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Listeria monocytogenes Strains Selected on Ciprofloxacin or the Disinfectant Benzalkonium Chloride Exhibit Reduced Susceptibility to Ciprofloxacin, Gentamicin, Benzalkonium Chloride, and Other Toxic Compounds▽

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***Listeria monocytogenes* is a leading agent for severe food-borne illness and death in the United States and other nations. Even though drug resistance has not yet threatened therapeutic interventions for listeriosis, selective pressure associated with exposure to antibiotics and disinfectants may result in reduced susceptibility to these agents. In this study, selection of several *L. monocytogenes* strains on either ciprofloxacin (2 μg/ml) or the quaternary ammonium disinfectant benzalkonium chloride (BC; 10 μg/ml) led to derivatives with increased MICs not only to these agents but also to several other toxic compounds, including gentamicin, the dye ethidium bromide, and the chemotherapeutic drug tetraphenylphosphonium chloride. The spectrum of compounds to which these derivatives exhibited reduced susceptibility was the same regardless of whether they were selected on ciprofloxacin or on BC. Inclusion of strains harboring the large plasmid pLM80 revealed that MICs to ciprofloxacin and gentamicin did not differ between the parental and plasmid-cured strains. However, ciprofloxacin-selected derivatives of pLM80-harboring strains had higher MICs than those derived from the plasmid-cured strains. Susceptibility to the antimicrobials was partially restored in the presence of the potent efflux inhibitor reserpine. Taken together, these data suggest that mutations in efflux systems are responsible for the multidrug resistance phenotype of strains selected on ciprofloxacin or BC.**

Listeria monocytogenes is a Gram-positive bacterium responsible for listeriosis, an illness that remains a leading cause of mortality and morbidity associated with food-borne infections in the United States and other industrialized nations (14, 20, 32). At risk are primarily pregnant women and their fetuses, the elderly, and those in immunocompromised states, including patients undergoing kidney dialysis or chemotherapy. Symptoms can be severe (septicemia, meningitis, and stillbirths), and the fatality rate is estimated at 16% (3, 26, 32).

L. monocytogenes is usually susceptible to a wide range of antibiotics, except cephalosporins and fosfomycin (10, 11, 22), and multidrug-resistant clinical isolates appear to be rare (8, 28, 29, 35). The treatment of choice for listeriosis consists of a β-lactam antibiotic (e.g., ampicillin), alone or in combination with an aminoglycoside (e.g., gentamicin), and clinical isolates of *L. monocytogenes* generally remain susceptible to these antibiotics (10, 22). However, trends toward reduced susceptibility to tetracyclines and fluoroquinolones have been noted (22). Even though these antibiotics are not typically used for treatment of listeriosis, their extensive use in empirical therapy and treatment of other infections could create selective pressure for *L. monocytogenes* mutants with reduced susceptibility. Fluoroquinolone-resistant isolates of *L. monocytogenes* from clinical cases were found to have enhanced transcription of *lde*, encoding a drug efflux transporter of the major facilitator superfamily (MFS) (6, 22). However, limited information is available on mutants obtained upon exposure of *L. monocytogenes* to fluoroquinolones.

In addition to antibiotic-related exposures, *L. monocytogenes* is expected to be frequently subjected to selection pressures associated with the extensive use of disinfectants such as quaternary ammonium compounds in food processing plants and health care settings. Limited information is currently available on possible cross-resistance following exposure to antibiotics such as ciprofloxacin and disinfectants such as the quaternary ammonium compound benzalkonium chloride (BC), extensively used in the food processing industry (19, 21). Similarly, exposure to BC may result in variants with reduced susceptibility to BC but also reduced susceptibility to antibiotics. Indeed, exposure of *L. monocytogenes* to progressively increasing concentrations of BC resulted in BC-resistant mutants that also exhibited reduced susceptibility to gentamicin and kanamycin (31, 34). However, cross-resistance to fluoroquinolones (e.g., ciprofloxacin) was not reported. The potential for coselection of antibiotic and disinfectant resistance upon exposure of *L. monocytogenes* to antimicrobial agents has important food safety and public health implications and needs to be further investigated.

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TABLE 1. *L. monocytogenes* strains employed in this study

Strain	Resistance[a]		Characteristic(s)	Source (reference)
	Cd	BC		
H7550 Cd^r	+	+	Serotype 4b strain from 1998–1999 hot dog outbreak	4
H7550 Cd^r C_1	+	+	Ciprofloxacin-selected derivative of H7550 Cd^r	This study
H7550 Cd^r C_2	+	+	Ciprofloxacin-selected derivative of H7550 Cd^r	This study
H7550 Cd^s	−	−	Plasmid-cured derivative of H7550 Cd^r	4
H7550 Cd^s C_1	−	+	Ciprofloxacin-selected derivative of H7550 Cd^s	This study
H7550 Cd^s C_2	−	+	Ciprofloxacin-selected derivative of H7550 Cd^s	This study
H7550 Cd^s BC_1	−	+	BC-selected derivative of H7550 Cd^s	This study
H7550 Cd^s BC_2	−	+	BC-selected derivative of H7550 Cd^s	This study
SK2802	+	−	Serotype 4b strain, sporadic case of listeriosis, USA, 2005	L. Wolf
SK2802 C_1	+	+	Ciprofloxacin-selected derivative of SK2802	This study
SK2802 C_2	+	+	Ciprofloxacin-selected derivative of SK2802	This study
SK2802 BC_1	+	+	BC-selected derivative of SK2802	This study
SK2802 BC_2	+	+	BC-selected derivative SK2802	This study
J0161 Cd^r	+	+	Serotype 1/2a strain from turkey deli meats outbreak, 2001	25
J0161 Cd^r C_1	+	+	Ciprofloxacin-selected derivative of J0161 Cd^r	This study
J0161 Cd^r C_2	+	+	Ciprofloxacin-selected derivative of J0161 Cd^r	This study
J0161 Cd^s	+	+	Plasmid-cured derivative of J0161 Cd^r	This study
J0161 Cd^s C_1	−	+	Ciprofloxacin-selected derivative of J0161 Cd^s	This study
J0161 Cd^s C_2	−	+	Ciprofloxacin-selected derivative of J0161 Cd^s	This study
J0161 Cd^s BC_1	−	+	BC-selected derivative of J0161 Cd^s	This study
J0161 Cd^s BC_2	−	+	BC-selected derivative of J0161 Cd^s	This study

