
lmo1173	1173 F	AAGAGGACGAGCAGGAATG	This study
	1173 R	CGCAATAAGGAAACAACAGA	This study
lmo1378 (*lisK*)	1378 F	TGATGGGCAGAAGATGATGAC	This study
	1378 R	GGAAGTGAGCGGATTTACCA	This study
lmo1508	1508 F	CTGCGGATGATAAGAAAAAG	This study
	1508 R	AGCACGATAGAACCGACA	This study
lmo1741	1741 F	TCTCGTGCTGGGCTATTTCT	This study
	1741 R	GTTTCTTCGCCATTAGTTGGT	This study
lmo1947 (*resE*)	1947 F	GAACGGTGAAACTGGTGTTG	This study
	1947 R	ATGCGAGAAGAAAAGACGAAAG	This study
lmo2011	2011 F2	CAAATGGGTTATCTGCTCCTTTAC	This study
	2011 R2	TGTTTCGTTGGATGGTTGG	This study
lmo2421 (*cesK*)	2421 F2	CCCAGCAAGCCAGAAATG	This study
	2421 R2	AAAATGGATGTAAGTGGTGTCC	This study
lmo2500 (*phoR*)	2500 F	TGGTAGAGGTGGACGAAG	This study
	2500 R	AATAGTAACGGATGGGATTG	This study
lmo2582	2582 F	CAACTACTCACACTCGCTTCACTT	This study
	2582 R	CCAACTCCACTCGGTCATTT	This study
lmo2679 (*kdpD*)	2679 F	GCTAATGGGAGCGTTTGGT	This study
	2679 R	TGTGTGAGCGGACCTTCTTC	This study
rrn	16S rRNA Forward	GATGCATAGCCGACCTGAGA	31
	16S rRNA Reverse	CTCCGTCAGACTTTCGTCCA	31

optical density than those of the wild-type strain (Fig. 3A; Table 5). The Δ*resE* deletion mutant showed a slightly later onset of growth (by roughly 2 days) and a 29% ($P < 0.001$) lower growth rate than those of the wild type at 3°C (Fig. 3A; Table 5). At 3°C, other mutant strains showed no prolonged growth onset or impaired growth at low temperature compared to wild-type EGD-e. At 37°C, the growth of all the mutant strains was similar to that of wild-type EGD-e (Fig. 3B; Table 5).

Complementation of the Δ*yycG* HK deletion strain restored the phenotype of the mutant strain at low temperature to the wild-type level (Fig. 4). The growth curves for the vector controls were similar to those for the corresponding parental mutant strains. Complementation of the Δ*lisK* mutation only slightly increased the maximum optical density from the mutant level and thus did not fully restore growth at low temperature (Fig. 4).

The minimum growth temperature of the Δ*yycG* strain was 3.7°C and that of the Δ*lisK* strain 2.7°C higher ($P < 0.001$) than that of wild-type EGD-e after a 21-day incubation. Furthermore, the minimum growth temperatures of the Δ*cesK* and Δ*kdpD* strains were 2.3°C and 0.5°C ($P < 0.05$) higher, respectively, than that of wild-type EGD-e. The minimum growth temperatures of the other mutants did not differ from that of the wild type, nor was there any significant difference between the Δ*yycGc* complemented strain and wild-type EGD-e. However, the Δ*lisKc* complemented strain showed a 3.1°C higher ($P < 0.01$) minimum growth temperature than wild-type EGD-e.

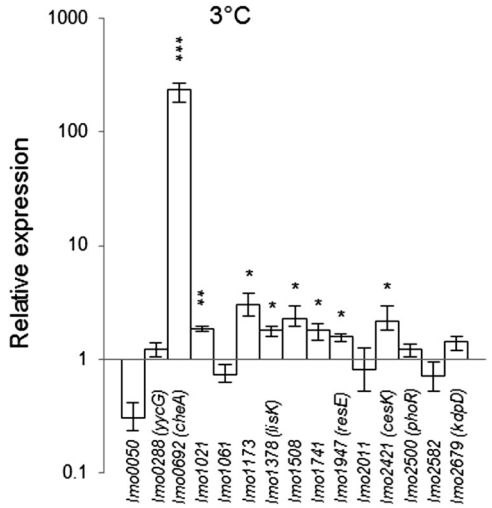

FIG 1 Relative expression levels of histidine kinase-encoding genes in *Listeria monocytogenes* EGD-e during the logarithmic growth phase at 3°C, on a log scale, each calibrated to the expression levels during logarithmic growth at 37°C. Gene expression was normalized to the 16S rRNA gene (*rrn*). Error bars represent the minimum and maximum ratios for three replicate cultures. Significantly increased relative expression levels (paired *t* test) are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$).

