

CesRK, a Two-Component Signal Transduction System in *Listeria monocytogenes*, Responds to the Presence of Cell Wall-Acting Antibiotics and Affects β -Lactam Resistance

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Listeria monocytogenes is a food-borne pathogen that can cause a variety of illnesses ranging from gastroenteritis to life-threatening septicemia. The β -lactam antibiotic ampicillin remains the drug of choice for the treatment of listeriosis. We have previously identified a response regulator of a putative two-component signal transduction system that plays a role in the virulence and ethanol tolerance of *L. monocytogenes*. Here we present evidence that the response regulator, CesR, and a histidine protein kinase, CesK, which is encoded by the gene downstream from *cesR*, are involved in the ability of *L. monocytogenes* to tolerate ethanol and cell wall-acting antibiotics of the β -lactam family. Furthermore, CesRK controls the expression of a putative extracellular peptide encoded by the *orf2420* gene, located immediately downstream from *cesRK*. Inactivation of *orf2420* revealed that it contributes to ethanol tolerance and pathogenesis in mice. Interestingly, we found that transcription of *orf2420* was strongly induced by subinhibitory concentrations of various cell wall-acting antibiotics, ethanol, and lysozyme. The induction of *orf2420* expression was abolished in the absence of CesRK. Our data suggest that CesRK is involved in regulating aspects of the cell envelope architecture and that changes in cell wall integrity provide a potent stimulus for CesRK-mediated regulation. These results further our understanding of how *L. monocytogenes* senses and responds to antibiotics that are used therapeutically in the treatment of infectious diseases.

Listeria monocytogenes is a gram-positive food-borne pathogen that can give rise to listeriosis, a serious disease with symptoms that include septicemia, encephalitis, meningitis, abortion, and febrile gastroenteritis. While listeriosis may occur in otherwise healthy individuals, persons primarily at risk are immunocompromised patients, pregnant women, the very young, and the elderly (37). The mortality rate is >20%, thus making listeriosis one of the most deadly bacterial infections. Individuals who develop listeriosis are usually treated with ampicillin, alone or in combination with gentamicin (33). *L. monocytogenes* has previously been reported to be susceptible to a wide range of antibiotics, but recently it has been found that strains of *Listeria* isolated from food, the environment, or patients with listeriosis have acquired resistance to a variety of antibiotics (40). It is of concern that the range of less effective antibiotics now includes ampicillin and gentamicin (30, 39). The emergence of antibiotic resistance among bacterial pathogens, including *L. monocytogenes*, emphasizes the need to understand how bacteria sense and respond to the presence of antimicrobial agents.

Two-component signal transduction systems allow bacteria to recognize and interpret a variety of signals from the environment (15). Two-component systems typically consist of a

membrane-bound sensor histidine protein kinase and a response regulator-transcription factor. The sensor kinase allows the bacterium to sense signals from the external environment and transfer this information to the response regulator inside the cell through a phosphorylation cascade. In response to specific signals, the sensor kinase becomes phosphorylated at a conserved histidine residue through an autophosphorylation event. The phosphoryl group is subsequently transferred from the kinase to a conserved aspartic acid residue in the response regulator. Phosphorylation of the response regulator alters its DNA-binding activity, resulting in the up- or down-regulation of genes under its control.

Certain two-component signal transduction systems have been shown to contribute to the resistance or sensitivity to antibiotics. In enterococci the VanRS two-component system mediates resistance to the glycopeptide antibiotic vancomycin by controlling the expression of genes required for the synthesis of modified cell wall precursors (3). The replacement of D-alanyl-D-alanine with D-alanyl-D-lactate results in a modified peptidoglycan with a lower binding affinity for vancomycin. The presence of vancomycin or other cell wall-acting agents in the growth medium appears to generate a signal sensed by VanS; however, the reports on the specificity of induction of the VanRS two-component system are conflicting (1, 4, 12, 14, 35). In *Streptococcus pneumoniae*, the VncRS two-component system mediates vancomycin-induced cell death (24), thus demonstrating that signal transduction is required for the bactericidal activity of vancomycin in this pathogen. How-

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ever, these conclusions were not confirmed in a more recent study (31). A second two-component system of *S. pneumoniae*, CiaHR, plays a role in β -lactam resistance and responds to changes in the external concentration of Ca^{2+} ions (8).

A previous study (20) identified three response regulators of two-component systems in *L. monocytogenes* LO28 that are important for virulence and growth under stress conditions found in the host environment. One of these response regulators, CesR (the cephalosporin sensitivity response regulator, formerly designated RR96), is homologous to VanR of enterococci (20). Inactivation of *cesR* resulted in reduced pathogenesis in mice and improved resistance to ethanol, a widely used disinfectant and food preservative. In the present study, we have investigated in more detail the molecular mechanisms underlying the reduced pathogenesis and increased ethanol resistance displayed by the mutant with the *cesR* insertion. We show that in-frame deletions of either *cesR* or the gene located downstream from *cesR*, *cesK* (cephalosporin sensitivity histidine protein kinase), which encodes a histidine protein kinase, increased the organism's sensitivity to cell wall-acting antibiotics of the β -lactam family. We have also identified a small open reading frame, *orf2420*, regulated by the CesRK two-component system. The transcription of *orf2420* is strongly induced during the transition into stationary phase. Furthermore, various antimicrobial agents that affect the bacterial cell wall act as inducers of *orf2420* expression in a CesRK-dependent manner. These results suggest a role for CesRK in sensing and responding to changes in cell wall integrity.

MATERIALS AND METHODS

Bacterial strains and growth media. *L. monocytogenes* serotype 1/2c strain LO28 (36) was routinely grown in brain heart infusion (BHI) medium (Oxoid) at 37°C with shaking. BK96 is an LO28 derivative carrying an insertion in *cesR* (formerly *r96*) (20). When required, erythromycin or kanamycin was added (final concentrations, 5 and 50 $\mu\text{g ml}^{-1}$, respectively). *Escherichia coli* strain TOP10 (Invitrogen) was grown in Luria-Bertani medium. When required, 150 μg of erythromycin ml^{-1} was added to the medium.