[a] For Cd, + indicates confluent growth on Iso-Sensitest agar containing 70 μg/ml anhydrous cadmium chloride; for BC, + indicates confluent growth on medium containing 10 μg/ml BC, prepared as described in Materials and Methods; − indicates absence of growth.

In this study, we determined the extent to which adaptation of *L. monocytogenes* to ciprofloxacin and to BC altered susceptibility to a panel of antimicrobial agents, including several antibiotics, BC, and other toxic compounds. Outbreak-derived strains harboring a large plasmid (pLM80) and their cured derivatives were included, to assess possible impact of the plasmid on the susceptibility profiles of the adapted strains.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. monocytogenes* strains employed in this study are listed in Table 1. The serotype 4b strain H7550 Cd^r is resistant to cadmium (Cd^r) and BC and was associated with the 1998-1999 hot dog-related multistate outbreak (4); it harbors pLM80 (ca. 80 kb), with genes for resistance to cadmium (*cadAC*) and to BC (*bcrABC*) (4, 24). *L. monocytogenes* J0161 Cd^r is a serotype 1/2a strain associated with a listeriosis outbreak in 2001, linked to consumption of turkey deli meats (25). It is also resistant to cadmium and BC and harbors a large plasmid highly similar to pLM80 (15). *L. monocytogenes* SK 2802 was from a sporadic case of listeriosis in 2005; this strain is resistant to cadmium and sodium arsenite but susceptible to BC and lacks *bcrABC*. H7550 Cd^s and J0161 Cd^s were plasmid-cured derivatives of strains H7550 Cd^r and J0161 Cd^r, respectively; these derivatives were obtained following repeated passages of the bacteria at 42°C and were susceptible both to cadmium and to BC (4). Strains were routinely propagated at 37°C on tryptic soy agar plates containing 5% sheep blood (Remel, Lenexa, KS), and long-term storage was at −80°C in brain heart infusion broth (BHI; Becton Dickinson and Co., Sparks, MD) with 20% glycerol (Fisher Scientific, Fair Lawn, NJ).

Isolation of derivatives selected on ciprofloxacin or BC. Bacteria were grown overnight in BHI at 37°C and spotted (3 μl) on Mueller-Hinton agar (MHA) (Mueller-Hinton broth [Becton Dickinson and Co.] with 1.2% Bacto agar [Becton Dickinson and Co.]) containing 2 μg/ml ciprofloxacin (Sigma Chemical Co., St. Louis, MO). Colonies growing following 48 h of incubation at 37°C were purified on MHA containing 2 μg/ml ciprofloxacin. Overnight cultures of the BC-susceptible strains H7550 Cd^s, J0161 Cd^s, and SK2802 were also spotted (3 μl) on MHA containing 10 μg/ml BC (Acros; catalog no. 263820010) and 2% defibrinated sheep blood (Becton Dickinson and Co.) (23). Single colonies obtained following incubation at 37°C for 48 h were subcultured on MHA containing 10 μg/ml BC and 2% defibrinated sheep blood for purification. Each experiment has been repeated in at least three separate trials.

Antimicrobial agents, susceptibility testing, and MIC determinations. Antibiotics used in this study are listed in Table 2. They represented major antibiotic classes and included those used for treatment of listeriosis as well as others commonly used in human and veterinary medicine. Ampicillin (catalog no. A9518), ciprofloxacin (catalog no. 17850), rifampin (catalog no. R3501), tetracycline (catalog no. T8032), fosfomycin (catalog no. P7902), and trimethoprim (catalog no. T7883) were purchased from Sigma; gentamicin (catalog no. 091676045), moxifloxacin (catalog no. 231757), and kanamycin (catalog no. BP 906-5) were from Fisher Scientific; erythromycin (catalog no. 0219452901) and streptomycin (catalog no. 0219454125) were from MP Biomedicals, Inc. (Solon, OH). Resistance to BC, cadmium chloride (Cd) (Sigma), and sodium arsenite (As) (Fluka, Buchs, Steinheim, Germany) was determined as described previously (23). Concentrations used for determinations of MIC values were 5, 10, 20, 35, 70, and 140 μg/ml for Cd and 5, 10, 20, 30, and 40 μg/ml for BC. Ethidium bromide (EthBr; catalog no. BP 102-5) (Fisher Scientific) resistance was determined as previously described (6). Concentrations for EthBr MIC determinations were 20, 25, 50, 100, and 200 μg/ml. Tetraphenylphosphonium chloride (TPP) (Sigma) was used at 50, 100, 200, 400, and 800 μM for MIC determina-

TABLE 2. Antibiotics employed in this study

Antibiotic	Drug class	Concn range (μg/ml)	MIC[a] (μg/ml)
Ampicillin	β-Lactam	0.125–2.0	≤1.0
Ciprofloxacin	Fluoroquinolone	0.5–16.0	2.0–8.0
Moxifloxacin	Fluoroquinolone	5.0	ND
Erythromycin	Macrolide	0.25–8.0	0.5
Gentamicin	Aminoglycoside	0.5–4.0	≤4.0
Kanamycin	Aminoglycoside	1.0–20.0	2.5
Streptomycin	Aminoglycoside	6.25–400.0	25.0
Rifampin	Rifamycin	0.5–4.0	0.5
Tetracycline	Tetracycline	1.0–10.0	2.5
Trimethoprim	Dihydrofolate reductase	0.5–4.0	0.5
Fosfomycin	Phosphonic acid derivative	64.0–2,048.0	≥2,048.0

[a] MICs for parental strains used in the study. MIC for ciprofloxacin was 2 μg/ml for all wild-type strains, except for SK2802 (MIC, 8 μg/ml). ND, not determined; moxifloxacin susceptibility was assessed by the disk diffusion method as described in Materials and Methods.

