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Coselection of Cadmium and Benzalkonium Chloride Resistance in Conjugative Transfers from Nonpathogenic *Listeria* spp. to Other Listeriae

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Resistance to the quaternary ammonium disinfectant benzalkonium chloride (BC) may be an important contributor to the ability of *Listeria* spp. to persist in the processing plant environment. Although a plasmid-borne disinfectant resistance cassette (*bcrABC*) has been identified in *Listeria monocytogenes*, horizontal transfer of these genes has not been characterized. Nonpathogenic *Listeria* spp. such as *L. innocua* and *L. welshimeri* are more common than *L. monocytogenes* in food processing environments and may contribute to the dissemination of disinfectant resistance genes in listeriae, including *L. monocytogenes*. In this study, we investigated conjugative transfer of resistance to BC and to cadmium from nonpathogenic *Listeria* spp. to other nonpathogenic listeriae, as well as to *L. monocytogenes*. BC-resistant *L. welshimeri* and *L. innocua* harboring *bcrABC*, along with the cadmium resistance determinant *cadA2*, were able to transfer resistance to other nonpathogenic listeriae as well as to *L. monocytogenes* of diverse serotypes, including strains from the 2011 cantaloupe outbreak. Transfer among nonpathogenic *Listeria* spp. was noticeably higher at 25°C than at 37°C, whereas acquisition of resistance by *L. monocytogenes* was equally efficient at 25 and 37°C. When the nonpathogenic donors were resistant to both BC and cadmium, acquisition of cadmium resistance was an effective surrogate for transfer of resistance to BC, suggesting coselection between these resistance attributes. The results suggest that nonpathogenic *Listeria* spp. may behave as reservoirs for disinfectant and heavy metal resistance genes for other listeriae, including the pathogenic species *L. monocytogenes*.

Listeria spp. are Gram-positive bacteria commonly found in the environment. The single human pathogen in this genus, *Listeria monocytogenes*, continues to be associated with significant disease burden due to its high morbidity and mortality toward vulnerable populations such as the elderly, pregnant women and their fetuses, neonates, and immunocompromised patients (10, 21, 34, 46). *L. monocytogenes* remains largely susceptible to antibiotics, with a common course for treatment being a combination of ampicillin and gentamicin (20). However, reported cases of listeriosis involving strains with multidrug resistance (6, 17, 38, 40, 52) suggest the potential for enhanced resistance through horizontal gene transfer with accompanying increases in public health concerns associated with this pathogen.

Colonization of the processing plant environment by *Listeria* spp. is a major contributor to contamination of processed, ready-to-eat foods (23, 53). Exposure to disinfectants commonly used in food processing plants, such as the quaternary ammonium compound benzalkonium chloride (BC), may provide selective pressures for disinfectant resistance with an accompanying increase in fitness within the processing plant environment. Several studies have investigated prevalence and mechanisms of BC resistance in *L. monocytogenes* (12, 31, 43, 50). However, less is known about the behavior and response of nonpathogenic *Listeria* spp. to selection pressures in food processing plants and other environments.

The processing plant environment may present many opportunities for nonpathogenic *Listeria* spp. to interact with *L. monocytogenes*. Nonpathogenic species such as *L. innocua* and *L. welshimeri* have been found to be more common than *L. monocytogenes* in food processing environments and in foods and to grow faster than *L. monocytogenes* in foods and other media (2, 8, 19, 30, 36, 47, 49, 54). These *Listeria* spp. are also more likely than *L. monocytogenes* to exhibit antibiotic resistance and to harbor plasmids (1, 6, 9, 13, 14, 35, 42, 44). Such findings suggest the potential for horizontal gene transfer among listeriae, with nonpathogenic strains serving as reservoirs for resistance determinants, potentially altering the fitness of the pathogenic species, *L. monocytogenes*.

In *L. monocytogenes* the efflux system encoded by *bcrABC* confers high-level resistance to BC and other quaternary ammonium compounds (12). The *bcrABC* cassette was first identified on a large plasmid (pLM80) harbored by strains implicated in the 1998–1999 hotdog outbreak of listeriosis. The cassette on these plasmids appears to be part of a composite transposon that also includes genes conferring resistance to cadmium (12, 24, 33).

Further evidence for the association between BC resistance and resistance to cadmium was obtained from characterization of *L. monocytogenes* from turkey processing plants. All BC-resistant strains were found to be also resistant to cadmium, leading to the speculation that BC resistance determinants (e.g., *bcrABC*) were acquired by plasmids that already harbored cadmium resistance determinants (31, 32).