DNA sequence analysis of the *cesR* region in *L. monocytogenes* LO28 and computer analyses of DNA and protein sequences. On the basis of the genome sequence of *L. monocytogenes* EGD, we designed PCR primers (Table 1) for the amplification of regions in *L. monocytogenes* LO28 up- and downstream from *cesR*. For the PCRs we used LO28 chromosomal DNA as the template and *Pfu* DNA polymerase (Invitrogen), which possesses proofreading 3' to 5' exonuclease activity. DNA sequence analysis of the PCR fragments was carried out by using the CEQ dye terminator cycle sequencing kit (Beckman Coulter). Homology searches were performed with the BLAST program (2). For the identification of signal peptides, we used the Signal P program (23). For the identification of transmembrane regions, we used the TMpred program (17).

Construction of strains with *cesR*, *cesK*, *orf2420*, *lmo2423*, and *lmo2424* deletions. For construction of in-frame mutants with deletions of the *cesR*, *cesK*, *orf2420*, *lmo2423*, and *lmo2424* genes, *L. monocytogenes* LO28 chromosomal DNA was used as the template for PCR amplification of DNA fragments containing either the 5' end of the gene and upstream sequences or the 3' end of the gene and downstream sequences. The primers used for the PCRs are listed in Table 1. Primers RRA and RRB (210 bp) and primers RRC and RRD (232 bp) were used for the *cesR* gene. For the *cesK* gene we used primers HKA and HKB (365 bp) and primers HKC and HKD (435 bp). Primers OrfA and OrfB (455 bp) and primers OrfC and OrfD (420 bp) were used for the *orf2420* gene, whereas primers KatA and KatB (456 bp) and primers KatC and KatD (423 bp) were used for the *lmo2423* gene. Finally, we used primers ThioA and ThioB (241 bp) and primers ThioC and ThioD (242 bp) for the *lmo2424* gene. In a second round of PCR, the *cesR*, *cesK*, *orf2420*, *lmo2423*, and *lmo2424* fragments were joined by using the method of splicing by overlap extension (18), creating PCR fragments of each fragment containing in-frame deletions of 495 bp in *cesR*, 492 bp in *cesK*, 141 bp in *orf2420*, 498 bp in *lmo2423*, and 171 bp in *lmo2424*. The $\Delta cesR$, $\Delta cesK$, and $\Delta orf2420$ fragments were digested with *Xba*I and *Eco*RI, whereas the

TABLE 1. Primers used in this study

Primer	Sequence (5'-3')
RRA.....	GGGGTCTAGAAAGTGTACAATAGGTAGAA ^a
RRB.....	TAATATCATAAAAGTTGGCTG
RRC.....	CAGCCAACCTTAGTGATATTAGTGATTAAGACT ATTTGGG
RRD.....	GGGGGAATTCATACCTCCCGTAAAAACAAAG ^b
RR-PRIM.....	TCCGCACCATCATAAGCTTG
HKA.....	GGGGTCTAGACAAGAAAAGGCCCTTGGAG ^a
HKB.....	CCAAAAAGATTGCATGATTCCGG
HKC.....	CGCGAATCATGCAATCTTTTGGCGGCAATTAG TGCGGAG
HKD.....	GGGGGAATTCGACGATAAACTGGTTTGGCG ^b
OrfA.....	GGGGTCTAGATGGCAACCAATCATGCGC ^a
OrfB.....	CGTAATACAAGCAACAACAATTAAC
OrfC.....	GTTTAAATGTTGTGCTTATTACGAAATAA ATTAACATAAGTGAGAAA
OrfD.....	CCCCGAATTCATGATCGACTGCGAGAAC ^b
Orf7.....	TTTGAACATGTACATAAATACCGC
KatA.....	GGGGGATCCCGTCAACCGGCCCTTAAGC ^c
KatB.....	GGCCGTTGATTAGAAATCCG
KatC.....	CGGATTTCTAATCAACCGGCCCATGATTTTCGC TCGAGACG
KatD.....	GGGGTCTAGATAAACGCCATACTTCCGCAC ^a
Kat8.....	CCTGTAAAAAGCCGACAATC
ThioA.....	GGGGTCTAGACGAAACATTAGAAGATGAAC CAG ^a
ThioB.....	GACCGCAAACCTTTCTTTTCCC
ThioC.....	GGGAAAAAGAAAGTTTGGCGACCACATCAGTA CCGGCTCTAG
ThioD.....	CCCCGGATCCAGCAGCTGAATTGTGTCTATAG ^c
Thio1.....	GCTTCTATAACCACGTCCAC
Thio-For.....	GGGGAAATTCCTTGAAGAAGTAGAAGAAGCGG ^b
Thio-Rev.....	GGGGATCCCGCTTCTATAACCACGTCCAC ^c
Kat-For.....	GGGGAAATTCCTGTATAGTGAGTGATAC ^b
Kat-Rev.....	GGGGATCCGGTTCATAGAAAAAGCCTCCG ^c
RR-For.....	GGGGAAATTCGCTATGTGACGAAATGAGGC ^b
RR-Rev.....	GGGGGATCCCGCACCATATAAGCTTG ^c
Orf-For.....	GGGGAAATTCGTGAAGAGATTCTAATGTGCG ^b
Orf-Rev.....	GGGGGATCCGTAATACAAGCAACAACAATTA AAAC ^c

^a The underlined sequence is the *Xba*I restriction enzyme site.

^b The underlined sequence is the *Eco*RI restriction enzyme site.

^c The underlined sequence is the *Bam*HI restriction enzyme site.

$\Delta lmo2423$ and $\Delta lmo2424$ fragments were digested with *Bam*HI and *Xba*I. The fragments were then cloned into temperature-sensitive shuttle vector pAUL-A and digested with *Xba*I-*Eco*RI or *Bam*HI-*Xba*I (5). The resulting plasmids were introduced into *L. monocytogenes* LO28 by electroporation (25), and integration of the plasmid was achieved by growing the transformed strains at 42°C in the presence of erythromycin. To allow allelic exchange between the intact genes and the deleted genes to take place, the strains containing the integrated plasmids were then grown at 30°C in the absence of erythromycin. Finally, strains carrying the desired deletions were identified by PCR, and correct deletion events were verified by DNA sequence analysis of the resulting PCR products.

Ethanol tolerance assays. Overnight cultures of the *L. monocytogenes* strains in BHI medium were diluted 1:100 in fresh BHI medium containing 5% ethanol. Bacterial growth was monitored over a period of 24 h by measuring the optical density at 600 nm (OD₆₀₀).