TABLE 3. Primers used in this study

Primer	Sequence (5′ to 3′)	Reference
s1	ATTACAGATGTGAGATTACGACG	4
s2	ACGTTTACTTGGCATAGCTAC	4
mdrL_F	CTCCACTCGTTACACTTCT	This study
mdrL_R	CAGACAAGGAAATGAACAC	This study
mdrM_F	GTACATCAGTGAAGCGTAACG	This study
mdrM_R	CTAGAACACAAGCGACTACAG	This study
mdrT_F	CGGCCCGTTGATGTTAACG	This study
mdrT_R	CATTCCGTCCAAACTAGCATC	This study
ldeF	GAAGAAGAATTTGTATGTTGTC	This study
ldeR	TCTCTCCATGCATTTTTCGG	This study

tions. Susceptibility to moxifloxacin was determined by the disk diffusion method using disks with 5 μg moxifloxacin (Fisher Scientific) on MHA. The diameter of the zone of inhibition was measured to the nearest whole millimeter.

For MIC determinations, one colony from a blood agar plate (37°C, 24 h) was resuspended in 0.1 ml BHI and 3 μl of the suspension was spotted in duplicate on MHA plates (supplemented with 2% blood, for BC) with antibiotic or other antimicrobial and incubated at 37°C for 48 h. The ranges of concentrations used for antibiotic MIC determinations are indicated in Table 2. MIC was defined as the concentration at which growth was prevented. MICs were determined in at least two separate trials (each time in duplicate).

The efflux inhibitor reserpine (Sigma) was used at 10 μg/ml as described previously (6). When MICs were determined in the presence of the reserpine, MICs in the absence of reserpine were always determined as control.

RNA extraction and RT-PCR. Expression of selected multidrug resistance (MDR) genes (*mdrL*, *mdrM*, *mdrT*, and *lde*) was assessed by using reverse transcription-PCR (RT-PCR) analysis. H7550 Cd^s and its BC-selected derivative H7550 Cd^s BC_1 were grown to logarithmic phase in BHI at 30°C (optical density at 600 nm [OD_{600}], ~0.7 to 0.9; determined with a spectrophotometer [SmartSpec 3000; Bio-Rad, Hercules, CA]). RNA was extracted from 1 ml of culture, and RT-PCR was performed as described previously (4). Primers employed are listed in Table 3. PCR was carried out using the Takara Ex *Taq* kit (Takara, Madison, WI) and a T1 thermal cycler (Biometra, Goettingen, Germany) as described previously (4). The housekeeping gene *spoVG* was used as internal control in the RT-PCRs, as described previously (4). RT-PCR for each gene was done in at least two independent trials. The image processing software ImageJ (http://rsbweb.nih.gov/ij/) was employed to quantify bands, and fold increase in transcript levels was determined as described previously (4).

RESULTS

Presence of pLM80 does not impact MICs to antibiotics. *L. monocytogenes* H7550 Cd^r harbors the 80-kb plasmid pLM80, which contains a cadmium resistance cassette as well as a cassette (*bcrABC*) mediating resistance to BC (4, 24). As expected, MICs to Cd and BC were markedly reduced in the cured derivatives, from 140 to 10 μg/ml and from 40 to 10 μg/ml, respectively. However, the cured derivatives exhibited the same MICs to the panel of antibiotics, EthBr, and TPP as those of the parental strains (data not shown). Similar data were obtained with the plasmid-cured derivative of the serotype 1/2a strain J0161, which harbors a pLM80-like plasmid that confers resistance to Cd and BC (15). The findings indicated that these plasmids conferred resistance to Cd and BC but did not influence MICs to the other antimicrobial agents that were tested.

***L. monocytogenes* strains selected on ciprofloxacin also exhibit reduced susceptibility to gentamicin.** Colonies growing at inhibitory concentrations of ciprofloxacin were selected for further characterization, using two independent ciprofloxacin-selected derivatives (C_1 and C_2) for strains H7550, J0161, and SK2802, respectively. Derivatives selected on ciprofloxacin exhibited reduced susceptibility to this antibiotic, with 4- to 32-fold (8 to 64 μg/ml) increases in MICs, depending on the strain (Fig. 1A and B); independent derivatives of the same strain exhibited similar increases in MIC values (data not shown).

Interestingly, even though as described above the presence of the plasmid in H7550 Cd^r and J0161 Cd^r did not impact MICs to any of the tested antibiotics, ciprofloxacin-selected derivatives of the plasmid-harboring strains consistently exhibited higher MICs to ciprofloxacin than did derivatives of the corresponding plasmid-cured strains (Fig. 1A and B). This was especially pronounced with ciprofloxacin-selected derivatives of strain J0161 Cd^r, which exhibited a 32-fold increase in MIC, in contrast to the 8-fold increase in MIC observed with ciprofloxacin-selected derivatives of J0161 Cd^s (Fig. 1A).

