APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2010, p. 8231–8238 Vol. 76, No. 24
0099-2240/10/$12.00 doi:10.1128/AEM.02056-10

Genetic Characterization of Plasmid-Associated Benzalkonium Chloride Resistance Determinants in a *Listeria monocytogenes* Strain from the 1998-1999 Outbreak▽

Driss Elhanafi,[1,3]† Vikrant Dutta,[2]† and Sophia Kathariou[3]*

Biomanufacturing Training and Education Center[1] and Departments of Microbiology[2] and Food, Bioprocessing and Nutrition Sciences,[3] North Carolina State University, Raleigh, North Carolina 27695-7624

Received 31 August 2010/Accepted 18 October 2010

Quaternary ammonium compounds such as benzalkonium chloride (BC) are widely used as disinfectants in both food processing and medical environments. BC-resistant strains of *Listeria monocytogenes* have been implicated in multistate outbreaks of listeriosis and have been frequently isolated from food processing plants. However, the genetic basis for BC resistance in *L. monocytogenes* remains poorly understood. In this study, we have characterized a plasmid (pLM80)-associated BC resistance cassette in *L. monocytogenes* H7550, a strain implicated in the 1998-1999 multistate outbreak involving contaminated hot dogs. The BC resistance cassette (*bcrABC*) restored resistance to BC (MIC, 40 μg/ml) in a plasmid-cured derivative of H7550. All three genes of the cassette were essential for imparting BC resistance. The transcription of H7550 BC resistance genes was increased under sublethal (10 μg/ml) BC exposure and was higher at reduced temperatures (4, 8, or 25°C) than at 37°C. The level of transcription was higher at 10 μg/ml than at 20 or 40 μg/ml. *In silico* analysis suggested that the BC resistance cassette was harbored by an IS*1216* composite transposon along with other genes whose functions are yet to be determined. The findings from this study will further our understanding of the adaptations of this organism to disinfectants such as BC and may contribute to the elucidation of possible BC resistance dissemination in *L. monocytogenes*.

Listeria monocytogenes is a food-borne pathogen associated with severe illness (listeriosis) in at-risk individuals, including those in extremes of age, pregnant women and their fetuses, and those with compromised immunity. Environmental contamination with this pathogen plays a key role in the eventual contamination of ready-to-eat foods and subsequent food-borne illness (16, 19, 30). Biofilm formation and persistence, resistance to disinfectants, resistance to *Listeria*-specific viruses, and the ability to replicate at low temperatures are among the attributes contributing to the organism's prevalence and persistence in food processing environments (7, 18, 19).

Resistance to quaternary ammonium disinfectants such as benzalkonium chloride (BC) is especially relevant to *Listeria*'s adaptations in food-related environments, as these compounds are used extensively in food processing, in retail, and for household or personal use (24, 26). BC resistance of *L. monocytogenes* isolated from foods and from the processing plant environment has been found to range from 10% (1) to as much as 42 to 46% (25, 27, 37). A study of strains from turkey processing plants revealed that resistance to BC was especially high among those of serotype 1/2a (or 3a) and 1/2b (or 3b) (60% and 51%, respectively) and that all BC-resistant strains were also resistant to the heavy metal cadmium (27).

Mechanisms underlying BC resistance in *L. monocytogenes* remain poorly understood. Several studies have provided evidence for chromosomal determinants (6, 34, 35, 37, 38), and evidence for plasmid-mediated resistance to BC also exists (21, 34, 35), even though plasmid-associated genes mediating BC resistance were not identified. Genome sequencing of several *L. monocytogenes* strains has identified numerous efflux systems on the chromosome as well as on plasmids (8, 9, 28; http://www.broadinstitute.org/annotation/genome/listeria_group/MultiHome.html), and some of these are potential candidates for BC resistance.

In this study, we have identified a plasmid-associated BC resistance system on an IS*1216* composite transposon in *L. monocytogenes* H7550, implicated in the 1998-1999 multistate outbreak of listeriosis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. *L. monocytogenes* strain H7550 was isolated from the 1998-1999 hot dog outbreak (4). Its pulsed-field gel electrophoresis profile with AscI and ApaI was indistinguishable from that of the sequenced strain H7858 (28; R. M. Siletzky and S. Kathariou, unpublished data). Both H7550 and H7858 are resistant to BC and to cadmium (27; data not shown). H7550-Cd^s is a cadmium-susceptible, plasmid-free derivative of *L. monocytogenes* H7550. The plasmid-cured derivative was obtained following repeated passages of the bacteria at 42°C. H7550-Cd^{s}S is a spontaneous mutant of H7550-Cd^s with resistance to streptomycin and was isolated on plates containing streptomycin (150 μg/ml). *L. monocytogenes* was grown either in Trypticase soy broth with 0.6% yeast extract (TSBYE) (Becton, Dickinson and Company, Sparks, MD) or in brain heart infusion (BHI) broth (Becton, Dickinson and Company). Agar media were BHI agar (1.2% Bacto agar; Becton, Dickinson and Company) or TSBYE with 1.2% agar (1.2% Bacto agar). *Escherichia coli* strains were grown at 30°C in Luria Bertani (LB) broth (Becton, Dickinson and Company) or on LB broth supplemented with 1.2% Bacto agar.

BC susceptibility and determinations of MIC. BC susceptibility of *L. monocytogenes* was assessed as described previously (27). Strains H7550 and H7550-Cd^s were used as resistant and susceptible controls, respectively. For MIC de-

* Corresponding author. Mailing address: Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624. Phone: (919) 513-2075. Fax: (919) 513-0014. E-mail: sophia_kathariou@ncsu.edu.

† D. Elhanafi and V. Dutta equally contributed to this work.

▽ Published ahead of print on 22 October 2010.

TABLE 1. Bacterial strains used in this study[a]

Strain	BC[r]	Source or reference
Listeria monocytogenes (serotype 4b)		
H7550	+	1998-1999 hot dog outbreak (4)
H7550-Cd[s]	−	Plasmid-cured derivative of H7550
H7550-Cd[s]S	−	Streptomycin-resistant mutant of H7550-Cd[s]
H7550-Cd[s]S(pDS195)	+	This study
H7550-Cd[s]S(pDS202)	+	This study
H7550-Cd[s]S(pDS201)	−	This study
H7550-Cd[s]S(pBEC59)	−	This study
H7550-Cd[s]S(pBEC57)	−	This study
H7550-Cd[s]S(pCON-1)	−	This study
Escherichia coli		
SM10 *thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km		36

[a] BC[r], resistance to BC determined by growth on MHA plates with an added BC concentration of 20 μg/ml as described in Materials and Methods. +, growth; −, no growth.