Antibiotic assays. The sensitivities of the wild-type strain and mutant strains $\Delta cesR$, $\Delta cesK$, and $\Delta orf2420$ to a variety of antibiotics were assayed by agar diffusion. Overnight cultures were diluted to 10⁷ CFU/ml and swabbed onto BHI agar. Filter disks (diameter, 6 mm; Oxoid) containing the antibiotics to be studied were placed on the surfaces of the agar plates. The plates were incubated overnight at 37°C. On the following day, the diameters of the zones of bacterial growth inhibition surrounding the disks were measured. The filter disks contained 30 μg (unless otherwise stated) of the following antibiotics: gentamicin, erythromycin, streptomycin (25 μg), rifampin, vancomycin, novobiocin, tetracycline, polymyxin B (300 μg), colistin sulfate (25 μg), minocycline, clindamycin (10 μg), kanamycin, spectinomycin (25 μg), fusidic acid (10 μg), chloramphenicol, oxytetracycline, ampicillin (10 μg), penicillin G (10 μg), cephalothin, cefaclor, cefoperazone, cephalixin, ceftriaxone, cefoxitin, cefuroxime, cefotetan, and cefta-

zidime. For each antibiotic, at least three independent disk diffusion assays were performed per strain.

RNA techniques. Total RNA was prepared by a hot acid phenol procedure (28). Primer extension analysis was performed as described previously (22) by using 15 µg of total RNA per reaction mixture. Primers Thio1, Kat8, RR-PRIM, and Orf7 (Table 1) labeled with ^{32}P at the 5' end were used for detection of the *lmo2424*, *lmo2423*, *cesR*, and *orf2420* transcription start sites, respectively. For detection of transcription start sites, RNA was prepared from wild-type cell cultures at an OD_{600} of 0.6. For measurement of *orf2420* induction by ethanol and cefuroxime, RNA was prepared from wild-type and $\Delta cesR$ cell cultures with and without treatment with 1% ethanol or 4 µg of cefuroxime/ml. The inducers were added to the cultures at an OD_{600} of 0.3, and samples for RNA preparations were collected 20 min after induction. Controls without ethanol and cefuroxime treatment were included in the experiment.

Mouse virulence assay. Overnight cultures were diluted 100-fold and grown to an OD_{600} of 0.6. The bacteria were washed and resuspended in phosphate-buffered saline prior to infection. Six-week-old female BALB/C mice were infected intragastrically with 2×10^9 bacteria or intraperitoneally with 1×10^{-4} bacteria. Three days after infection, the mice were killed and the spleen of each mouse was homogenized. Tenfold serial dilutions of the homogenized spleen in physiological saline buffer were plated onto BHI agar plates. Colonies were counted after overnight incubation at 37°C. The data were tested for significance by using the Student *t* test.

Construction of *lacZ* fusions to *cesR*, *orf2420*, *lmo2423*, and *lmo2424* promoters and β -galactosidase assays. DNA fragments containing the promoter regions of *cesR*, *orf2420*, *lmo2423*, and *lmo2424* were amplified by PCR with primers RR-For and RR-Rev, Orf-For and Orf-Rev, Kat-For and Kat-Rev, and Thio-For and Thio-Rev, respectively (Table 1). The PCR fragments were digested with *EcoRI* and *BamHI* and cloned into *EcoRI*-*BamHI*-digested pTCV-*lac*, a shuttle vector for the construction of transcriptional fusions (29). Correct insertion of the fragments into pTCV-*lac* was verified by DNA sequencing analyses. The resulting plasmids containing fusions of the promoters to *lacZ* were electroporated into the wild-type and mutant strains.

For measurement of *orf-lacZ* expression in the presence of a variety of antimicrobial agents, wild-type, $\Delta cesR$, and $\Delta cesK$ strains carrying the *orf2420-lacZ* fusion were grown in BHI medium to an OD_{600} of 0.2. The cultures were split, and the potential inducers were added at subinhibitory concentrations. Controls to which no inducers were added were included in the experiment. Cells were collected 1 h after the addition of the potential inducers and assayed for β -galactosidase activity. The antimicrobials tested and their final concentrations were as follows: ethanol, 1%; cephalixin, 4 µg/ml; cefuroxime, 4 µg/ml; penicillin G, 0.05 µg/ml; ampicillin, 0.05 µg/ml; vancomycin, 0.1 µg/ml; bacitracin, 4 µg/ml; and D-cycloserine, 20 µg/ml. For the lysozyme induction experiments, cells were collected 2 h after the addition of lysozyme to a final concentration of 0.2, 0.5, or 1 mg/ml.

For the β -galactosidase assay, cells were permeabilized by treatment with 0.5% toluene and 4.5% ethanol, and β -galactosidase activities were determined as described previously (21). The specific activity of β -galactosidase was calculated as $[(\text{OD}_{420}$ of the reaction mixture - OD_{550} of the reaction mixture)/(reaction time in minutes \times OD_{600} of the cells used in the reaction mixture)]. The specific β -galactosidase activities presented are the averages of three independent experiments, in which the observed variations did not exceed 10%.

Nucleotide sequence accession number. The nucleotide sequence of the locus in *L. monocytogenes* LO28 encoding the putative proteins Lmo2424, Lmo2423, CesR, CesK, and Orf2420 was submitted to GenBank and given accession number AY281157.

RESULTS

Genetic organization of *cesR* region in *L. monocytogenes* LO28. A gene encoding a putative response regulator, CesR (formerly designated RR96), which is involved in the virulence and ethanol tolerance of *L. monocytogenes* LO28, was identified previously (20). Analysis of the *L. monocytogenes* EGD-e genome sequence (9) revealed that *cesR* lies in the center of a five-gene cluster. DNA sequence analysis of the corresponding region in *L. monocytogenes* LO28 showed a gene organization identical to that of the *cesR* region in EGD-e (Fig. 1A). The product encoded by *lmo2424* is a 94-amino-acid (aa) protein that shares homology with the thioredoxins of the thioredoxin-