DISCUSSION

The growth of the Δ*lisK* and Δ*yycG* deletion mutant strains was impaired considerably in relation to wild-type EGD-e at low temperature (Fig. 3A; Table 5). The growth of neither of these strains differed from that of the wild type at 37°C (Fig. 3B; Table 5), suggesting that these genes are associated with growth of *L. monocytogenes* at low temperature. The restored phenotype of the Δ*yycG*c complemented strain (Fig. 4) confirmed the cold sensitivity of this mutant to be due specifically to the deletion of *yycG*.

Supporting the impaired growth at 3°C (Fig. 3A; Table 5) and the high minimum growth temperature of the Δ*yycG* mutant, *yycG* of wild-type EGD-e was induced upon cold shock (Fig. 2A to C). Moreover, the main growth defects of the Δ*yycG* strain were observed during the early phase of incubation at 3°C, suggesting that *yycG* plays a role in immediate cold stress but not in cold acclimation. Indeed, wild-type *yycG* was not induced during logarithmic-phase growth at 3°C (Fig. 1). According to Chan et al., the relative expression level of the cognate RR-encoding *yycF* gene of *L. monocytogenes* wild-type strain 10403S was significantly increased at low temperature (25). The fact that both of the TCS YycGF components were activated at low temperature suggests that this TCS is an important player in the cold tolerance of *L. monocytogenes*. While several studies suggest that YycF is essential (24, 26, 41), we were able to delete the cognate *yycG* gene, suggesting that *yycG* is not essential for the survival of *L. monocytogenes*. Because the *yycF* orthologue in *Streptococcus pneumoniae* regulates genes involved in fatty acid biosynthesis and the consistency of membrane lipids (42), YycF was suggested to have a role in structural alteration of the *L. monocytogenes* cell membrane (24). Thus, YycG may respond to the decreasing cell membrane fluidity at low temperature, and maintaining the optimal membrane structure may be a major means for YycGF to enhance the survival of *L. monocytogenes* at low temperature.

The growth of the Δ*lisK* mutant was restricted at low temper-

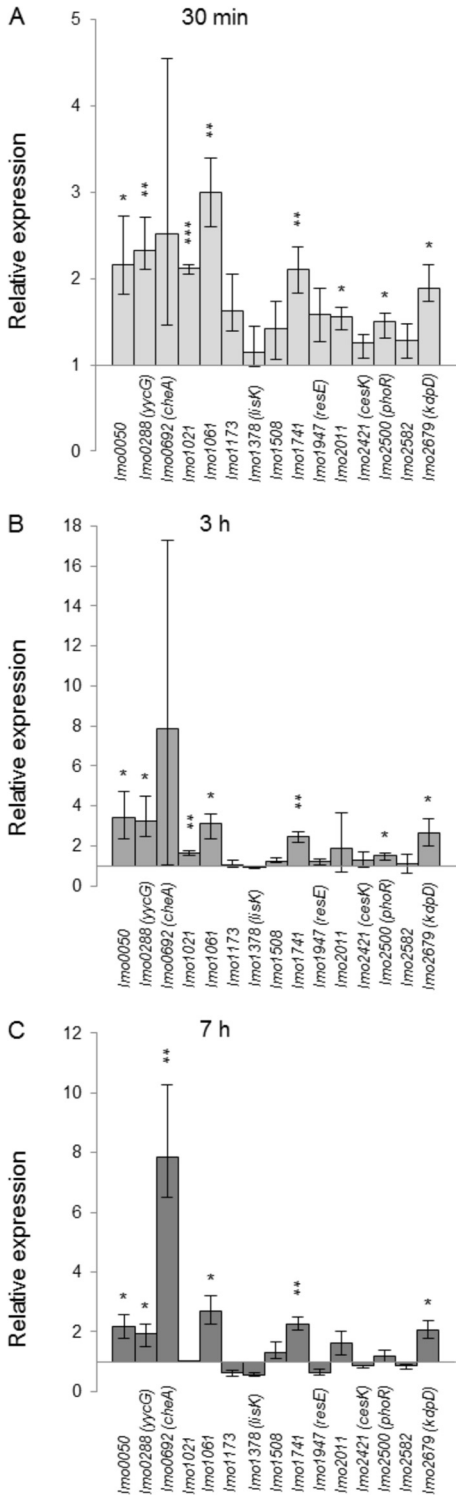

FIG 2 Relative expression levels of histidine kinase-encoding genes in *Listeria monocytogenes* EGD-e 30 min (A), 3 h (B), and 7 h (C) after cold shock from 37°C to 5°C, each calibrated to t_0 at 37°C. Gene expression was normalized to the 16S rRNA gene (*rrn*). Error bars represent the minimum and maximum ratios for three replicate cultures. Significantly increased relative expression levels (paired *t* test) are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$).