TABLE 2. Primers used in this study

Primer	Sequence (5′ to 3′)[a]
BcF	GAATGGATCCTTCAATTAGATCGAGGCACG
BcR	GTATGAATTCGTATAATCCGGATGCTGCCC
BcR2	GTATGAATTCCATCAACCTTCCTTTAATGAGG
BcF2	GACTGGATCCGATTCTGGAACATCCCTATC
BcF5	GAATGGATCCGGAGGGTAATCATGTCAG
BcF7	GACTGGTACCCTCATTAAAGGAAGGTTGATGG
BcR3	GACTGGTACCCATATGGATTCATGTG
p1	CATTAGAAGCAGTCGCAAAGCA
p2	GTTTTCGTGTCAGCAGATCTTTGA
p3	GTTATCAGGATCTACGACTGTC
p4	GTAATTCCTGCATTACGCATAACTG
p5	GTCATAGATCGCTGCCGTAATTTCAG
p6	CAATATGGCTATTGTATTCCCATGCTG
p7	CAGAGTGAACGTGATCAGCATC
s1	ATTACAGATGTGAGATTACGACG
s2	ACGTTTACTTGGCATAGCTAC
c1	ACAAGTTAGATCAAAAGAGTCTTTTATTAAACG
c2	ATCTTCTTCATTTAGTGTTCCTGCAAATACTTC

[a] Underlined sequences correspond to restriction enzyme sites as follows: GGATCC, BamHI; GAATTC, EcoRI; GGTACC, KpnI.

terminations, a single colony from a blood agar plate (Remel, Lenexa, KS) was suspended in 100 μl of Mueller Hinton broth (Becton, Dickinson and Company), 5 μl of the suspension was spotted in duplicate on Mueller Hinton agar (1.2% agar) plates with 1.2% defibrinated sheep blood (Becton, Dickinson and Company) and variable concentrations of BC (0, 5, 10, 15, 20, 25, 30, 35, and 40 μg/ml), and the plates were incubated at 30°C for 48 h. MIC was defined as the lowest assessed concentration of BC that prevented growth. All MICs were determined in at least two independent trials.

Recombinant plasmid constructs. Primers used to construct the recombinant plasmids are listed in Table 2, and their locations are indicated in Fig. 1. PCR analysis was performed with the Takara Ex *Taq* kit (Takara, Madison, WI) and a T1 thermal cycler (Biometra, Göttingen, Germany). Primers BcF and BcR were used to produce a PCR fragment containing the entire *bcrABC* cassette along with the ca. 800-nucleotide (nt) upstream intergenic region. Primers BcF2 and BcR were employed to amplify the *bcrABC* cassette with 105 nt upstream of the putative start codon of *bcrA*, including the putative promoter region. The *bcrABC* cassette without the putative promoter was amplified with primers BcF5 and BcR. A PCR fragment containing the ca. 800-nt upstream intergenic region, *bcrA* and *bcrB*, but lacking *bcrC* was amplified with BcF and BcR2 (Table 2 and Fig. 1). The PCR fragments obtained with BcF and BcR, BcF2 and BcR, BcF5 and BcR, and BcF and BcR2 were digested with BamHI and EcoRI (New England Biolabs, Beverly, MA), ligated into the temperature-sensitive shuttle vector pCON-1 (3), and similarly digested with BamHI and EcoRI, resulting in pDS195, pDS202, pBEC59, and pDS201, respectively. The recombinant plasmids were electroporated into *E. coli* SM10 (36), and transformants were selected on LB agar supplemented with ampicillin (100 μg/ml). For the plasmid construct pBEC57 harboring an in-frame deletion of *bcrB*, pDS202 was used as template for inverse PCR analysis with primers BcF7 and BcR3 (both harboring a KpnI restriction site) (Fig. 1). The PCR amplicon was digested with KpnI, self-ligated with T4 DNA ligase (Promega, Madison, WI), and electroporated into *E. coli* SM10.

Recombinant plasmids were mobilized into H7550-Cd[s]S via conjugation with *E. coli* SM10 harboring the recombinant plasmids, as described previously (20). Transconjugants were selected on BHI agar plates supplemented with chloramphenicol (6 μg/ml) and streptomycin (1,200 μg/ml) at 30°C for 2 to 3 days and confirmed using PCR. BC susceptibility of the transconjugants was assessed as described above.

RNA isolation and RT-PCR. *L. monocytogenes* H7550 was grown in TSBYE at 4, 8, 25, and 37°C until the mid- to late logarithmic phase (optical density at 600 nm [OD_{600}], ~0.7 to 0.9), with the growth phase being monitored with a spectrophotometer (SmartSpec 3000; Bio-Rad, Hercules, CA). Cultures were then divided into two portions, one of which was treated at the indicated temperature for 30 min with a sublethal concentration (10 μg/ml) of BC or other concentrations as indicated. The other portion (control) remained untreated at the indicated temperature for 30 min. Total RNA was isolated using the SV total RNA isolation system (Promega). RNA was then subjected to DNase treatment using Turbo DNA-*free* (Ambion, Austin, TX). The concentration and quality of the RNA were determined by measuring the absorbance at 260 nm (NanoDrop, Wilmington, DE). RNA was stored at −80°C when necessary. Reverse transcription-PCR (RT-PCR) analysis for *cadA* involved a similar approach with cells treated for 30 min with sublethal concentrations (10 μg/ml) of cadmium chloride. Reverse transcription was carried out with primer c2, and PCR was done with primers c1 and c2 (Table 2).

RT-PCR experiments included *spoVG* as a housekeeping gene control, as previous studies indicated that expression of this gene was constitutive in *L. monocytogenes*, including at low temperatures (22). Total RNA was reverse transcribed to produce cDNA using 200 ng of RNA and the ImProm-II reverse transcription system (Promega) according to the manufacturer's protocol, using extension temperatures of 46°C and 43°C for *bcrABC* and *spoVG* transcripts, respectively. Self-priming controls (reverse transcription without a gene-specific primer) and negative controls (reverse transcription with no RNA) were included with all RT-PCR analyses. Sense and antisense primers for *bcrABC* RT-PCR were p1 and p2, respectively (Table 2), whereas those for *spoVG* RT-PCR were s1 and s2, respectively (Table 2). Each RT-PCR analysis was done in duplicate and in at least two independent experiments. For band quantifications we used the image processing software ImageJ (http://rsbweb.nih.gov/ij/). In order to calculate the fold increase in the transcript levels of the genes in response to presence of BC or cadmium, the gel density values obtained from the RT-PCR gel images using ImageJ were normalized to those of the control (culture without BC or cadmium). The transcript levels of the reference gene *spoVG* in the presence and absence of BC or cadmium were similarly normalized. The ratio of the normalized *bcrABC* levels to that of the normalized *spoVG* levels corresponded to the fold increase. To determine the impact of temperature, *bcrABC* levels were first normalized to those of *spoVG* from the same culture, and the fold increase upon addition of BC was determined as the ratio of the normalized levels in the absence or presence of BC.