Strains selected on ciprofloxacin exhibited no change in their susceptibility to ampicillin, rifampin, streptomycin, trimethoprim, erythromycin, tetracycline, fosfomycin, or kanamycin (data not shown). However, susceptibility to gentamicin was consistently reduced in all tested ciprofloxacin-selected derivatives. As observed with ciprofloxacin MICs, ciprofloxacin-selected derivatives of plasmid-harboring strains exhibited higher MICs to gentamicin than did derivatives of their plasmid-cured counterparts (Fig. 1A and B). This difference was again especially noticeable with J0161 Cd^r C_1, which exhibited a 32-fold increase in MIC to gentamicin in comparison to J0161 Cd^r, whereas J0161 Cd^s C_1 exhibited an 8-fold increase in MIC in comparison to J0161 Cd^s (Fig. 1A). The same trend was observed with independently isolated derivatives of the same strain (data not shown). Increases in both ciprofloxacin and gentamicin MICs were also observed with ciprofloxacin-selected derivatives of strain SK2802 (Fig. 1C).

***L. monocytogenes* strains selected on ciprofloxacin also exhibit reduced susceptibility to BC, EthBr, and TPP.** As discussed above, the plasmid-cured strains H7550 Cd^s and J0161 Cd^s exhibited reduced tolerance to BC (MIC, 10 μg/ml). However, BC susceptibility determinations of ciprofloxacin-selected derivatives H7550 Cd^s C_1 and J0161 Cd^s C_1 revealed that they had markedly increased MIC (30 μg/ml) for BC (Fig. 1A and B); identical results were obtained with H7550 Cd^s C_2 and J0161 Cd^s C_2 (data not shown). MICs were not altered in ciprofloxacin-selected derivatives of the plasmid-harboring strains (Fig. 1A and B). Ciprofloxacin-selected derivatives of SK2802, which lacks resistance to BC, exhibited increases in MIC to BC from 10 to 30 μg/ml (Fig. 1C).

Resistance to ciprofloxacin was also accompanied by reduced susceptibility to EthBr and the toxic compound TPP, both of which are MDR efflux substrates. EthBr MICs markedly increased from 20 μg/ml in the parental strains to 200 μg/ml in the ciprofloxacin-selected mutants, regardless of whether the strains harbored plasmids or not (data not shown). A 4-fold increase in MIC was also observed with TPP (from 100 μM in the parental strains to 400 μM in the ciprofloxacin-selected derivatives) (data not shown). However, no impact was detected on MICs of Cd or sodium arsenite (data not shown).

***L. monocytogenes* strains selected on BC also exhibit reduced susceptibility to ciprofloxacin, gentamicin, EthBr, and TPP.** The plasmid-cured derivatives H7550 Cd^s and J0161 Cd^s as well as strain SK2802 lack the BC resistance cassette *bcrABC* and exhibited relatively low tolerance to BC (MIC, 10 μg/ml).