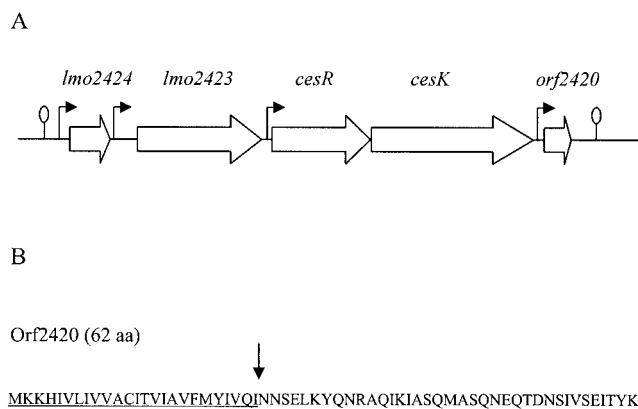


FIG. 1. *cesRK* locus in *L. monocytogenes* LO28. (A) Genetic organization of the *cesRK* locus. Transcription start sites are indicated by arrows. Putative transcription terminators are indicated by lollipops. See text for a detailed description of the putative gene products. (B) The putative protein encoded by *orf2420* contains a signal peptide sequence of 25 aa (underlined). The putative cleavage site is indicated by an arrow.

thioredoxin reductase systems implicated in the regulation of the redox homeostasis (10). *lmo2423* encodes a 291-aa protein homologous to Co^{2+} , Zn^{2+} , and Cd^{2+} transporters of the cation diffusion facilitator family (26). As published previously, *cesR* encodes a putative response regulator (231 aa) of a two-component signal transduction system with homology to VanR of *Enterococcus faecium* (20). CesR contains an N-terminal receiver domain and a C-terminal DNA-binding domain characteristic of response regulator proteins of the OmpR-PhoB subfamily (15). *cesK*, located just downstream from *cesR*, encodes a putative histidine protein kinase (381 aa) of a two-component signal transduction system and shows homology to VanS of *E. faecium*. The CesK protein contains two potential membrane-spanning regions in the N-terminal region and a transmitter domain in the C-terminal portion of the protein, typical of sensor proteins of two-component regulatory systems (15). *orf2420* encodes a 62-aa protein with no homology to any known proteins. Orf2420 possesses an N-terminal signal peptide, suggesting that it is secreted from the cell (Fig. 1B). Reverse transcription-PCR analyses suggested the presence of putative transcription terminators upstream from *lmo2424* and downstream from *orf2420* (Fig. 1A; data not shown). When the sequence information for the CesRK locus from *L. monocytogenes* LO28 and EGD-e was compared with the genome sequence of the nonpathogenic species *Listeria innocua*, we observed that only four of the five coding sequences were predicted in *L. innocua* (9). *lmo2424*, *lmo2423*, *CesR*, and *CesK* of *L. monocytogenes* LO28 correspond to Lin2518, Lin2517, Lin2516, and Lin2516 of *L. innocua*, respectively. However, an open reading frame corresponding to Orf2420 in *L. monocytogenes* LO28 and Lmo2420 in *L. monocytogenes* EGD-e has not been defined in *L. innocua* (9).

In order to identify the promoters at the *cesRK* locus, we performed primer extension analysis with RNA samples from wild-type cells in the exponential growth phase (OD_{600} = 0.6). We found that the transcription start sites were located upstream from *lmo2424*, *lmo2423*, *cesR*, and *orf2420* (Fig. 2A to

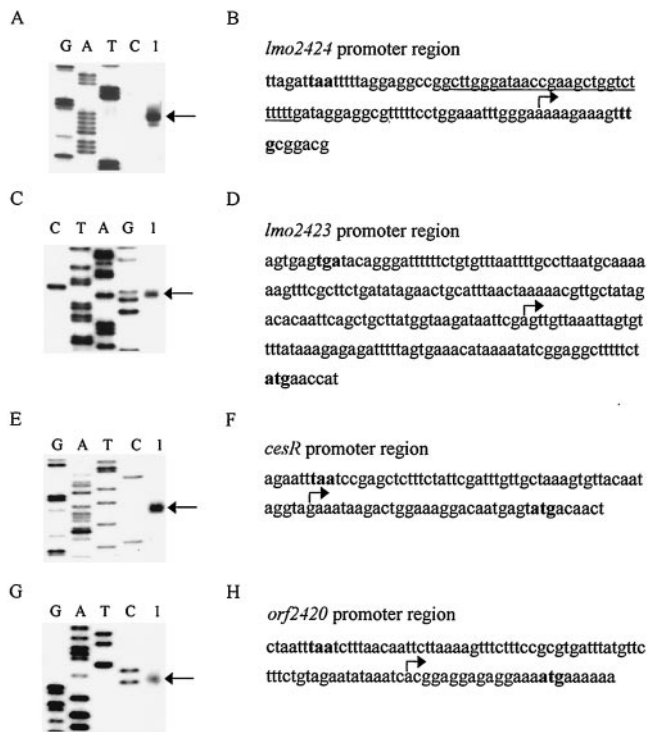


FIG. 2. Primer extension analysis of promoters in the *cesRK* locus. Identification of transcription initiation sites at the promoters of *lmo2424* (A), *lmo2423* (C), *cesR* (E), and *orf2420* (G). The primer extension products are indicated by arrows. Lanes G, A, T, and C are sequencing ladders. The promoter region sequences are shown for *lmo2424* (B), *lmo2423* (D), *cesR* (F), and *orf2420* (H). Translation start and stop codons are indicated in boldface. The transcription start site is indicated by an arrow. A putative transcription terminator upstream from *lmo2424* is underlined in panel B.

H). No transcription initiation site was observed in the 34-bp intergenic region between *cesR* and *cesK* (data not shown).

Deletion of *cesR*, *cesK*, and *orf2420* results in ethanol tolerance. We have previously shown that insertional inactivation of *cesR* results in ethanol resistance. In order to determine the contribution of the individual genes at the *cesRK* locus to this phenotype, we constructed mutants with in-frame deletions of each of the five genes (*lmo2424*, *lmo2423*, *cesR*, *cesK*, and *orf2420*). In the absence of ethanol we did not detect any differences in growth between the wild-type and mutant strains (data not shown). Next, we examined the growth of the wild-type and mutant strains with deletions in 5% ethanol, together with the original mutant with the *cesR* insertion, BK96 (20). As expected, BK96 was capable of growing in the presence of this concentration of ethanol, whereas growth of the wild-type strain was restricted (Fig. 3). Mutants carrying in-frame deletions of *cesR*, *cesK*, and, to a lesser extent, *orf2420*, were also able to grow in the presence of 5% ethanol, whereas mutants with in-frame deletions of *lmo2424* or *lmo2423* were not ethanol resistant. Our data show that *cesR*, *cesK*, and *orf2420* increase the ethanol tolerance of *L. monocytogenes*.