RESULTS

Identification of BC resistance determinants on *L. monocytogenes* plasmid pLM80. *L. monocytogenes* strain H7550 from the 1998-1999 hot dog outbreak was resistant to both cadmium and BC and harbored a large, ca. 80-kb plasmid, pLM80 (28). Plasmid curing of H7550 rendered the bacteria susceptible to the heavy metal cadmium. This was expected since pLM80 harbored genes for cadmium resistance (28). Testing of H7550-Cd[s] for BC resistance revealed that the derivative was also susceptible to BC (Table 1). These findings suggested that,

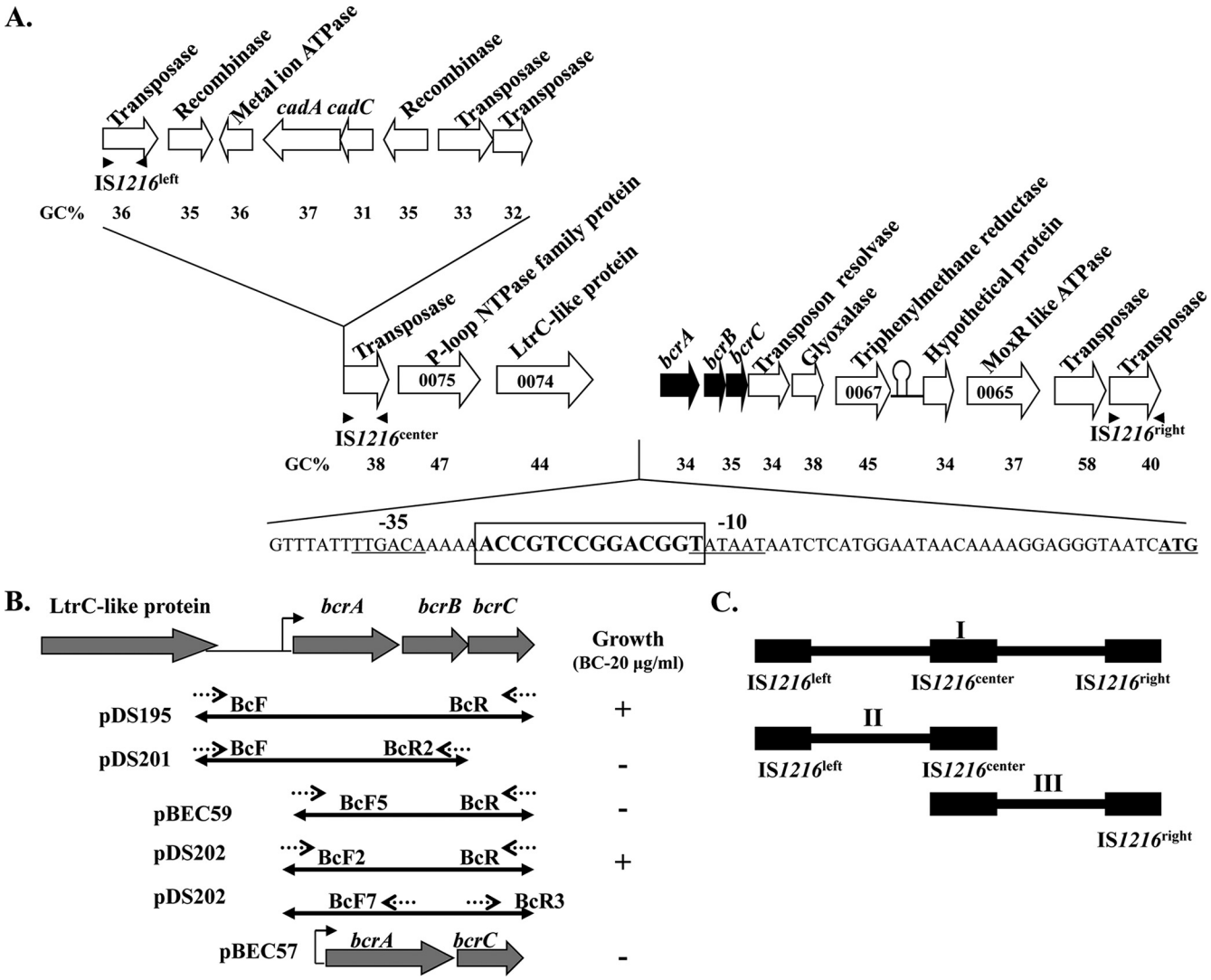

FIG. 1. BC resistance cassette *bcrABC* in *L. monocytogenes* H7858. (A) Genetic organization and putative annotation of the *bcrABC* resistance cassette region in plasmid pLM80. Arrows indicate the direction of transcription. Genes implicated in BC resistance (*bcrABC*) are in black. The 67-nt sequence upstream of the start codon of the *bcrA* gene harboring a canonical promoter is shown along with the −10 and −35 regions (underlined). A palindromic sequence between the −10 and −35 regions, and partially overlapping with the −10 region, is shown in bold and boxed. Three copies of IS*1216* are indicated as IS1216^{left}, IS1216^{center}, and IS1216^{right} with solid arrows indicating the orientation of IS*1216* inverted repeats. (B) Recombinant plasmids harboring different *bcrABC* segments. Dotted arrows indicate the position and orientation of the primers used for recombinant plasmid construction. The plasmids were introduced in the pLM80-cured derivative H7550-Cd^{S}S, and the resulting strains harboring these plasmids were tested for growth on BC (20 μg/ml), as described in Materials and Methods. Growth or lack of growth are indicated by + and −, respectively. (C) Schematic diagram of the possible transposable units harboring both cadmium and BC resistance genes (I), cadmium resistance genes (II), and BC resistance genes (III).

in addition to cadmium resistance determinants, pLM80 also harbored determinants for resistance to BC. However, unlike BC-resistant derivatives of originally BC-susceptible strains obtained following prolonged exposure to BC, which were resistant both to BC and to ethidium bromide (34, 35), the ethidium bromide MICs for H7550 and H7550-Cd^{s} were the same (both strains grew at 32 but not at 64 μg/ml).

Analysis of the annotation of pLM80 (RefSeq accession no. NZ_AADR00000000), identified three open reading frames (ORFs) as possible determinants for BC resistance (Fig. 1). These included a putative transcriptional regulator of the TetR family harboring a helix-turn-helix (HTH) DNA binding motif (*bcrA*; Pfam, PF00440). This ORF was followed by two putative small multidrug resistance (SMR) genes, *bcrB* and *bcrC* (Pfam: PF00893). Sequence analysis of the region suggested a long (850 bp; GC, 36%) intergenic region between *bcrA* and its upstream ORF (LtrC-like protein). Upstream (31 nt) of the putative start codon of *bcrA* we identified a canonical promoter (−10, TATAAT; −35, TTGACA) (Fig. 1). A perfect palindrome (ACCGTCCGGACGGT) was identified between the −10 and −35 promoter sequences (Fig. 1), suggesting a possible binding site for a transcriptional regulator. Highly similar (99 to 100% identity) *bcrABC* sequences (including the upstream region harboring the putative promoter) were detected

in two *L. monocytogenes* strains among those with sequenced genomes: J0161, of serotype 1/2a (NZ_AARW02000017.1) and FSL F2-515, also of serotype 1/2a (NZ_AARI02001718). The genes were located on a large plasmid in J0161 (19a); even though strain FSL F2-515 harbored a plasmid, the genes were absent from that plasmid (19a), suggesting a chromosomal location in that strain.

With the exception of J0161 and FSL F2-515, no other sequences with significant homology to *bcrABC* were detected in the nucleotide database. The deduced BcrB and BcrC polypeptides, however, exhibited similarity (44 to 73%) to multiple proteins of the SMR family in diverse Gram-positive and Gram-negative bacteria. Furthermore, a chromosomal cassette (SugE1-E2) with 33 to 34% identity at the amino acid sequence level was identified in the genomes of all screened *L. monocytogenes* strains, including H7858 (data not shown).