Such findings suggest the need to characterize horizontal

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TABLE 1 Bacterial strains used in this study

Strain	Resistance[a]	Genotype[b]			Source or reference
		cadA1	*cadA2*	*bcrABC*	
Donor strains					
L. innocua					
L0910	Cd^r	–	–	–	This study
L1214B	Cd^r	+	–	–	This study
L1306A	Cd^r	+	–	–	This study
CLIP 11262	Cd^r	–	+	–	16
L1221	Cd^r BC^r	–	+	+	This study
L1333a	Cd^r	–	+	–	This study
L0921	Cd^r	+	–	–	This study
L. welshimeri					
L0725	Cd^r BC^r	–	+	+	This study
L1325	Cd^r BC^r	–	+	+	This study
L0918	Cd^r BC^r	–	+	+	This study
L0926	Cd^r BC^r	–	+	+	This study
Recipient strains					
L. innocua					
L1206S	Str^r	–	–	–	This study
L. welshimeri					
L1316S	Str^r	–	–	–	This study
L0927S	Str^r	–	–	–	This study
L. monocytogenes (serotype)					
1/2a3 (1/2a)	Str^r	–	–	–	22
10403S (1/2a)	Str^r	–	–	–	37
2381L (4b)	Str^r	–	–	–	39
2857S (1/2a)	Str^r	–	–	–	This study
2858S (1/2b)	Str^r	–	–	–	This study

[a] Cd^r, BC^r, and Str^r indicate resistance to cadmium, BC, and streptomycin, respectively, determined as described in Materials and Methods.
[b] Determined by PCR with the respective primers as described in Materials and Methods.

transfer of *bcrABC* in *Listeria* spp., including transfer from nonpathogenic species that may act as reservoirs. However, efforts to assess the conjugative transfer of BC resistance in *Listeria* spp. can be thwarted by the frequent occurrence of spontaneous mutants exhibiting high-level resistance to BC (41, 51; M. Rakic-Martinez and S. Katharios, unpublished data). On the other hand, spontaneous mutants with high levels of resistance to cadmium appear to be extremely rare (S. Katharios-Lanwermeyer and M. Rakic-Martinez, unpublished data). Therefore, we hypothesized that BC-resistant, cadmium-resistant strains of *L. innocua* and *L. welshimeri* harbored the corresponding resistance determinants on plasmids (similarly to *L. monocytogenes* strains with pLM80 and related plasmids, described above), and we used cadmium resistance transfer as a surrogate for assessments of conjugative transfer of BC resistance. We examined the transfer of such resistance among *L. innocua* and *L. welshimeri*, as well as from these nonpathogenic species to *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Listeria* spp. strains used in the present study are listed in Table 1. Five BC-resistant strains of nonpathogenic *Listeria* spp. (four strains of *L. welshimeri* and *L. innocua* L1221) were used as potential donors of BC resistance to other nonpathogenic listeriae (*L. welshimeri* L1316S, *L. welshimeri* L0927S, and *L. innocua* L1206S). All BC-resistant donor strains were also resistant to cadmium (Table 1). To further assess conjugative transfer of resistance we used as potential donors of cadmium resistance six *L. innocua* strains resistant to cadmium but susceptible to BC (Table 1). With the exception of the reference strain *L. innocua* CLIP 11262 (16), the nonpathogenic *Listeria* spp. strains were isolated from the environment of turkey processing plants in the United States between 2003 and 2005. Isolation and characterization of *Listeria* spp. from these processing plants will be described elsewhere.

Nonpathogenic *Listeria* spp. used as recipients were streptomycin-resistant derivatives of *L. welshimeri* and *L. innocua* (Table 1). Spontaneous mutants with resistance to streptomycin (MIC > 600 μg/ml) were isolated on brain heart infusion agar (BHIA) plates (BHI broth [BHI; Becton Dickinson, Sparks, MD] plus 1.2% Bacto agar [Becton Dickinson]) with streptomycin sulfate (600 μg/ml; Sigma, St. Louis, MO). In addition, five *L. monocytogenes* strains of diverse serotypes were used as potential recipients. The strains included the serotype 1/2a laboratory reference strains 10403S and 1/2a3 (22, 25, 37), serotype 4b strain 2381L, a streptomycin-resistant derivative of a strain from the 1985 California outbreak (39), and strains 2857S and 2858S of serotypes 1/2a and 1/2b, respectively, streptomycin-resistant derivatives of two strains from the 2011 cantaloupe outbreak (5) (Table 1). These streptomycin-resistant derivatives (streptomycin MIC > 600 μg/ml) were obtained as described above. Bacteria were grown in BHI or on BHIA and preserved at −80°C as described previously (12).