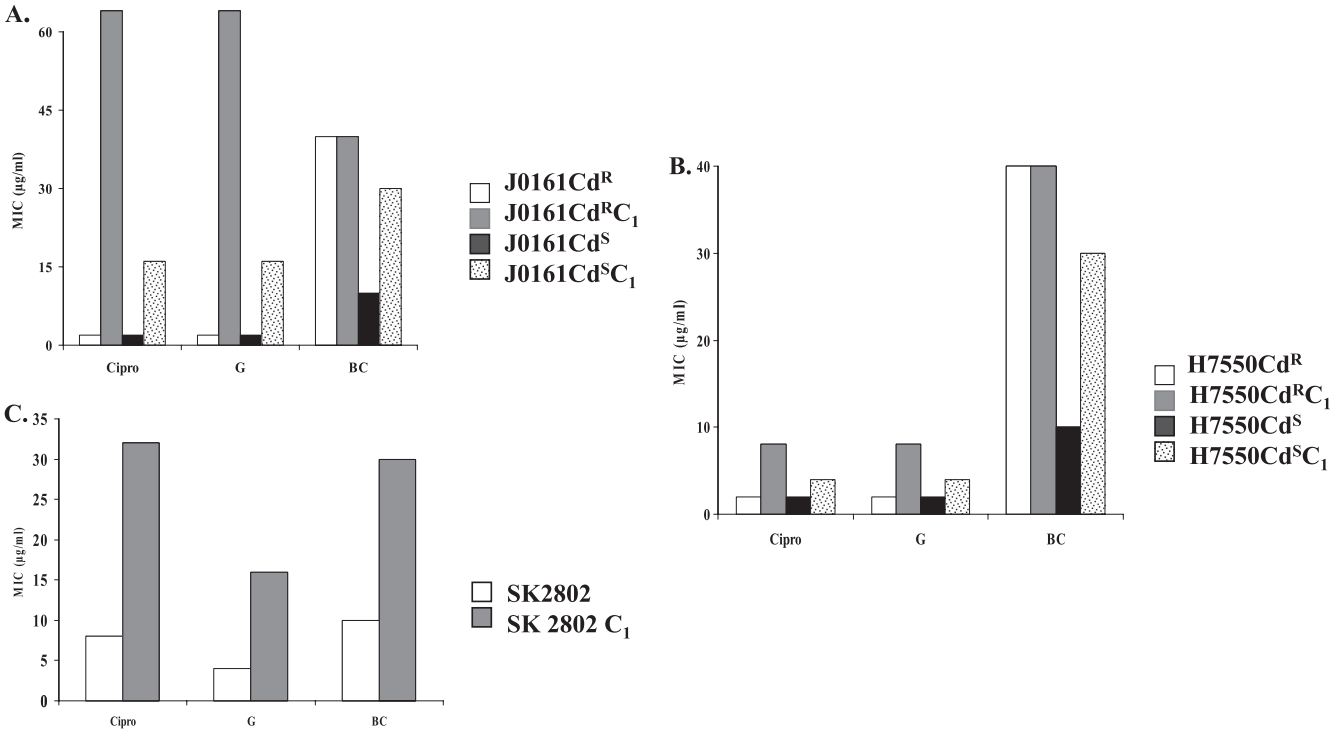

FIG. 1. Ciprofloxacin-selected strains also exhibit reduced susceptibility to gentamicin and BC. MICs for ciprofloxacin (Cipro), gentamicin (G), and benzalkonium chloride (BC) were determined for plasmid-harboring strain J0161 Cd^r (white bars) and its ciprofloxacin-selected derivative J0161 Cd^r C_1 (gray bars), as well as for the plasmid-cured strain J0161 Cd^s (black bars) and its ciprofloxacin-selected derivative J0161 Cd^s C_1 (stippled bars) (A); plasmid-harboring strain H7550 Cd^r (white bars) and its ciprofloxacin-selected derivative H7550 Cd^r C_1 (gray bars), as well as for the plasmid-cured strain H7550 Cd^s (black bars) and its ciprofloxacin-selected derivative H7550 Cd^s C_1 (stippled bars) (B); and strain SK2802 (white bars) and its ciprofloxacin-selected derivative SK2802 C_1 (gray bars) (C). MICs were determined as described in Materials and Methods in two independent trials (no variation was seen).

At least two independent derivatives of each strain were isolated on medium with BC and designated BC_1 and BC_2. BC-selected derivatives of all strains exhibited increases in their MIC for BC (from 10 to 30 μg/ml) (Fig. 2 and data not shown). However, these strains also exhibited 4- to 8-fold-increased resistance to ciprofloxacin and 2- to 8-fold-increased resistance to gentamicin (Fig. 2). In addition, these BC-selected strains exhibited higher tolerance to EthBr and TPP, with the increases in MICs being identical to those described above with ciprofloxacin-selected derivatives (MICs increasing from 20 to

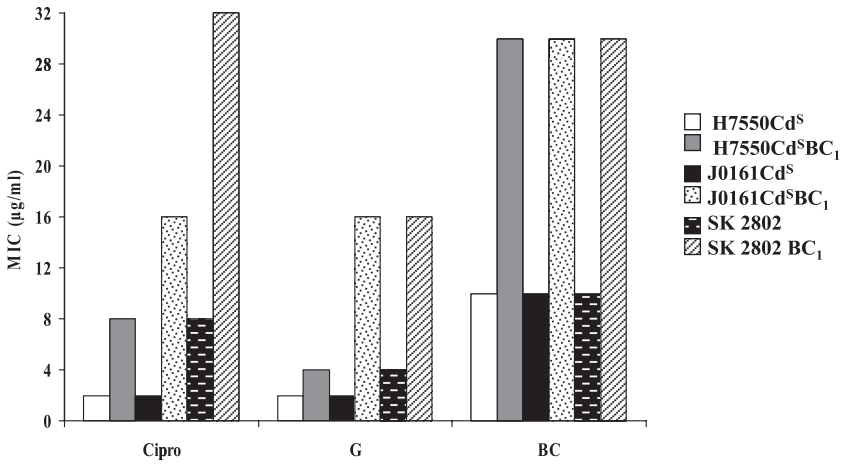

FIG. 2. BC-selected strains also exhibit reduced susceptibility to ciprofloxacin and gentamicin. Antimicrobial susceptibilities to ciprofloxacin (Cipro), gentamicin (G), and benzalkonium chloride (BC) were determined for plasmid-cured strain H7550 Cd^s (white bars) and its BC-selected derivative H7550 Cd^s BC_1 (gray bars), plasmid-cured strain J0161 Cd^s (black bars) and its BC-selected derivative J0161 Cd^s BC_1 (stippled bars), and strain SK2802 (horizontally dashed bars) and its BC-selected derivative SK2802 BC_1 (diagonally dashed bars). MICs were determined as described in Materials and Methods in two independent trials (no variation was seen).