Sequence analysis of the *bcrABC* region provided evidence for a composite transposon flanked by two IS*1216* elements (IS*1216* center and IS*1216* right, each containing a transposase [75.3% nt identity; 83.2% amino acid identity to each other] flanked by IS*1216* inverted repeats [5′ GGTTCTGTTGCAA AGTTT 3′]) (Fig. 1) (12). The putative composite transposon was ca. 12.4 kb long. In addition to the two IS*1216* elements and the *bcrABC* cassette, the transposon harbored genes with putative conjugative functions: plm80_0074 and plm80_0075 were annotated as an LtrC-like protein and a member of the P-loop NTPase domain superfamily, respectively. Downstream of the *bcrABC* cassette we identified one ORF with 87 to 99% identity to a putative resolvase gene in plasmids of *Bacillus cereus* and in Tn*1546* of *Enterococcus faecium*. The putative resolvase gene was followed by four ORFs encoding a putative glyoxalase superfamily protein, a putative triphenylmethane reductase, a hypothetical protein, and a MoxR-like protein, respectively. A stem-loop structure ($\Delta G = -18.7$ kcal/mol) was identified 110 nt downstream of the plm80_0067 stop codon, possibly corresponding to a Rho-independent transcriptional terminator (Fig. 1).

The overall GC content of the ca. 12.4-kb region is 40%. However, pronounced diversity in GC content was observed within this region: the GC content of the *bcrABC* cassette was 34%, significantly lower than the average for the genome of *L. monocytogenes* (38%); other ORFs had widely variable GC contents, ranging from 34 to 58% (Fig. 1). It was of interest that plm80_0067, downstream of the putative glyoxalase and encoding a putative triphenylmethane reductase (TMR), had an unusually high GC content (45%) and 99 to 100% identity to putative TMR genes from *Pseudomonas* sp., *Aeromonas* sp., and *Citrobacter* sp.

The IS*1216* composite transposon harboring *bcrABC* was immediately downstream of another putative transposon harboring the cadmium resistance cassette *cadAC* and flanked by IS*1216* left and IS*1216* center (Fig. 1A). The genomic region in pLM80 suggests the presence of three possible transposable units, with one carrying both cadmium and BC resistance genes (Fig. 1C, I) and others carrying either cadmium (Fig. 1C, II) or BC (Fig. 1C, III) resistance genes.

***bcrABC* cassette confers BC resistance to a plasmid-cured derivative of *L. monocytogenes* H7550.** Subcloning of the entire *bcrABC* cassette along with 900 nt upstream of the *bcrA* start codon in pCON-1 (including the 850-bp intergenic region between *bcrA* and pLM80_0074) resulted in pDS195, whereas pDS202 harbored *bcrABC* and 105 nucleotides upstream of *bcrA*, including the canonical promoter (Fig. 1B). Transfer of pDS195 and pDS202 into H7550-Cd^{s}S rendered the bacteria able to grow at 35 μg/ml, whereas MICs of BC were markedly lower for H7550-Cd^{s}S harboring the empty vector (Table 3). Constructs pDS201 (lacking *bcrC*), pBEC57 (lacking *bcrB*), and pBEC59 (*bcrABC* with only 11 nt upstream of *bcrA* and thus lacking the canonical promoter) (Fig. 1B) were not able to restore BC resistance (Table 3). In spite of repeated efforts, a construct harboring *bcrB* and *bcrC*, but lacking *bcrA*, could not be obtained. These data suggested that BC resistance could be conferred by the *bcrABC* cassette and that the 105-nt upstream region that included the canonical −10 and −35 promoter sequences was both required and sufficient for expression.

TABLE 3. Benzalkonium chloride (BC) MICs for *L. monocytogenes* strain H7550 and its derivatives used in this study

Strain	MIC[a] (μg/ml)
H7550	40
H7550-Cd^s	10
H7550-Cd^{s}S	10
H7550-Cd^{s}S(pCON-1)	10
H7550-Cd^{s}S(pDS195)	40
H7550-Cd^{s}S(pDS201)	10
H7550-Cd^{s}S(pDS202)	40
H7550-Cd^{s}S(pBEC59)	10
H7550-Cd^{s}S(pBEC57)	10

[a] MIC was defined as the lowest assessed concentration of BC that prevented growth. MICs were determined as described in Materials and Methods at 30°C. Strains with MICs of 10 μg/ml had impaired growth at 2.5 and 5 μg/ml and did not grow at all at 10 μg/ml.

Cotranscription of *bcrABC* with downstream genes and increase in *bcrABC* transcript levels in response to BC. RT-PCR data suggested that *bcrA*, *bcrB*, and *bcrC* were cotranscribed and were also transcribed together with the downstream ORFs encoding a putative transposon resolvase, a putative glyoxalase, and a putative triphenylmethane reductase (Fig. 2). The ORF upstream of *bcrA*, plm80_0074, was not in this transcriptional unit and neither was plm80_0066, downstream of the putative triphenylmethane reductase (Fig. 2). These results were confirmed in five independent trials and are in congruence to the GC-rich stem-loop structure found downstream of plm80_0067 (Fig. 1). Thus, *bcrABC* was part of a polycistronic message of ca. 3.8 kb, with the putative triphenylmethane reductase gene being the last gene in the operon.

RT-PCR data also suggested that levels of the transcript that included *bcrABC* were higher in the presence of BC (10 μg/ml) than without the disinfectant (Fig. 3). To further assess the impact of BC on expression of *bcrABC*, transcript levels were assessed via RT-PCR at different concentrations of BC (0, 10, 20, and 40 μg/ml) using the housekeeping gene *spoVG* as a reference. RT-PCR data suggested that transcript levels of *bcrABC* were increased in the presence of 10 μg/ml BC (ca. 1.4- to 2-fold) but were similar to baseline levels at higher BC concentrations (20 and 40 μg/ml) (Fig. 3). RT-PCR with *spoVG* suggested that there were no significant changes in levels of *spoVG* transcripts when cells were grown in the presence of different concentrations of BC (0, 10, 20, and 40 μg/ml) (Fig. 3).

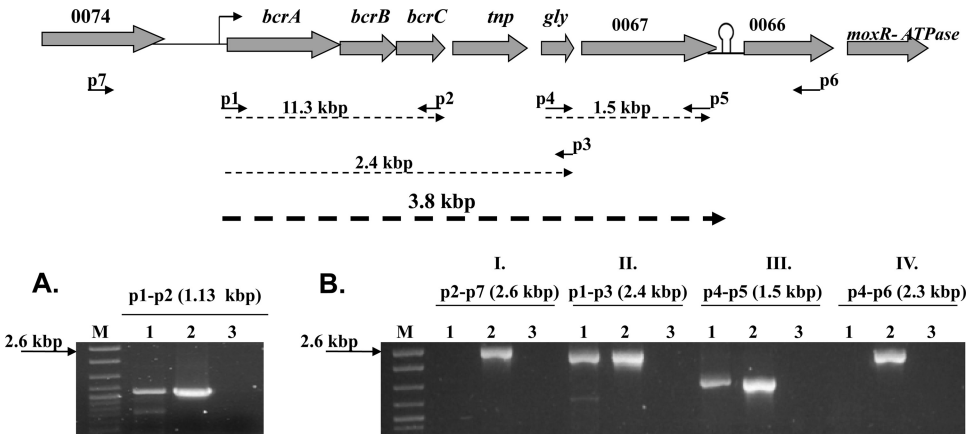

FIG. 2. Cotranscription of BC resistance cassette and downstream genes assessed by reverse transcription-PCR in *L. monocytogenes* strain H7550 grown at 25°C. (A) Total RNA was reverse transcribed into cDNA using primer p2, and PCR analysis was performed using a cDNA template and primers p1 and p2. (B) Reverse transcription with primer p2 and PCR with cDNA template and primers p7 and p2 (I); reverse transcription with primer p3 and PCR with cDNA template and primers p1 and p3 (II); reverse transcription with primer p5 and PCR with cDNA template and primers p4 and p5 (III); and reverse transcription with primer p6 and PCR with cDNA template and primers p4 and p6 (IV). Lanes: 1, RT-PCR of H7550 exposed to BC (10 μg/ml); 2 and 3, H7550 genomic DNA and total RNA, respectively, used as positive and negative controls for RT-PCR; and M, 100- to 2,686-bp DNA molecular marker XIV (Roche, Indianapolis, IN).