Cadmium and BC susceptibility and determinations of MIC. BC and cadmium susceptibility assessments were performed as described previously (31). Strains were considered resistant to cadmium when they yielded confluent growth in the presence of 35 μg of cadmium chloride anhydrous (Sigma)/ml after incubation at 37°C for 48 h. For cadmium MIC determinations, 10 μl of an overnight culture was spotted onto BHIA plates with variable concentrations of cadmium chloride (2.5, 5, 10, 20, 35, 70, 140, and 200 μg/ml), and the plates were incubated at 25°C and observed daily for 5 days. The MIC was defined as the lowest concentration of cadmium that prevented visible growth. MIC of BC (Acros, New

Jersey) was determined as described previously (31) using variable concentrations of BC (0.1, 0.5, 2.5, 5, 10, 20, 35, and 40 μg/ml) and after incubation of the plates at 37°C for 48 h.

Conjugations. Cultures of recipient and donor strains were grown overnight (18 h) at 37°C and mixed in a 1:10 donor/recipient ratio for conjugations. Filter matings were done as described previously (22). Briefly, the mixture (100 μl of donor and 900 μl of recipient) was centrifuged (6,000 rpm, 3 min) and resuspended in 100 μl of BHI, which was then spotted onto sterile membrane filters (0.45-μm pore size; Millipore Corp., Bedford, MA), followed by incubation at the indicated temperature for 24 h. For agar matings, after centrifugation and resuspension in BHI (100 μl), the mixture was spotted (50 μl) onto BHIA and incubated at the indicated temperature for 24 h. To isolate transconjugants, mating mixtures were rinsed off the membrane filter or, for agar matings, removed from the surfaces of the agar plates with a sterile glass rod and plated on double-selective medium (BHIA with 600 μg of streptomycin/ml and 35 μg of cadmium chloride/ml), incubated at 25°C, and observed for up to 96 h. Controls included each of the parental strains plated on the double-selective medium and incubated similarly. The conjugation frequency was determined as the ratio of the number of transconjugants over CFU of the recipient strain at the end of the conjugation period. CFU were determined by plating dilutions on BHIA with streptomycin (600 μg/ml) and incubation at 37°C for 36 h. Experiments were performed in duplicate and in at least three independent trials.

PCR. Primers used for PCR are listed in Table S1 in the supplemental material. Identification of the three different *cadA* determinants used the primers cadA-Tn5422F and cadA-Tn5422R for *cadA1*, associated with Tn*5422*, the primers cadA-pLM80F and cadA-pLM80R for *cadA2*, harbored on pLM80, and the primers cadA-EGDeF and cadA-EGDeR for *cadA3*, harbored by EGDe (32). Primers BcF and BcR were used to produce a PCR fragment containing the entire *bcrABC* cassette along with the ~800-nucleotide (nt) upstream intergenic region (12). PCR for *hly*, encoding the *L. monocytogenes* virulence determinant listeriolysin O, used the primers hlyAF and hlyAR (15) (see Table S1 in the supplemental material). *L. welshimeri* was differentiated from *L. innocua* using the primers Lw_0908_F and Lw_0908_R (*L. welshimeri*-specific), as well as the primers Li_0558_F and Li_0558_R (*L. innocua* specific) (Table S1), derived from genome sequences specific to the corresponding species (16, 18). In addition to *cadA2* and *bcrABC* (harbored on pLM80), we used a panel of several additional primer pairs to screen for other pLM80 open reading frames (ORFs) representing diverse locations on both fragments of the plasmid (see Table S1 in the supplemental material) (33). PCR was performed as previously described (12, 31) using an ExTaq kit (TaKaRa, Madison, WI).

RESULTS

PCR analysis of the strains used as donors revealed that all BC-resistant strains, regardless of species (*L. welshimeri* or *L. innocua*) harbored *bcrABC* as well as the plasmid-associated cadmium resistance determinant *cadA2*. The *cadA2* determinant was also harbored by *L. innocua* 1333a and *L. innocua* CLIP 11262, which were resistant to cadmium but susceptible to BC and lacking *bcrABC*. Three strains of *L. innocua* harbored an alternative plasmid-associated cadmium resistance determinant, *cadA1*, and one cadmium-resistant strain (*L. innocua* L0910) was found to be negative for both *cadA1* and *cadA2* (Fig. 1 and Table 1). None of the strains harbored the chromosomal cadmium resistance determinant *cadA3* (data not shown). The strains used as recipients (*L. welshimeri* L1316S, *L. welshimeri* L0927S, and *L. innocua* L1206S) were susceptible to BC and cadmium and lacked *bcrABC*, *cadA1*, *cadA2*, or *cadA3* (Table 1).