TABLE 5 Mean maximum growth rates and optical densities of *Listeria monocytogenes* wild-type EGD-e and histidine kinase deletion mutant strains at 37°C and 3°C

Strain	Mean maximum growth rate ± SD (OD_{600} units/h)[a]		Mean maximum optical density ± SD (OD_{600} units)[a]	
	37°C	3°C	37°C	3°C
EGD-e	0.24 ± 0.019	0.0035 ± 0.0001	0.85 ± 0.006	0.71 ± 0.004
Δ*lmo0050*	0.27 ± 0.022	0.0057 ± 0.0005	0.82 ± 0.054***	0.83 ± 0.010
Δ*lmo0288* (Δ*yycG*)	0.24 ± 0.021	0.0024 ± 0.0003***	0.85 ± 0.007	0.65 ± 0.039**
Δ*lmo0692* (Δ*cheA*)	0.25 ± 0.020	0.0033 ± 0.0001	0.84 ± 0.006	0.82 ± 0.006
Δ*lmo1021*	0.20 ± 0.013**	0.0055 ± 0.0007	0.83 ± 0.005*	0.81 ± 0.013
Δ*lmo1061*	0.26 ± 0.023	0.0050 ± 0.0004	0.84 ± 0.007	0.84 ± 0.010
Δ*lmo1173*	0.24 ± 0.020	0.0044 ± 0.0003	0.85 ± 0.006	0.82 ± 0.009
Δ*lmo1378* (Δ*lisK*)	0.25 ± 0.019	0.0005 ± 0.0003***	0.84 ± 0.006	0.19 ± 0.023***
Δ*lmo1508*	0.24 ± 0.021	0.0056 ± 0.0005	0.86 ± 0.007	0.87 ± 0.011
Δ*lmo1741*	0.25 ± 0.017	0.0044 ± 0.0002	0.86 ± 0.005	0.79 ± 0.007
Δ*lmo1947* (Δ*resE*)	0.25 ± 0.016	0.0025 ± 0.0001***	0.85 ± 0.005	0.76 ± 0.006
Δ*lmo2011*	0.24 ± 0.022	0.0036 ± 0.0001	0.86 ± 0.007	0.80 ± 0.007
Δ*lmo2421* (Δ*cesK*)	0.25 ± 0.023	0.0036 ± 0.0002	0.88 ± 0.007	0.77 ± 0.007
Δ*lmo2500* (Δ*phoR*)	0.22 ± 0.015	0.0032 ± 0.0002	0.83 ± 0.005	0.77 ± 0.009
Δ*lmo2582*	0.25 ± 0.019	0.0040 ± 0.0002	0.84 ± 0.006	0.79 ± 0.009
Δ*lmo2679* (Δ*kdpD*)	0.25 ± 0.018	0.0079 ± 0.0007	0.84 ± 0.005	0.96 ± 0.011

[a] Significantly decreased values compared to the corresponding value of the wild type are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$) (Student's *t* test).