Transcription of *bcrABC* is higher at lower temperatures (4, 8, and 25°C) than at 37°C. Temperature was found to impact *bcrABC* transcript levels, with levels at 4 to 25°C being greater than those at 37°C. Following normalization (ratio of *bcrABC* band density to the *spoVG* band from the same sample), levels in the absence of BC (baseline levels) at 37°C were ca. 57% those at 4°C (Fig. 4). The impact of temperature was even more noticeable in the presence of BC, with normalized levels at 8, 25, and 37°C being ca. 83, 92, and 42% those at 4°C, respectively (Fig. 4). Comparison of normalized values in the absence or presence of BC revealed that the presence of the disinfectant resulted in higher transcript levels at all tested temperatures. However, the impact of BC was greater at 4 and 25°C (ca. 1.8- and 2.0-fold increase, respectively) than at 8 and 37°C (ca. 1.2-fold increase each) (Fig. 4). The observed impact of temperature on increased and baseline levels of *bcrABC* transcription was consistently observed in independent experiments (data not shown).

Lack of cross-induction of pLM80 genes mediating resistance to BC and to cadmium. As described above, pLM80 harbors genes mediating resistance to BC (*bcrABC*) as well as to cadmium (*cadAC*), and the *cadAC* cassette (ORFs plm80_0082 and plm80_0083) is in the vicinity of *bcrABC* (Fig. 1A). To determine whether *bcrABC* expression may also be

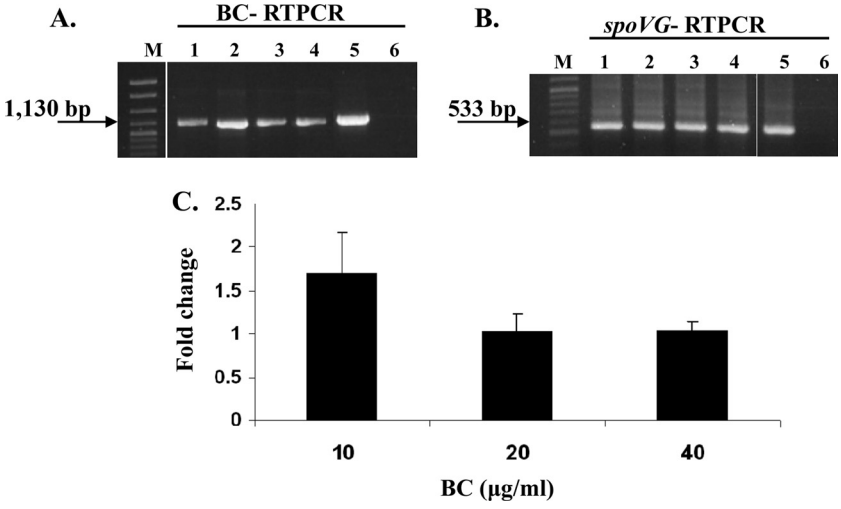

FIG. 3. Impact of BC concentration on transcription of *bcrABC*. (A) RT-PCR of *bcrABC* using primers p2 for cDNA and p1 and p2 for PCR. Lanes: 1 to 4, H7550 exposed to 0, 10, 20, and 40 μg/ml of BC, respectively, at 25°C for 30 min; 5 and 6, H7550 genomic DNA and total RNA, respectively, used as positive and negative controls for RT-PCR; and M, 100- to 2,686-bp DNA molecular marker XIV (Roche). The arrow points to the expected *bcrABC* PCR product of 1,130 bp. (B) Transcript levels of housekeeping gene *spoVG* in the same cultures as those described for panel A. RT-PCR using primer s2 (for cDNA) and s1 and s2 for PCR. Lanes are as described for panel A. The arrow points to the expected *spoVG* PCR product of 533 bp. (C) Fold change in the transcript levels of *bcrABC* after exposure to 10, 20, and 40 μg/ml of BC. Fold change was determined as described in Materials and Methods, and the data are averages from two independent trials.

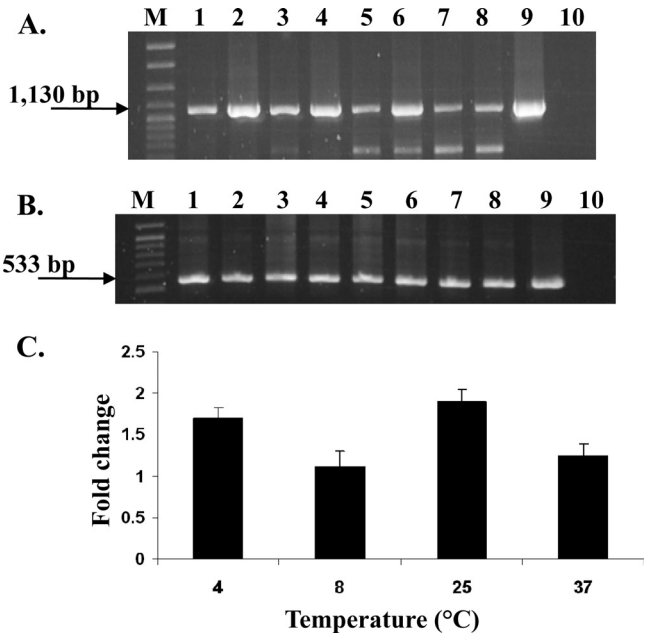

FIG. 4. Temperature regulation of *bcrABC*. (A) RT-PCR of *bcrABC* using primers p2 for cDNA and p1 and p2 for PCR. Lanes: 1, 3, 5, and 7, H7550 grown at 4, 8, 25, and 37°C, respectively; 2, 4, 6, and 8, H7550 grown at 4, 8, 25, and 37°C and exposed to BC (10 μg/ml) for 30 min as described in Materials and Methods; 9 and 10, H7550 genomic DNA and total RNA, respectively, used as positive and negative controls for RT-PCR; and M, 100- to 2,686-bp DNA molecular marker XIV (Roche). The arrow points to the expected *bcrABC* PCR product of 1,130 bp. Bottom bands in lanes 5 to 8 represent unspecific PCR products (confirmed with Southern blotting). (B) Transcript levels of housekeeping gene *spoVG* in the same cultures as those described for panel A. RT-PCR using primers s2 for cDNA and s1 and s2 for PCR. Lanes are as described for panel A. The arrow points to the expected *spoVG* PCR product of 533 bp. (C) Fold change in transcript levels of *bcrABC* at 4, 8, 25, and 37°C after exposure to BC (10 μg/ml). Fold change was determined as described in Materials and Methods, and the data are averages from two independent trials.

enhanced by cadmium, and reversely whether BC may increase expression of *cadAC*, RT-PCR was employed using *spoVG* as a reference. The data clearly indicated that *bcrABC* expression was increased (ca. 2-fold) by BC but not by sublethal exposure to cadmium (10 μg/ml $CdCl_2$), and similarly *cadA* expression was enhanced by cadmium (ca. 4-fold) but not by BC (Fig. 5). RT-PCR data indicated that *spoVG* expression was stable under the conditions tested (Fig. 5).