Deletion of *cesR*, *cesK*, and *orf2420* affects pathogenicity in mice. Disruption of *cesR* leads to a significant reduction in the virulence potential in the mouse model of intragastric (i.g.) infection but not in the mouse model of intraperitoneal (i.p.)

infection (20). To determine whether this effect can be attributed to the lack of a functional CesRK system, we tested the virulence of the mutants carrying in-frame deletions of *cesR* and *cesK* in i.g. infection assays. Mice were infected with the wild-type strain, the $\Delta cesR$ strain, or the $\Delta cesK$ strain. On day 3, the number of bacteria in the spleens of the mice was estimated as described in Materials and Methods. The counts of the $\Delta cesR$ and $\Delta cesK$ mutants in the spleens on day 3 corresponded to the levels previously observed for BK96, i.e., a 10-fold reduction in the number of CFU per spleen relative to the number of CFU of the wild-type strain ($P < 0.03$) (Fig. 4A). These results support previous observations, suggesting that the CesRK two-component system contributes to the pathogenicity of *L. monocytogenes* in mice.

We also infected mice through the i.g. or i.p. route with the *orf2420* deletion mutant. In both these assays, the $\Delta orf2420$ mutant was significantly impaired in its ability to colonize the spleen (Fig. 4A and B; $P < 0.03$). These results suggest that Orf2420 also plays a role in murine listeriosis.

Antibiotic resistance of $\Delta cesR$, $\Delta cesK$, and $\Delta orf2420$ strains. The ethanol resistance phenotypes observed for the *cesR*, *cesK*, and *orf2420* in-frame deletion mutants suggested that either the cell membrane or cell wall integrity might be affected by the mutations. To address this possibility we compared the levels of resistance of wild-type and mutant strains to a variety of antibiotics by using a disk diffusion assay. No significant differences in inhibition zone sizes between wild-type and mutant strains were observed for a large range of antibiotics (gentamicin, erythromycin, streptomycin, rifampin, vancomycin, novobiocin, tetracycline, polymyxin B, colistin, minocycline, clindamycin, kanamycin, spectinomycin, fusidic acid, chloramphenicol, oxytetracycline; data not shown). However,

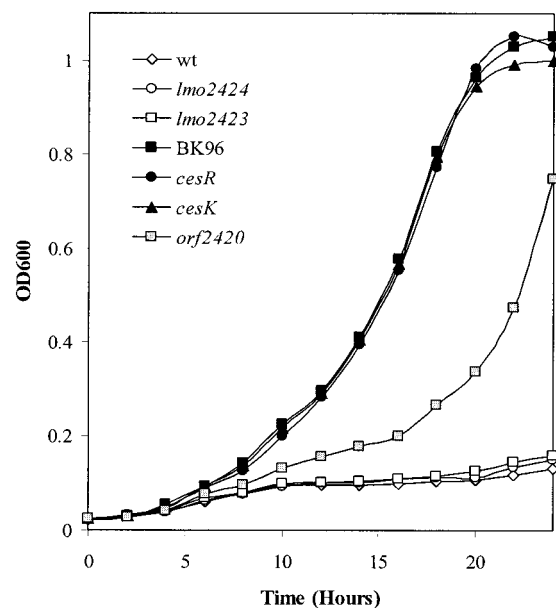
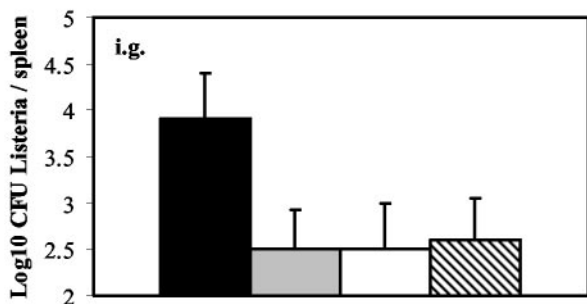


FIG. 3. Ethanol tolerance of wild-type and mutant strains. The growth of the wild-type (wt) strain, *cesR* insertion mutant BK96, and strains carrying in-frame deletions of *lmo2424*, *lmo2423*, *cesR*, *cesK*, and *orf2420* was monitored in BHI medium containing 5% ethanol. The data represent the means of three experiments, in which the observed variation did not exceed 10%.

A



B

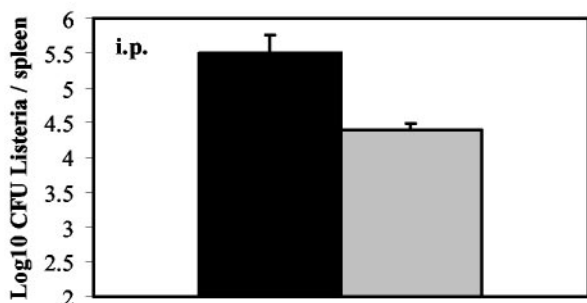


FIG. 4. Mouse virulence assays with wild-type, $\Delta orf2420$, $\Delta cesR$, and $\Delta cesK$ strains. The growth and survival of wild-type (black bars), $\Delta orf2420$ (gray bars), $\Delta cesR$ (white bars), and $\Delta cesK$ (hatched bars) strains in the spleens of infected mice were evaluated on day 3 after i.g. (A) or i.p. (B) injection. The \log_{10} CFU per spleen is the mean average for five mice. The experiments were repeated twice, with similar results each time.

in the presence of cell wall-acting antibiotics of the β -lactam family, including ampicillin, penicillin G, and the cephalosporins, the $\Delta cesR$ and $\Delta cesK$ mutants were significantly more sensitive (Table 2). The most dramatic difference was observed for the cephalosporin cefuroxime. In this case, the size of the inhibition zone for the wild-type strain was 14.9 mm, whereas the inhibition zone sizes for the $\Delta cesR$ and $\Delta cesK$ strains were considerably larger (29.7 and 29.5 mm, respectively). No significant difference between the inhibition zone sizes of the wild-type strain and the $\Delta orf2420$ strain was observed (Table 2). These results show that the CesRK two-component system plays an important role in the resistance of *L. monocytogenes* to antibiotics of the β -lactam family.