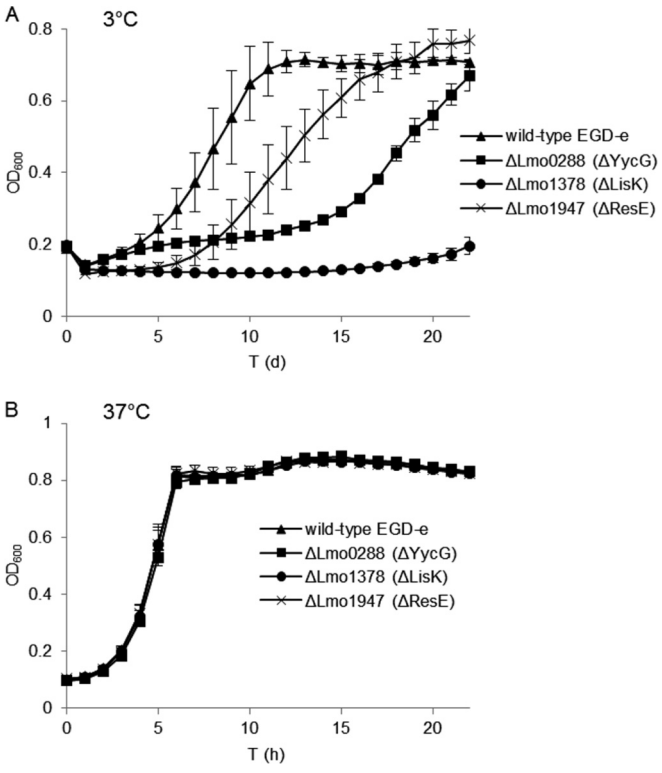

FIG 3 (A) Growth curves for *Listeria monocytogenes* Δ*lmo0288* (Δ*yycG*), Δ*lmo1378* (Δ*lisK*), and Δ*lmo1947* (Δ*resE*) histidine kinase deletion mutant strains and the wild-type strain EGD-e in BHI broth at 3°C. All the strains were grown for 23 days, and the OD_{600} was measured every hour. The other histidine kinase mutant strains showed no significantly impaired growth compared to the wild type. (B) Growth curves for *L. monocytogenes* Δ*yycG*, Δ*lisK*, and Δ*resE* histidine kinase deletion mutant strains and the wild-type strain EGD-e in BHI broth at 37°C. All the strains were grown for 24 h, and the OD_{600} values were measured every 15 min. The data shown represent the mean OD_{600} values and standard deviations for five replicate cultures. The correspondence between the OD_{600} values and viable cell numbers was verified by plate counts.

ature (Fig. 3A; Table 5), and this observation was supported by the upregulation of wild-type *lisK* at 3°C (Fig. 1). Nevertheless, *lisK* was not induced upon cold shock (Fig. 2A to C), suggesting that *lisK* plays a role in cold acclimation of *L. monocytogenes*. LisK has been described to contribute to the resistance of *L. monocytogenes* strain LO28 to cephalosporins and its sensitivity to nisin (43) and to have a growth phase-dependent role in acid tolerance (44). Moreover, the deletion of *lisK* in LO28 resulted in reduced osmotolerance (45) and in reduced virulence in mice (44). The reduced growth of the Δ*lisR* strain at low temperature reported by Chan et al. (26) supports our findings of impaired growth at 3°C and a high minimum growth temperature of the Δ*lisK* strain, indicating that the TCS LisKR plays an important role in the cold tolerance of *L. monocytogenes*. LisK has been proposed to positively regulate *htrA* (*lmo0292*), encoding a serine protease (46); *lmo2229*, encoding the penicillin-binding protein PBPA2; and *lmo1021*, encoding an HK (43). LisK is thus probably involved in cell envelope-related stress

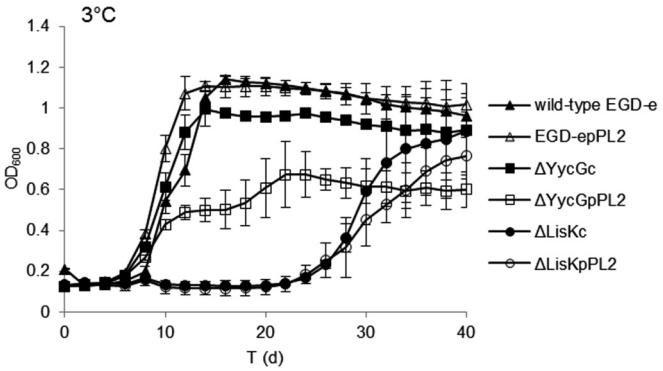

FIG 4 Growth curves for *Listeria monocytogenes* wild-type EGD-e, the Δ*yycGc* and Δ*lisKc* complemented strains, and the vector controls EGD-epPL2, Δ*yycG*pPL2, and Δ*lisK*pPL2 at 3°C. All the strains were grown for 40 days, and the OD_{600} was measured every hour. The data shown represent the mean OD_{600} values and standard deviations for five replicate cultures.

responses, as previously suggested by Gottschalk et al. and Nielsen et al. (47, 48).

The failure to complement the Δ*lisK* mutation at low temperature could be explained by the intentional integration of pPL2 into a chromosomal target position at the $tRNA^{Arg}$ prophage attachment site in the EGD-e genome (37). In this atypical location, transcription or translation of the newly introduced *lisK* gene could be disrupted, leading to non- or dysfunctional LisK. Alternatively, the disrupted phenotype of the Δ*lisK*c strain may have stemmed from possible overexpression of *lisR*. Since *lisK* and *lisR* are carried in an operon, with *lisR* lying upstream of *lisK*, complementation was attempted by using the whole operon in order to restore the putative promoter. Thus, the presence of two copies of functional *lisR* may have led to production of excess LisR, which could either be lethal to the cells or hamper the expression or function of LisK. Indeed, Cotter et al. demonstrated that overexpression of *lisR* reversed the elevated nisin resistance observed for a Δ*lisK* mutant (43).