DISCUSSION

In this study we have described a BC resistance mechanism of *L. monocytogenes* H7550 (1998-1999 multistate outbreak strain) associated with a gene cassette harbored on the plasmid of this strain, pLM80. This gene cassette was also detected in two other sequenced *Listeria* genomes. Evidence for chromosomal efflux pumps implicated in quaternary ammonium compound resistance was provided for *L. monocytogenes* (6, 34, 35, 37, 38). Even though there was also evidence for plasmid-mediated resistance to BC (21, 34, 35), the plasmid-associated genes responsible for such resistance have not yet been identified.

The BC-resistance cassette described here was composed of one TetR family transcriptional regulator (*bcrA*) with a helix-turn-helix (HTH) DNA binding motif and two SMR genes (*bcrB* and *bcrC*). SMR proteins are proton-dependent multidrug efflux systems that typically require coexpression of two genes (2, 31) and have been characterized in several Gram-positive and Gram-negative bacteria, including *E. coli* EmrE and *Bacillus subtilis* EbrA and EbrB (14, 17, 29).

Members of the TetR family of regulators control transcription of multidrug efflux systems (33), suggesting a likely role for *bcrA* in transcriptional control of the *bcrABC* cassette. The palindrome between the putative −10 and −35 promoter regions of *bcrABC* may serve as a recognition sequence for the

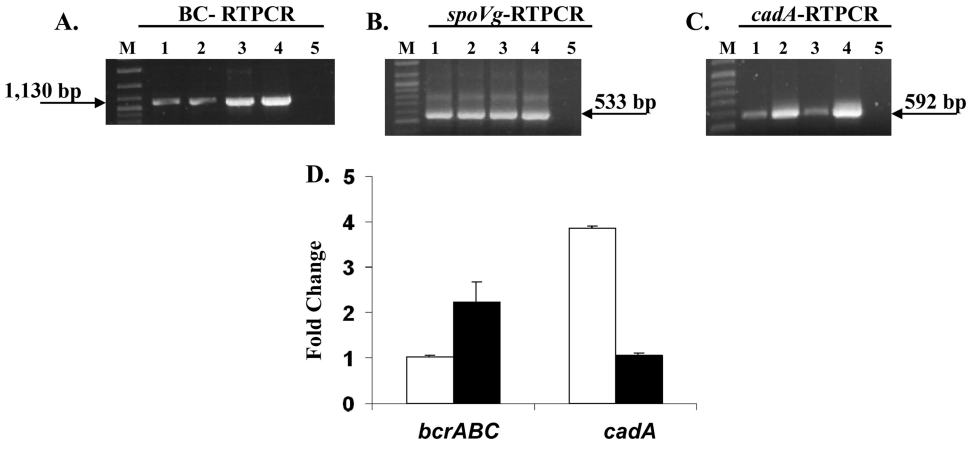

FIG. 5. Increased transcript levels of *bcrABC* and *cadA* by BC and cadmium in *L. monocytogenes* H7550 grown at 25°C. (A) RT-PCR of *bcrABC* using primer p2 for cDNA and primers p1 and p2 for PCR. Lanes: 1, H7550 in the absence of BC or cadmium; 2, H7550 exposed to cadmium; 3, H7550 exposed to BC; 4 and 5, H7550 genomic DNA and total RNA, respectively, used as positive and negative controls for RT-PCR; and M, 100- to 2,686-bp DNA molecular marker XIV (Roche). The arrow points to the expected *bcrABC* PCR product of 1,130 bp. Cadmium and BC exposures were for 30 min as described in Materials and Methods. (B) Transcript levels of housekeeping gene *spoVG* in the same cultures as those described for panel A. RT-PCR using primer s2 for cDNA and s1 and s2 for PCR. Lanes are as described for panel A. The arrow points to the expected *spoVG* PCR product of 533 bp. (C) RT-PCR of *cadA* using primer c2 for cDNA and primers c1 and c2 for PCR. Lanes are as described for panel A. The arrow points to the expected *cadA* PCR product of 592 bp. (D) Fold change in the transcript levels of *bcrABC* and *cadA* after exposure to BC (black bars) and cadmium (white bars). Fold change was determined as described in Materials and Methods, and the data are averages from two independent trials.

repressor. This palindrome overlaps with the putative −10 promoter sequence, thus possibly interfering with the transition of the RNA polymerase-promoter complex into a transcribing state. A palindrome overlapping the −10 region (but of different length and sequence content) was also described upstream of *qacR*, implicated in transcriptional control of the multidrug efflux pump *qacA* of *Staphylococcus aureus* (10, 32). Further studies are needed to confirm that *bcrA* functions as a repressor by binding to this region of dyad symmetry. A deletion mutant of this gene could not be obtained, suggesting that constitutive overexpression of *bcrBC* might be lethal to the cells.

Transcription of *bcrABC* was increased by sublethal levels of BC. No increase in the gene transcript level was noted in response to treatment with cadmium. Inversely, an increase in the gene transcript level of *cadA* was observed upon exposure to cadmium, but not BC. Thus, in spite of their genomic proximity on pLM80, the cassettes mediating resistance to BC and to cadmium are not regulated by the same substrates.

In spite of the increased *bcrABC* transcript levels upon exposure to BC, transcripts were also readily detected even in the absence of the disinfectant. This may suggest either that repression of transcription is weak or that other, currently unidentified molecules result in the observed baseline transcription levels in the absence of BC. Further studies are needed to determine how transcription is enhanced in the presence of sublethal levels of BC (e.g., by binding to BcrA and prevention of repression, as described in other efflux systems) (13, 40) and to assess whether additional molecules also increase transcript levels. Preliminary data suggest that, besides BC, other quaternary ammonium disinfectants (benzethonium chloride, cetyl-trimethyl ammonium bromide) also enhance the transcription of *bcrABC* in H7550 (V. Dutta and S. Kathariou, unpublished), but it is not known whether transcript levels can also be increased by other, structurally unrelated molecules that may be exported by this efflux system.

Transcript levels of *bcrABC* were increased at sublethal concentrations (10 μg/ml) of BC, but not at higher concentrations (e.g., 20 or 40 μg/ml). It is possible that BcrA conformation and the ability to bind to operator sites is different at low concentrations (10 μg/ml) compared to that at high concentrations (20 or 40 μg/ml) of BC. It is also possible that general toxicity associated with exposure to high concentrations of BC (even with a relatively short exposure time of 30 min) resulted in the observed loss of transcript level increase. However, we think this is less likely, as *bcrABC* transcripts were at baseline levels and *spoVG* levels were not impacted.