Stationary-phase induction of *orf2420* expression depends on CesRK. Two-component systems are often found to be autoregulated at the transcriptional level and/or located in close proximity to their target genes. To determine whether any of the promoters at the *cesRK* locus are regulated by the CesRK two-component system, we fused the promoter regions of *lmo2424*, *lmo2423*, *cesR*, and *orf2420* to *lacZ*. Plasmids containing the promoter-*lacZ* fusions were introduced into wild-type, $\Delta cesR$, and $\Delta cesK$ strains; and the specific β -galactosidase activities were determined at various time points during growth. The expression of *lmo2424-lacZ*, *lmo2423-lacZ*, and

TABLE 2. β -Lactam resistance of *L. monocytogenes* LO28 wild-type, $\Delta cesR$, $\Delta cesK$, and $\Delta orf2420$ strains

β -Lactam	Avg zone of inhibition (mm) \pm SD ^a			
	Wild type	$\Delta cesR$	$\Delta cesK$	$\Delta orf2420$
Ampicillin	35.4 \pm 0.2	40.0 \pm 0.1 ^b	39.6 \pm 0.3 ^b	35.5 \pm 0.2
Penicillin G	33.4 \pm 0.2	37.7 \pm 0.6 ^b	37.7 \pm 0.3 ^b	33.8 \pm 0.5
Cephalothin (I) ^c	28.8 \pm 0.3	32.0 \pm 0.1 ^c	32.1 \pm 0.2 ^c	ND ^d
Cefaclor (I)	21.2 \pm 0.5	25.3 \pm 0.3 ^c	25.0 \pm 0.1 ^c	ND
Cefoperazone (III)	20.2 \pm 0.1	22.8 \pm 0.3 ^c	23.2 \pm 0.2 ^c	ND
Cephalexin (I)	15.9 \pm 0.1	20.9 \pm 0.1 ^b	20.9 \pm 0.1 ^b	ND
Ceftriaxone (III)	15.7 \pm 0.2	22.4 \pm 0.2 ^b	22.2 \pm 0.7 ^b	ND
Cefoxitin (II)	15.6 \pm 0.8	21.8 \pm 0.1 ^b	21.6 \pm 0.1 ^b	ND
Cefuroxime (II)	14.9 \pm 0.4	29.7 \pm 0.2 ^b	29.5 \pm 0.2 ^b	15.5 \pm 0.5
Cefotetan (III)	12.7 \pm 0.2	15.6 \pm 0.1 ^c	15.4 \pm 0.1 ^c	ND
Ceftazidime (III)	6.2 \pm 0.1	7.8 \pm 0.1 ^c	7.5 \pm 0.1 ^c	ND

^a The results presented are averages of triplicate experiments.
^b Significant difference ($P < 0.01$) between the mutant strain and the wild type.
^c Significant difference ($P < 0.05$) between the mutant strain and the wild type.
^d ND, not determined.
^e The classification of the cephalosporins is indicated in parentheses (I, narrow spectrum; II, expanded spectrum; III, broad spectrum).

cesR-lacZ was unaffected by the growth phase (data not shown). Moreover, the expression profile was identical in wild-type, $\Delta cesR$, and $\Delta cesK$ strains. However, expression of *orf2420* varied with the growth phase and reached a maximum in stationary phase (Fig. 5). Specifically, the β -galactosidase activity in stationary phase was approximately 10-fold higher than the activity in early exponential growth phase. Furthermore, the activity of the *orf2420-lacZ* fusion was very low in $\Delta cesR$ and $\Delta cesK$ strains. The activity of *orf2420-lacZ* in the $\Delta orf2420$ strain was comparable to the activity measured in wild-type cells, suggesting that Orf2420 does not affect its own expression (Fig. 5). These data show that the CesRK two-component system is responsible for the induction of *orf2420* expression.

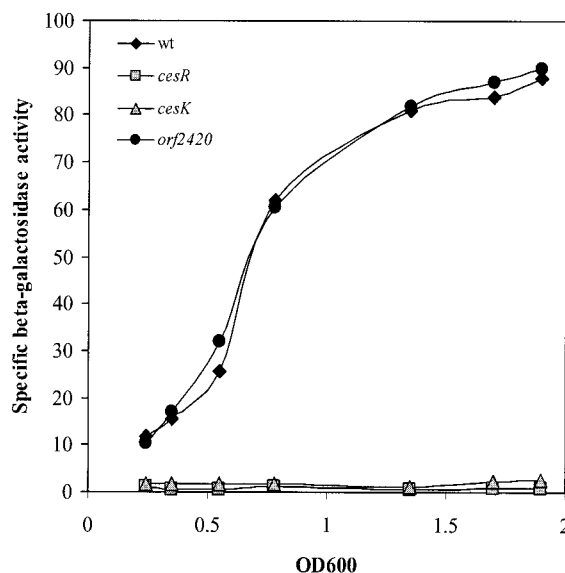


FIG. 5. Transcription of *orf2420* requires CesRK. The levels of expression of *orf2420-lacZ* in the wild-type (wt) strain and strains containing *cesR*, *cesK*, and *orf2420* deletions were determined. Cells were grown in BHI medium. At various cell densities, the cells were harvested and subjected to β -galactosidase assays.

Antimicrobial agents affecting the bacterial cell wall induce expression of *orf2420* in a CesRK-dependent manner. The observations that in-frame deletions in *cesR* or *cesK* result in an increased sensitivity to β -lactam antibiotics and an increased resistance to ethanol suggested that CesRK might be responding to changes in cell envelope integrity. To address this hypothesis, we analyzed the transcription of *orf2420*, which is controlled by CesRK, in the presence of cefuroxime and ethanol. Total RNA was extracted from wild-type and $\Delta cesR$ cultures after 20 min of treatment with subinhibitory concentrations of cefuroxime or ethanol. Next, the expression of *orf2420* was examined by primer extension analysis. In the wild-type strain, cefuroxime and ethanol were found to strongly induce transcription of *orf2420* (Fig. 6A). Importantly, no induction of *orf2420* was observed in the $\Delta cesR$ strain. This result strongly indicates that the CesRK two-component system responds to the presence of cefuroxime and ethanol in the growth medium.