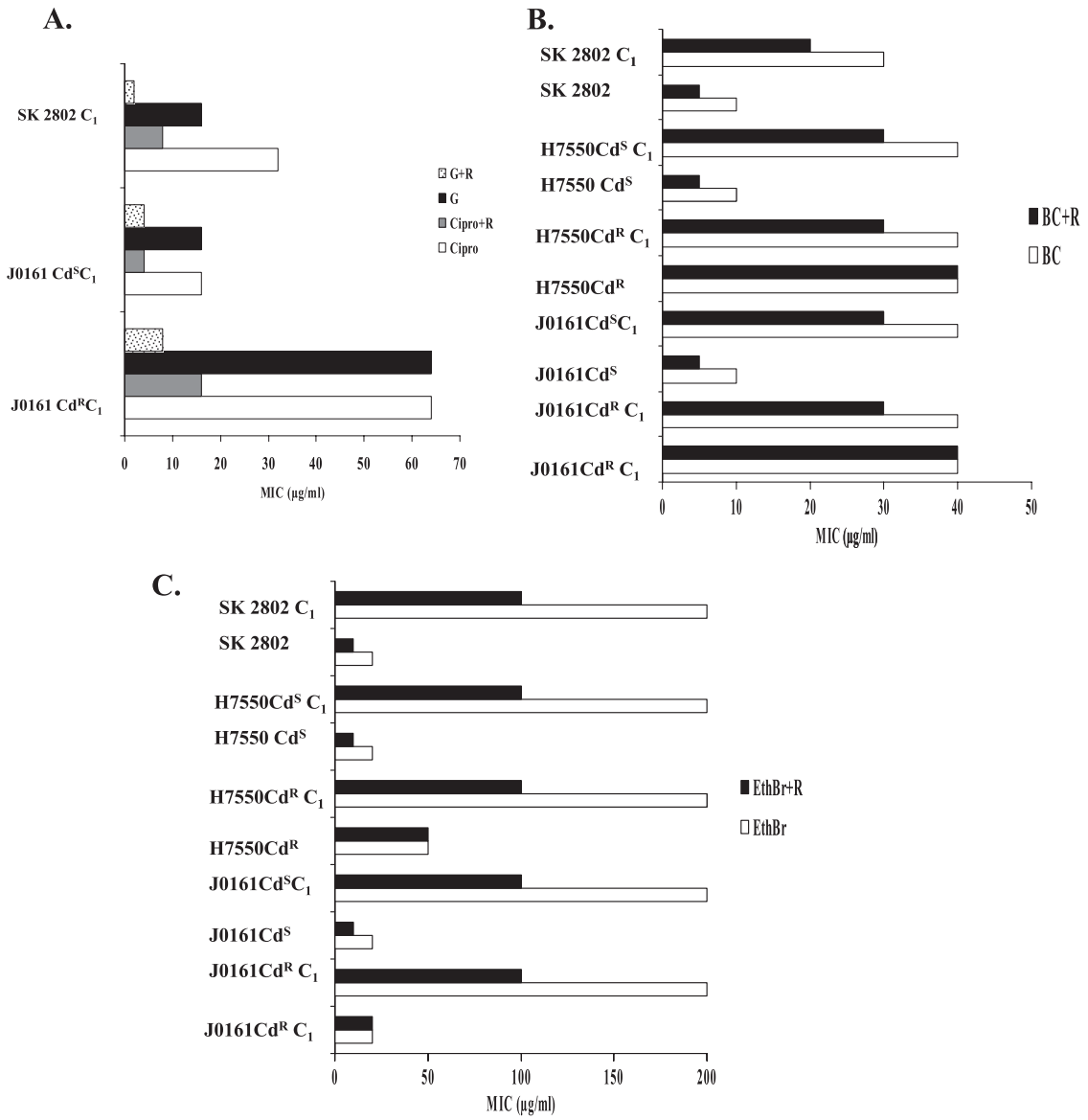

FIG. 3. Ciprofloxacin-selected derivatives of *L. monocytogenes* exhibit reduced MICs to ciprofloxacin, gentamicin, benzalkonium chloride, and ethidium bromide in the presence of the efflux inhibitor reserpine. (A) Susceptibility to ciprofloxacin (Cipro) and gentamicin (G) was determined in the presence and absence of reserpine (R). Strains are as indicated in Table 1 and in legends to Fig. 1 and Fig. 2. MICs for ciprofloxacin were determined in the absence (white bars) and presence (gray bars) of reserpine. MICs for gentamicin were also determined in the absence (black bars) and presence (stippled bars) of reserpine. (B) MICs for benzalkonium chloride (BC) in the absence (white bars) and presence (black bars) of reserpine (R). (C) MICs for ethidium bromide (EthBr) in the absence (white bars) and presence (black bars) of reserpine (R). MICs were determined as described in Materials and Methods in two independent trials (no variation was seen).

200 μg/ml for EthBr and from 100 to 400 μM for TPP) (data not shown). There was no impact on MICs to the other antibiotics in the panel or to Cd and sodium arsenite (data not shown).

Antimicrobial agent MICs of ciprofloxacin-selected and BC-selected strains of *L. monocytogenes* are reduced in the presence of the efflux pump inhibitor reserpine. Ciprofloxacin MICs for all ciprofloxacin-selected derivatives were reduced at least 4-fold in the presence of the efflux inhibitor reserpine; the extent of reduction was more pronounced in derivatives with high MICs (32 μg/ml or 64 μg/ml), for which 8-fold MIC reductions were observed in the presence of reserpine (Fig. 3A and data not shown). Gentamicin MICs also declined in the presence of reserpine (e.g., from 16 to 2 μg/ml in the case of SK2802 C_1) (Fig. 3A). Ciprofloxacin and gentamicin MICs of the wild-type parental strains were also decreased (from 2 to 1 μg/ml or 8 to 4 μg/ml) in the presence of reserpine (data not shown).

In the presence of reserpine, the ciprofloxacin-selected strains had increased susceptibility to BC (Fig. 3B), EthBr (Fig. 3C), and TPP (data not shown). MIC reductions for BC and EthBr were also noticed for the parental strains that were free of the plasmid, whereas no impact was noted in the plasmid-harboring strains H7550 Cd^r and J0161 Cd^r (Fig. 3B and C).