Resistance of *Listeria* species to BC is of special relevance to the pathogen's ecology in food processing plants, where BC and other quaternary ammonium disinfectants are used extensively. As noted before, two classes of BC-resistant strains can be recognized in *L. monocytogenes*, namely, resistant derivatives of previously BC-susceptible strains, resulting from adaptation to sublethal BC levels, and strains that are naturally resistant to BC, such as H7550 (34, 35, 38). Resistant derivatives of previously susceptible strains appear to involve chromosomal efflux systems, such as *mdrL*, that also mediate efflux of ethidium bromide and that are inhibited by the efflux inhibitor reserpine (35). The adapted derivatives may result from mutations leading to overexpression of these efflux systems, even though such mutations still need to be identified. On the other hand, in strains such as H7550, resistance to high levels of BC is mediated by *bcrABC*, a plasmid-associated system which shows increased transcript levels following exposure to BC and at low temperatures (4 to 25°C). In naturally resistant strains, resistance to BC is not accompanied by resistance to ethidium bromide, and neither is it inhibited by reserpine (35). We have indeed found that in strain H7550 the BC MIC was not affected by this efflux inhibitor, whereas MICs of the plasmid-cured strains were reduced in the presence of reserpine (M. Rakik-Martinez and S. Kathariou, unpublished).

The impact of temperature on transcription of *bcrABC* may be of special relevance to environmental adaptations of *Listeria* species in food processing plants where low temperatures often prevail. Temperature has a profound impact on the gene expression of *L. monocytogenes*, including genes involved in virulence and in environmental adaptations (5, 11, 15, 23, 39). Further studies are needed to characterize the mechanism responsible for temperature-dependent expression of *bcrABC* in *L. monocytogenes* and to also determine whether a similar impact of temperature is observed at the proteomic level.

Given that quaternary ammonium compounds such as BC are manmade reagents, it will be of interest to identify compounds in nature that can be exported by *bcrABC* and conditions responsible for acquisition and retention of this gene cassette by *L. monocytogenes*. The cassette was found to be on putative composite transposable units flanked by IS*1216* inverted repeats. In addition to genes mediating resistance to BC, these putative composite transposable units harbor a cadmium resistance cassette, a putative glyoxalase, and a putative triphenylmethane reductase. The diversity of GC content within this region of pLM80 suggests acquisition of these genes through horizontal gene transfer, possibly from varied sources. The role of IS*1216* transposition in gene transfer has been well described in another Gram-positive bacterium, *Enterococcus faecium* (12).

The presence of *bcrABC* on a putative IS*1216* composite transposon harbored by pLM80 suggests possible mechanisms for the transfer of these genes among different *Listeria* genomes via transposition or plasmid mobilization. The presence of *bcrABC* in the vicinity of, and possibly in the same transposable unit as, *cadAC* may also partially explain the finding that BC-resistant strains of *L. monocytogenes* were also resistant to cadmium (27). Further work is needed to assess the ability of *bcrABC* to disseminate from H7550 to BC-susceptible strains of different serotypes of *L. monocytogenes* and different *Listeria* species.

In conclusion, we have characterized a plasmid-based BC resistance gene cassette on a putative composite transposon in a strain of *L. monocytogenes* associated with the 1998-1999 multistate outbreak. Such a resistance cassette draws attention to the possible role of disinfectants in adaptations and persistence of *L. monocytogenes* in food processing and other environments with frequent disinfectant use. Further studies are needed to elucidate the evolution and dissemination of these BC resistance genes in *L. monocytogenes* and to assess the possible role of these genes in environmental persistence as well as in the virulence of this pathogen.

ACKNOWLEDGMENTS

This work was partially supported by a grant from the American Meat Institute Foundation and USDA grant 2006-35201-17377.

We thank Mira Rakic-Martinez for information on MICs for ethidium bromide and on the impact of reserpine. We are thankful to all other members of our laboratory for their support and encouragement.

REFERENCES

1. **Aase, B., G. Sundheim, S. Langsrud, and L. V. Rørvik.** 2000. Occurrence of and a possible mechanism for resistance to a quaternary ammonium compound in *Listeria monocytogenes*. Int. J. Food Microbiol. **62:**57–63.
2. **Bay, D. C., K. L. Rommens, and R. J. Turner.** 2008. Small multidrug resistance proteins: a multidrug transporter family that continues to grow. Biochim. Biophys. Acta **1778:**1814–1838.
3. **Behari, J., and P. Youngman.** 1998. Regulation of *hly* expression in *Listeria monocytogenes* by carbon sources and pH occurs through separate mechanisms mediated by PrfA. Infect. Immun. **66:**3635–3642.
4. **Centers for Disease Control and Prevention.** 1998. Multistate outbreak of listeriosis—United States, 1998. MMWR Morb. Mortal. Wkly. Rep. **47:** 1085–1086.
5. **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.
6. **Earnshaw, A. M., and L. M. Lawrence.** 1998. Sensitivity to commercial disinfectants, and the occurrence of plasmids within various *Listeria monocytogenes* genotypes isolated from poultry products and the poultry processing environment. J. Appl. Microbiol. **84:**642–648.
7. **Gandhi, M., and M. L. Chikindas.** 2007. *Listeria*: a foodborne pathogen that knows how to survive. Int. J. Food Microbiol. **113:**1–15.
8. **Gilmour, M. W., M. Graham, G. Van Domselaar, S. Tyler, H. Kent, K. M. Trout-Yakel, O. Larios, V. Allen, B. Lee, and C. Nadon.** 2010. High-throughput genome sequencing of two *Listeria monocytogenes* clinical isolates during a large foodborne outbreak. BMC Genomics **11:**120.
9. **Glaser, P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend, F. Baquero, P. Berche, H. Bloecker, 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. Kurapkat, E. Madueno, A. Maitournam, J. M. Vicente, E. Ng, H. Nedjari, G. Nordsiek, S. Novella, B. de Pablos, J. C. Perez-Diaz, R. Purcell, B. Remmel, M. Rose, T. Schlueter, 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.
10. **Grkovic, S., M. H. Brown, N. J. Roberts, I. T. Paulsen, and R. A. Skurray.** 1998. QacR is a repressor protein that regulates expression of the *Staphylococcus aureus* multidrug efflux pump QacA. J. Biol. Chem. **273:**18665–18673.
11. **Gründling, A., L. S. Burrack, H. G. Bouwer, and D. E. Higgins.** 2004. *Listeria monocytogenes* regulates flagellar motility gene expression through MogR, a transcriptional repressor required for virulence. Proc. Natl. Acad. Sci. U. S. A. **101:**12318–12323.
12. **Heaton, M. P., L. F. Discotto, M. J. Pucci, and S. Handwerger.** 1996. Mobilization of vancomycin resistance by transposon-mediated fusion of a VanA plasmid with an *Enterococcus faecium* sex pheromone-response plasmid. Gene **171:**9–17.
13. **Hinrichs, K., C. Kisker, M. Duvel, A. Muller, K. Tovar, W. Hillen, and W. Saenger.** 1994. Structure of the Tet repressor-tetracycline complex and regulation of antibiotic resistance. Science **264:**418–420.
14. **Jack, D. L., M. L. Storms, J. H. Tchieu, I. T. Paulsen, and M. H. Saier, Jr.** 2000. A broad-specificity multidrug efflux pump requiring a pair of homologous SMR-type proteins. J. Bacteriol. **182:**2311–2313.
15. **Johansson, J., P. Mandin, A. Renzoni, C. Chiaruttini, M. Springer, and P. Cossart.** 2002. An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. Cell **110:**551–561.
16. **Kathariou, S.** 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. J. Food Prot. **65:**1811–1829.
17. **Kikukawa, T., T. Nara, T. Araiso, S. Miyauchi, and N. Kamo.** 2006. Two-component bacterial multidrug transporter, EbrAB: mutations making each component solely functional. Biochim. Biophys. Acta **1758:**673–679.
18. **Kim, J. W., and S. Kathariou.** 2009. Temperature-dependent phage resistance of *Listeria monocytogenes* epidemic clone II. Appl. Environ. Microbiol. **75:**2433–2438.
19. **Kornacki, J. L., and J. B. Gurtler.** 2007. Incidence and control of *Listeria* in food processing facilities, p. 681–766. *In* E. T. Ryser and E. H. Marth (ed.), *Listeria*, listeriosis and food safety, 3rd ed. CRC Press, Boca Raton, FL.