To identify more potential inducers of the CesRK two-component system, we took advantage of the *orf2420-lacZ* fusion. For these assays, wild-type and $\Delta cesR$ strains carrying the *orf2420-lacZ* fusion were grown in BHI medium to an OD₆₀₀ of 0.2. The cultures were split, and the potential inducers were added at subinhibitory concentrations. Cells were collected 1 h after the addition of the potential inducers and assayed for β -galactosidase activity. In the absence of inducers, the specific activity of *orf2420-lacZ* in the wild-type strain was largely the same at an OD₆₀₀ of 0.2 and 1 h later (OD₆₀₀ = 0.4; Fig. 6B). The addition of 1% ethanol resulted in a dramatic induction of *orf2420-lacZ* expression (76-fold) in the wild-type strain (Fig. 6B). No induction of *orf2420* was observed in the $\Delta cesR$ mutant strain (Fig. 6B). These results are consistent with the results of the primer extension analysis with *orf2420* (Fig. 6A). Next, we tested the inducing capabilities of seven antibiotics targeting cell wall synthesis. Cephalixin, cefuroxime, penicillin G, ampicillin, vancomycin, bacitracin, and D-cycloserine strongly induced the expression of *orf2420-lacZ*, from 10-fold (bacitracin) to 95-fold (vancomycin), in a CesR-dependent fashion (Fig. 6B). Similar results were obtained for the $\Delta cesK$ strain (data not shown). Subinhibitory concentrations of the membrane-active compounds Triton X-100, Tween 20, and sodium dodecyl sulfate did not affect the expression of the *orf2420-lacZ* fusion, suggesting that they do not function as inducers (data not shown).

Given that ethanol and a variety of cell wall-acting antibiotics induce the *orf2420* promoter, we wished to determine whether the cell wall hydrolytic enzyme lysozyme was capable of acting as an inducer. Indeed, subinhibitory concentrations of lysozyme induced *orf2420-lacZ* expression in a dose-dependent manner (Fig. 6C). The induction was dependent on CesR and CesK (data not shown). Collectively, these data suggest that cell wall-acting antibiotics, ethanol, and lysozyme all generate a potent inducing signal sensed by the CesRK two-component system.

DISCUSSION

We have identified a two-component system in *L. monocytogenes*, CesRK, which mediates sensitivity to ethanol and resistance to antibiotics of the β -lactam family and which contributes to the virulence of the organism in mice. Moreover, it

is demonstrated that CesRK enables *L. monocytogenes* to sense the presence of a variety of cell wall-acting antimicrobial agents, including ethanol, cell wall-acting antibiotics, and the cell wall lytic enzyme, lysozyme.

We observed that the virulence potential of *L. monocytogenes* lacking the CesRK two-component system is reduced in comparison to that of the wild-type strain. Consequently, the target genes controlled by the CesRK two-component system may represent a collection of genes that are important for survival and persistence during an infection. In the present study, we have identified a gene regulated by CesRK. This target gene, *orf2420*, contributes to virulence in mice and decreases ethanol resistance. Interestingly, an open reading frame corresponding to *orf2420* has not been defined in the avirulent species *L. innocua*, suggesting a specific role for Orf2420 in the virulence of *L. monocytogenes* (9). Although the sensitivity of the $\Delta orf2420$ mutant to cell wall-acting antibiotics was not significantly affected, the transcription of *orf2420* was highly induced in the presence of these agents in a CesRK-dependent manner. The increased expression of *orf2420* in the presence of cell wall-acting antibiotics, ethanol, and lysozyme suggests that Orf2420 may play a role in the response to cell wall damage. Studies are ongoing in our laboratory to identify the role(s) of Orf2420 in the response to alterations of cell wall integrity.

The identification of *orf2420* as a target gene for CesRK provided a tool for the identification of the signal sensed by CesRK. The level of transcription of *orf2420* increases about 10-fold in a CesRK-dependent manner in *L. monocytogenes* cells going from the early exponential phase into the stationary growth phase, suggesting that a signal sensed by CesRK is generated though this growth phase transition. In *E. coli*, the peptidoglycan layer is known to undergo considerable changes upon entry into stationary phase (27). Furthermore, the antimicrobial agents that induce the transcription of *orf2420* in a CesRK-dependent manner affect different stages of peptidoglycan synthesis (11, 19, 32). Thus, peptidoglycan appears to play an important role in the generation of the signal sensed by CesRK. Considering the diversity and broad specificity of the inducing factors, the actual signal sensed by the two-component system most likely accumulates as a consequence of some physical changes in the cell wall induced by these factors. The signal may be an intermediate in the biosynthesis or degradation of peptidoglycan; however, its true nature remains to be determined.

The opposite responses of the *cesRK* deletion mutants to ethanol and β -lactam antibiotics is a paradoxical finding. In *E. coli*, growth in the presence of ethanol is known to result in the production of un-cross-linked peptidoglycan (19). The β -lactam antibiotics prevent cross-linking of the peptidoglycan by binding to and inhibiting the activity of the penicillin-binding protein (PBP) transpeptidases (15). Both ethanol and β -lactams induce the expression of *orf2420* in a CesRK-dependent manner. However, the *orf2420* gene mediates only ethanol sensitivity and not β -lactam resistance, suggesting that other genes controlled by CesRK are involved in β -lactam resistance, but not necessarily in ethanol sensitivity. Thus, different genes under the control of CesRK may promote ethanol sensitivity and β -lactam resistance.

The cephalosporins are used in the medical treatment of bacterial meningitis; however, they have no effect in cases in