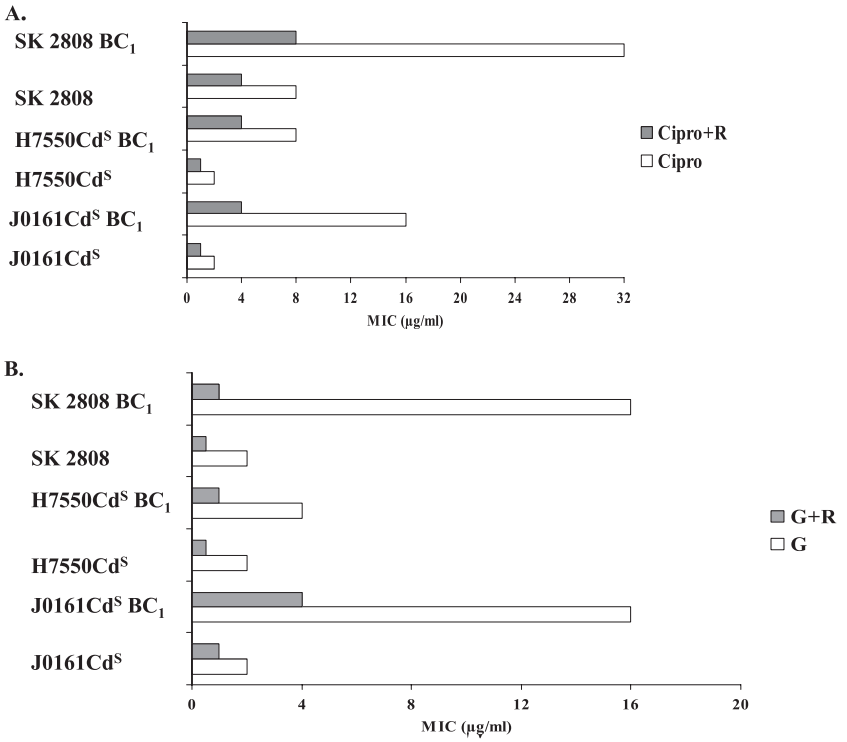

FIG. 4. BC-selected strains exhibit reduced MICs to ciprofloxacin and gentamicin in the presence of the efflux inhibitor reserpine. MICs of strains selected on BC were determined for ciprofloxacin (Cipro) (A) and gentamicin (G) (B). MICs were determined in the absence (white bars) and presence (gray bars) of reserpine (R). Strains are as indicated in Table 1 and in legends to Fig. 1 and Fig. 2. MICs were determined as described in Materials and Methods in two independent trials (no variation was seen).

Derivatives obtained following exposure to BC showed similar trends of reduction of MICs for ciprofloxacin, gentamicin, BC, EthBr, and TPP in the presence of reserpine (Fig. 4A and B and data not shown).

The disk diffusion method was employed to assess susceptibilities of the ciprofloxacin- or BC-selected strains to moxifloxacin, a fluoroquinolone that is not a substrate for drug efflux systems (6). None of the strains exhibited significant changes in their susceptibility to moxifloxacin, and MICs were also not significantly affected by reserpine (Table 4 and data not shown).

Expression of MDR transporter *lde* was enhanced in H7550 Cd^s BC$_1$, obtained following exposure to BC. Expression of *lde* was enhanced (5-fold) in BC-selected strain H7550 Cd^s BC$_1$ in comparison to the parental strain H7550 Cd^s (Fig. 5B and D). We failed to obtain evidence for expression of other chromosomal MFS transporters, including *mdrL*, *mdrM*, and *mdrT*, in either the wild-type parental strains or their BC-selected and

TABLE 4. Moxifloxacin susceptibility of *L. monocytogenes* strains selected on ciprofloxacin and BC

Strain	Inhibition zone diam (mm)[a]	
	Reserpine present	Reserpine absent
J0161 Cd^r	25	26
J0161 Cd^r C$_1$	25	26
J0161 Cd^s	26	27
J0161 Cd^s C$_1$	27	29
J0161 Cd^s BC$_1$	26	26
H7550 Cd^r	26	27
H7550 Cd^r C$_1$	25	26
H7550 Cd^s	25	27
H7550 Cd^s C$_1$	27	28
H7550 Cd^s BC$_1$	26	26

[a] Results are averages from two representative experiments, each done in duplicate.

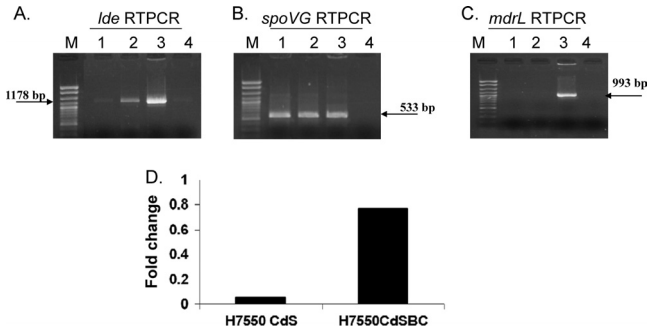

FIG. 5. BC-selected strain H7550 Cd^s BC$_1$ exhibits enhanced transcription of *lde* in comparison to that of the parental strain. (A to C) RT-PCR was employed to assess expression of *lde* (A); housekeeping gene *spoVG*, used as a control (B); and *mdrL* (C). Lanes: 1, H7550 Cd^s; 2, H7550 Cd^s BC$_1$; 3, H7550 Cd^s genomic DNA; 4, H7550 Cd^s RNA (to confirm absence of genomic DNA in the RNA used for RT-PCR); M, 100- to 2,686-bp DNA molecular marker XIV (Roche). Arrows indicate the sizes of the expected PCR products. (D) Expression of *lde* in BC-selected mutant H7550 Cd^s BC$_1$ (H7550CdSBC) in comparison to the parental strain H7550 Cd^s (H7550 CdS). RT-PCR and ImageJ analysis were done as described in Materials and Methods.

ciprofloxacin-selected derivatives (Fig. 5C and data not shown).

DISCUSSION

In this study, selection of several *L. monocytogenes* strains on either ciprofloxacin or BC led to derivatives with increased MICs not only to the agents employed in the selection but to several additional toxic compounds. The spectrum of compounds to which the derivatives exhibited reduced susceptibility was the same regardless of whether selection was on ciprofloxacin or on BC. This multidrug resistance phenotype suggested that these derivatives had increased expression of an MDR efflux system(s). In accord with this notion, the ciprofloxacin- and BC-selected strains exhibited higher MICs to EthBr and TPP, which are known to serve as substrates for MDR efflux systems (7). Moreover, at least 4-fold reductions in MICs were observed in the presence of the potent efflux inhibitor reserpine. In contrast, the strains did not have significant increases in MICs for the fluoroquinolone moxifloxacin, which does not serve as a substrate for efflux systems; our finding that moxifloxacin MICs were not impacted by reserpine and were not enhanced in strains selected on ciprofloxacin or BC was similar to earlier observations with ciprofloxacin-resistant clinical isolates (6). Taken together, these data suggest that MDR efflux system mutations are likely responsible for the phenotype of the strains in the current study. Enhanced efflux has also been implicated in multidrug resistance phenotypes of other bacteria (e.g., *Escherichia coli*, *Salmonella*, and *Pseudomonas*) following exposure to disinfectants, although other mechanisms (e.g., envelope changes) have also been implicated (1, 9, 12, 16, 30, 33).