19a. **Kuenne, C., S. Voget, J. Pischimarov, S. Oehm, A. Goesmann, R. Daniel, T. Hain, and T. Chakraborty.** 2010. Comparative analysis of plasmids in the genus *Listeria*. PLoS One **5:**e12511.

20. **Lauer, P., M. Y. N. 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.
21. **Lemaître, J. P., H. Echchannaoui, G. Michaut, C. Divies, and A. Rousset.** 1998. Plasmid-mediated resistance to antimicrobial agents among listeriae. J. Food Prot. **61:**1459–1464.
22. **Liu, Y., and A. Ream.** 2008. Gene expression profiling of *Listeria monocytogenes* strain F2365 during growth in ultrahigh-temperature-processed skim milk. Appl. Environ. Microbiol. **74:**6859–6866.
23. **Loh, E., O. Dussurget, J. Gripenland, K. Vaitkevicius, T. Tiensuu, P. Mandin, F. Repoila, C. Buchrieser, P. Cossart, and J. Johansson.** 2009. A trans-acting riboswitch controls expression of the virulence regulator PrfA in *Listeria monocytogenes*. Cell **139:**770–779.
24. **McDonnell, G., and A. D. Russell.** 1999. Antiseptics and disinfectants: activity, action, and resistance. Clin. Microbiol. Rev. **12:**147–179.
25. **Mereghetti, L., R. Quentin, N. Marquet-Van Der Mee, and A. Audurier.** 2000. Low sensitivity of *Listeria monocytogenes* to quaternary ammonium compounds. Appl. Environ. Microbiol. **66:**5083–5086.
26. **Merianos, J. J.** 1991. Quaternary ammonium antimicrobial compounds, p. 225–255. *In* S. S. Block (ed.), Disinfection, sterilization and preservation, 4th ed. Lea & Feigner, Malvern, PA.
27. **Mullapudi, S., R. M. Siletzky, and S. Kathariou.** 2008. Heavy-metal and benzalkonium chloride resistance of *Listeria monocytogenes* isolates from the environment of turkey-processing plants. Appl. Environ. Microbiol. **74:**1464–1468.
28. **Nelson, K. E., D. E. Fouts, E. F. Mongodin, J. Ravel, R. T. DeBoy, J. F. Kolonay, D. A. Rasko, S. V. Angiuoli, S. R. Gill, I. T. Paulsen, J. Peterson, O. White, W. C. Nelson, W. Nierman, M. J. Beanan, L. M. Brinkac, S. C. Daugherty, R. J. Dodson, A. S. Durkin, R. Madupu, D. H. Haft, J. Selengut, S. Van Aken, H. Khouri, N. Fedorova, H. Forberger, B. Tran, S. Kathariou, L. D. Wonderling, G. A. Uhlich, and D. O. Bayles.** 2004. Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. Nucleic Acids Res. **32:**2386–2395.
29. **Ninio, S., D. Rotem, and S. Schuldiner.** 2001. Functional analysis of novel multidrug transporters from human pathogens. J. Biol. Chem. **276:**48250–48256.
30. **Painter, J., and L. Slutsker.** 2007. Listeriosis in humans, p. 85–109. *In* E. T. Ryser and E. H. Marth (ed.), *Listeria*, listeriosis and food safety, 3rd ed. CRC Press, Boca Raton, FL.
31. **Paulsen, I. T., R. A. Skurray, R. Tam, M. H. Saier, Jr., R. J. Turner, J. H. Weiner, E. B. Goldberg, and L. L. Grinius.** 1996. The SMR family: a novel family of multidrug efflux proteins involved with the efflux of lipophilic drugs. Mol. Microbiol. **19:**1167–1175.
32. **Paulsen, I. T., M. H. Brown, T. G. Littlejohn, B. A. Mitchell, and R. A. Skurray.** 1996. Multidrug resistance proteins QacA and QacB from *Staphylococcus aureus*: membrane topology and identification of residues involved in substrate specificity. Proc. Natl. Acad. Sci. U. S. A. **93:**3630–3635.
33. **Ramos, J. L., M. Martínez-Bueno, A. J. Molina-Henares, W. Terán, K. Watanabe, X. Zhang, M. T. Gallegos, R. Brennan, and R. Tobes.** 2005. The TetR family of transcriptional repressors. Microbiol. Mol. Biol. Rev. **69:**326–356.
34. **Romanova, N., S. Favrin, and M. W. Griffiths.** 2002. Sensitivity of *Listeria monocytogenes* to sanitizers used in the meat processing industry. Appl. Environ. Microbiol. **68:**6405–6409.
35. **Romanova, N. A., P. F. Wolffs, L. Y. Brovko, and M. W. Griffiths.** 2006. Role of efflux pumps in adaptation and resistance of *Listeria monocytogenes* to benzalkonium chloride. Appl. Environ. Microbiol. **72:**3498–3503.
36. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Biotechnology **1:**784–791.
37. **Soumet, C., C. Ragimbeau, and P. Maris.** 2005. Screening of benzalkonium chloride resistance in *Listeria monocytogenes* strains isolated during cold smoked fish production. Lett. Appl. Microbiol. **41:**291–296.
38. **To, M. S., S. Favrin, N. Romanova, and M. W. Griffiths.** 2002. Postadaptational resistance to benzalkonium chloride and subsequent physicochemical modifications of *Listeria monocytogenes*. Appl. Environ. Microbiol. **68:**5258–5264.
39. **Toledo-Arana. A., O. Dussurget, G. Nikitas, N. Sesto, H. Guet-Revillet, D. Balestrino, E. Loh, J. Gripenland, T. Tiensuu, K. Vaitkevicius, M. Barthelemy, M. Vergassola, M. A. Nahori, G. Soubigou, B. Régnault, J. Y. Coppée, M. Lecuit, J. Johansson, and P. Cossart.** 2009. The *Listeria* transcriptional landscape from saprophytism to virulence. Nature **459:**950–956.
40. **Wang, L., B. Jeon, O. Sahin, and Q. Zhang.** 2009. Identification of an arsenic resistance and arsenic-sensing system in *Campylobacter jejuni*. Appl. Environ. Microbiol. **75:**5064–5073.