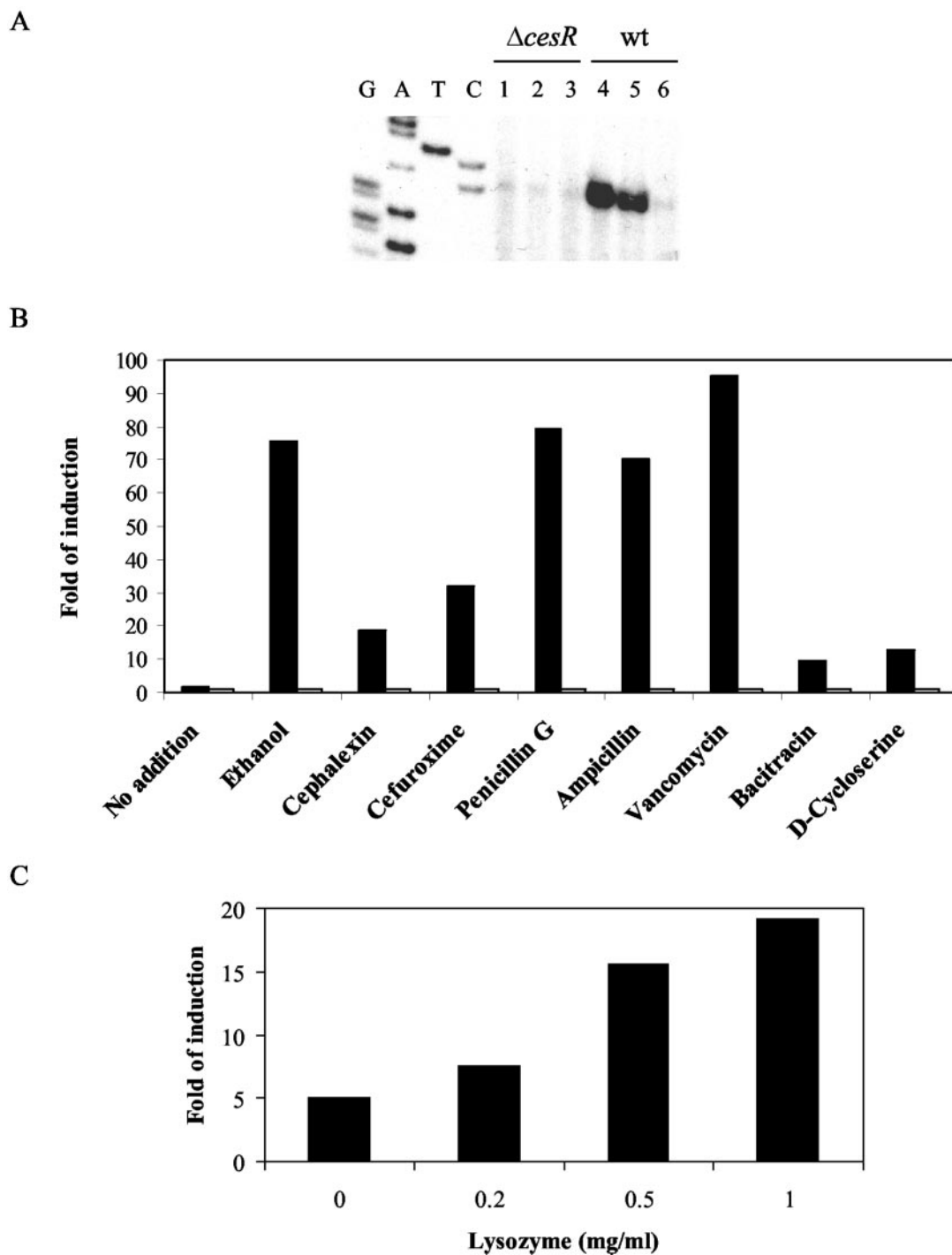


FIG. 6. Transcription of *orf2420* is induced by ethanol, cell wall-acting antibiotics, and lysozyme. (A) Expression of *orf2420* in response to ethanol and cefuroxime analyzed by primer extension. The analysis was performed with RNA purified from the $\Delta cesR$ mutant strain (lanes 1 to 3) or the wild-type (wt) strain (lanes 4 to 6). Cells were grown in BHI medium to an OD_{600} of 0.3. The cell cultures were split and treated with 1% ethanol (lanes 1 and 4) or 4 μg of cefuroxime per ml (lanes 2 and 5) for 20 min. Controls without treatment were included (lanes 3 and 6). (B) Expression of *orf2420-lacZ* in response to various antimicrobial agents. The wild-type strain (black bars) and the $\Delta cesR$ mutant strain (open bars) containing the *orf2420-lacZ* fusion were grown in BHI medium to an OD_{600} of 0.2. The cell cultures were split and treated with the indicated antibiotics for 1 h. Cell pellets were harvested and subjected to β -galactosidase assays. The final concentrations of the antimicrobial agents in the medium were as follows: No addition, no antimicrobial agent; ethanol, 1%; cephalixin, 4 $\mu\text{g}/\text{ml}$; cefuroxime, 4 $\mu\text{g}/\text{ml}$; penicillin G, 0.05 $\mu\text{g}/\text{ml}$; ampicillin, 0.05 $\mu\text{g}/\text{ml}$; vancomycin, 0.1 $\mu\text{g}/\text{ml}$; bacitracin, 4 $\mu\text{g}/\text{ml}$; and D-cycloserine, 20 $\mu\text{g}/\text{ml}$. The data represent the means of three experiments, in which the observed variation did not exceed 10%. (C) Expression of *orf2420-lacZ* in response to lysozyme. The wild-type strain containing the *orf2420-lacZ* fusion was grown in BHI medium to an OD_{600} of 0.2. The cell culture was split and treated with the indicated concentration of lysozyme for 2 h. Cell pellets were harvested and subjected to β -galactosidase assays. The data represent the means of three experiments, in which the observed variation did not exceed 10%.

which *L. monocytogenes* is the cause of disease. The reason for this natural resistance to cephalosporins is that they have only a low affinity for the primary lethal target for active β -lactams in *L. monocytogenes*, PBP 3 (16, 38). The cephalosporins were, however, found to be good inhibitors of PBPs 1, 2, and 4 (38). The LisRK two-component system has recently been shown to mediate the resistance of *L. monocytogenes* to cephalosporins. In addition, LisRK contributes to the response to the lantibiotic nisin, ethanol, acid, and hydrogen peroxide stress; and it plays a significant role in the virulence potential of *L. monocytogenes* (6, 7). The similar roles played by LisRK and CesRK in mediating ethanol sensitivity and cephalosporin resistance may reflect a regulatory connection between the two signal transduction systems.

In the present study, we have identified a two-component system involved in the ability of *L. monocytogenes* to sense and respond to a variety of antimicrobial agents present in the environment, foods, and the infected host. Importantly, some of these agents are used therapeutically in the treatment of infections caused by *L. monocytogenes* and other human pathogens. It is becoming increasingly clear that a number of two-component systems of important bacterial pathogens, such as VanRS of *Enterococcus faecalis* (3), CiaHR and VncRS of *S. pneumoniae* (13, 24), and LisRK and CesRK of *L. monocytogenes* (7; this study), may indirectly contribute to the pathogenicity of these organisms by mediating resistance or sensitivity to antibiotics. Furthermore, CiaHR, LisRK, and CesRK play direct roles in virulence in mouse models, suggesting important roles for these systems in directly contributing to the infection process (6, 20, 34; this study). Further studies on such two-component systems will improve our understanding of how human pathogens sense and respond to commonly used antimicrobial agents and will provide a better understanding of the molecular mechanisms by which antimicrobial resistance may arise.

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