In previous studies, mutants of *L. monocytogenes* strains selected on BC had phenotypes partially overlapping with those of the BC- or ciprofloxacin-selected strains in our study. Such mutants had reduced susceptibility to BC, EthBr, and gentamicin and, in several cases, kanamycin, but changes in ciprofloxacin MICs were not reported (31, 34). Additionally, these mutants exhibited increased transcription of *mdrL* (but not *lde*), and their phenotype was attributed to increased levels of the *mdrL* transporter (31). Inactivation of *mdrL* in *L. monocytogenes* LO28 resulted in increased susceptibility to macrolides, cefotaxime, and certain metals (zinc, cobalt, and chromium) (18). The different resistance phenotype of our strains, which did not have increased MICs to erythromycin (a macrolide) or kanamycin, suggests that *mdrL* is likely not involved in the multidrug resistance profile that we observed. Further support for this is provided by the fact that we failed to detect increased expression of *mdrL* in either the ciprofloxacin-selected or the BC-selected derivatives. One cannot exclude the possibility that different transporters will contribute to the multidrug resistance phenotype of BC-selected mutants in different studies, depending on strain background and experimental conditions employed for the selection.

Derivatives of *L. monocytogenes* selected on ciprofloxacin have not been described before. However, clinical isolates with enhanced resistance to ciprofloxacin have been identified as mentioned above and were found to also have increased resistance to EthBr and acridine orange, as well as increased expression of the *lde* transporter (6). Insertional inactivation of *lde* resulted in reduced MICs to ciprofloxacin, EthBr, and acridine orange, suggesting that this transporter mediated the observed resistance phenotype. However, susceptibility of these clinical isolates or the *lde* mutant to gentamicin or BC was not described (6). Our data showing increased expression of *lde* in the mutants also provide tentative evidence for involvement of this transporter, although involvement of additional transporters cannot be excluded. In addition to *mdrL* and *lde*, the *Listeria* genome harbors several other genes for putative MDR transporters (5, 24), some of which may contribute to the phenotype of mutants described here. Two new transporters of the major facilitator superfamily, *mdrT* and *mdrM*, were identified during a screen for genes impacting the elicitation of the innate immune response in macrophages infected with *L. monocytogenes* 10403S, and transcript levels of *mdrM* were markedly enhanced in the presence of rhodamine 6G and TPP (2). However, we failed to obtain evidence of increased expression of either *mdrT* or *mdrM* in the ciprofloxacin- or BC-selected strains, suggesting that these transporters are likely not involved in the phenotype of the strains. Further studies (e.g., comparative analysis of transcriptome profiles) would be valuable in the effort to identify other transporters that may be expressed at higher levels in the mutants than in the parental strains and to characterize the impact of exposure to antibiotics, disinfectant, or other toxic compounds such as TPP on expression of the genes.

H7550 Cd^r and J0161 Cd^r harbor large and closely related plasmids (15, 24). Even though MICs to ciprofloxacin and gentamicin did not differ between the parental and plasmid-cured strains, ciprofloxacin-selected derivatives had higher MICs for either ciprofloxacin or gentamicin than did those derived from the plasmid-cured counterparts of either strain. These findings were consistently obtained and were specific to MICs for these antibiotics; they were not observed in MICs to EthBr or TPP. Further studies are needed to determine whether the apparent impact of the plasmid is specific to pLM80 and closely related plasmids and to clarify the underlying mechanism. It is possible that a pLM80-associated efflux system for ciprofloxacin and gentamicin (but not for EthBr or TPP) was activated in pLM80-harboring strains selected on BC or ciprofloxacin, along with chromosomal transporters such as *lde*. Comparative transcriptome analyses would be valuable in assessing the validity of this hypothesis.

As has been discussed for other pathogens (9, 16), derivatives such as described in the current study have a number of clinical or environmental implications. Exposure to ciprofloxacin (e.g., during empirical treatment or in the course of treatment for other infections) may lead to *L. monocytogenes* strains with decreased susceptibility to gentamicin, currently one of the drugs of choice for treatment of listeriosis. Such strains may have an enhanced ability to persist in the environment, as they also exhibit enhanced tolerance to disinfectants such as BC. Similarly, BC and other quaternary ammonium disinfectants are extensively used in health care settings as well as in the food processing industry. Strains selected on BC can have enhanced resistance not only to BC but to a range of other antimicrobial compounds, including antibiotics such as ciprofloxacin and gentamicin.

Lastly, it is conceivable that in such strains additional phenotypes of public health and food safety relevance may be

impacted. There is increasing evidence that MDR transporters in bacterial pathogens can mediate not only resistance to antimicrobials but a number of other processes as well (7, 17, 27). In the case of *L. monocytogenes*, the study by Crimmins et al. (2) has provided clear evidence that MDR transporter systems may be involved in processes of fundamental importance to host-pathogen interactions, such as elicitation of the innate immune response. Furthermore, *L. monocytogenes* virulence genes *prfA* and *inlA* were shown to be upregulated in response to sublethal concentrations of quaternary ammonium compounds (13). It will be important to determine whether BC- or ciprofloxacin-selected strains are also impacted in their virulence in cell culture or animal models and whether attributes such as ability to persist in biofilms and persist in the environment may also be altered in such strains.

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