Low temperature induced the relative expression of *resE*, and the respective mutant strain displayed a lower mean maximum growth rate than wild-type EGD-e at 3°C (Fig. 1; Table 5). However, the growth onset of the Δ*resE* strain at 3°C was only slightly delayed (Fig. 3A). The ResDE homologue in *B. subtilis* was reported to contribute to both aerobic and anaerobic respiration (49), but no examples of this TCS affecting growth at low temperature have been presented. Our findings suggest that *resE* plays a minor role in an early growth phase of *L. monocytogenes* at low temperature. The cognate RR ResD was suggested to affect the composition of the cell envelope by responding to changes in cell wall integrity, leading to enhanced tolerance of *L. monocytogenes* to ethanol (24). Thus, the role of ResE at low temperature may also involve sensing the functionality of the cell envelope.

TCSs are known to hold the potential for cross-phosphorylation, either through cross talk or through branching signaling pathways (50). Our study revealed *L. monocytogenes* to cope at 3°C without the complete *yycG* gene, while *yycF* has been shown to be essential (24, 26, 41). This suggests that, at low temperature, YycF of *L. monocytogenes* may communicate with HKs other than YycG in order to overcome the stress. The TCS YycFG is highly conserved and presumed to be specific for Gram-positive bacteria with a low G+C content (51). Howell et al. demonstrated the HK PhoR to cross-phosphorylate the noncognate RR YycF in *B. subtilis* (52); thus, PhoR may be an interesting target for further studies on cross-phosphorylation of YycFG in *L. monocytogenes*. Intriguingly, the growth of the Δ*lmo1061* and Δ*lmo1173* strains did not differ from that of wild-type EGD-e at low temperature (Table 5). However, Chan et al. reported the Δ*lmo1060* and Δ*lmo1172* cognate RR mutants to have reduced growth at 4°C (26). This also alludes to the putative existence of an alternative phosphotransfer pathway for the RRs Lmo1060 and Lmo1173 at low temperature. Nonetheless, further studies are warranted to verify these possibly cross-phosphorylating TCSs of *L. monocytogenes*.

Some of the HK genes were upregulated at low temperature, whereas the corresponding mutant strains showed no trouble in growth at 3°C. The most prominently cold-induced HK gene was *cheA* (Fig. 1). Transcription of *cheA* has been suggested to be thermoregulated (53); it is thus plausible that low temperature was the main inducer of *cheA* in the present study. However, the observed growth of the Δ*cheA* mutant at 3°C indicates that *cheA* is not essential for *L. monocytogenes* at low temperature. Genes involved in bacterial chemotaxis signaling systems are highly conserved (54), and Palonen et al. also demonstrated that *cheA* of *Y. pseudotuberculosis* IP32953 was induced at 3°C in relation to 28°C (22). As opposed to our findings, insertional mutation of *cheA* also yielded a cold-sensitive phenotype of *Y. pseudotuberculosis* (22). In our study, the expression of *lmo1508* at 3°C was induced in relation to that at 37°C (Fig. 1), as detected previously in *L. monocytogenes* strain 10403S at 10°C (27). Conversely, we did not find the Δ*lmo1508* strain to struggle at 3°C, which suggests that Lmo1508 has no role in the growth of *L. monocytogenes* at low temperature. In addition to TCS cross-phosphorylation, these findings may also be due to posttranscriptional regulation. For instance, regulation of the cold shock protein-encoding genes occurs mainly at the posttranscriptional level (55). Renzoni et al. also showed *prfA* transcripts, but not the PrfA protein product, in cells at low temperature, suggesting that posttranscriptional events inhibit translation initiation of the main virulence gene regulator of *L. monocytogenes* (56). Hence, the role of HK genes, which show cold-induced expression but not cold-sensitive mutant phenotypes, in the cold tolerance of *L. monocytogenes* is tentative. The upregulation of *lmo1021* at 3°C (Fig. 1) and post-cold shock (Fig. 2A and B) may have been due to an increase in *lisK* expression, as *lmo1021* was suggested to be positively regulated by LisK (43). However, the growth of the Δ*lmo1021* strain at 3°C was unaffected, which suggests that *lmo1021* does not have a role in the growth of *L. monocytogenes* at low temperature.

In conclusion, we identified two HK-encoding genes, *yycG* and *lisK*, to be important for the growth of *L. monocytogenes* at 3°C. In addition, several HK-encoding genes of *L. monocytogenes* EGD-e were upregulated either at 3°C or post-cold shock, suggesting putative roles for them at low temperature.

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