***L. welshimeri* and *L. innocua* harboring *cadA2* can readily transfer cadmium resistance to other nonpathogenic listeriae.** Results from matings performed at 25°C on filter membranes and on agar were comparable, but the frequency of transfer was generally higher and more consistent for the latter (data not shown). The transfer frequency averaged between 10^{-7} and 10^{-8} (Table 2). Selected putative *L. welshimeri* transconjugants obtained using *L. innocua* strains as donors were evaluated with PCR using species-specific primers. The putative transconjugants were positive with the *L. welshimeri*-specific primers but negative for those specific for *L. innocua* (Fig. 2 and data not shown), confirming that they were derivatives of the recipient strain and not spontaneous streptomycin-resistant mutants of the donor.

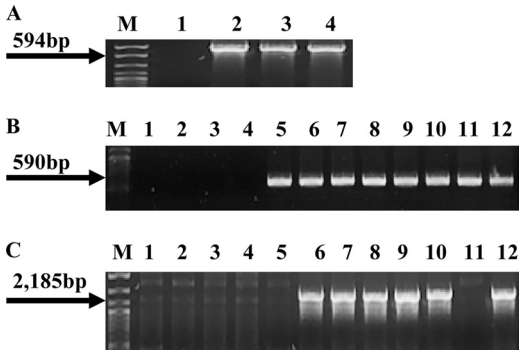

FIG 1 Presence of *cadA1*, *cadA2*, and *bcrABC* in nonpathogenic donor strains. (A) PCR-based detection of *cadA1* using the primers cadA-Tn5422F and cadA-Tn5422R. Lanes 1, 2, 3, and 4, *L. innocua* strains L0910, L0921, L1214B, and L1306A, respectively; lane M, DNA molecular marker XIV (Roche, Indianapolis, IN). The arrow points to the expected *cadA1* PCR product. (B) PCR-based detection of *cadA2* using the primers cadA-pLM80F and cadA-pLM80R. Lanes 1, 2, 3, and 4, *L. innocua* strains L0910, L0921, L1214B, and L1306A, respectively; lanes 5, 10, and 11, *L. innocua* strains CLIP 11262, L1221, and L1333a, respectively; lanes 6, 7, 8, and 9, *L. welshimeri* strains L0725, L1325, L0918, and L0926, respectively. Lane 12, *L. monocytogenes* H7550, used as a positive control (12). Lane M, same as in panel A. Arrow points to the expected *cadA2* PCR product. (C) PCR of *bcrABC* using primers BcF and BcR. Lanes are as defined in panel B. With the exception of *L. innocua* strains CLIP 11262 and L1333a in lanes 5 and 11, respectively, all donors harboring *cadA2* also contained *bcrABC*. Lane M, same as in panel A. Arrow points to the expected *bcrABC* product.

The efficiency of conjugative transfer depended markedly on the strains used as donors as well as recipients. Few or no transconjugants were obtained from certain *L. innocua* donors (*L. innocua* strains L0910, L1214B, L1306A, and L0921), regardless of the strain that was used as a potential recipient. Furthermore, one of the strains used as recipient, *L. innocua* L1206S, failed to yield transconjugants with any of the donors (Table 2).

Based on PCR analysis of the resistance determinants, all donor strains that could efficiently transfer resistance harbored *cadA2*. With the exception of *L. innocua* strains 1333a and CLIP 11262, these strains were also resistant to BC and harbored *bcrABC* (Table 1). In contrast, of the four other potential donors that failed to yield transconjugants three harbored *cadA1*, and one (*L. innocua* L0910) lacked *cadA1* or *cadA2* (Table 1).

The BC resistance determinant *bcrABC* is cotransferred with the cadmium resistance determinant *cadA2*. As mentioned above, most (five of seven) of the *cadA2*-harboring cadmium-resistant strains used as donors also harbored the BC resistance cassette *bcrABC*. PCR analysis of selected transconjugants obtained using these strains as donors revealed that, in addition to *cadA2*, they also harbored *bcrABC* (Fig. 3A and C). The transconjugants exhibited elevated MICs not only for cadmium (200 μg/ml) but also for BC (35 to 40 μg/ml), similar to the cadmium and

TABLE 2 Transfer frequency of cadmium resistance in conjugations between indicated donor and recipient strains[a]

	Transfer frequency (recipient)							
	L. welshimeri		*L. innocua*	*L. monocytogenes*				
Donor	L0927S	L1316S	L1206	1/2a3	2381L	10403S	2857S	2858S
L. welshimeri								
L0725	5.8×10^{-7}	1.4×10^{-7}	$<1.0 \times 10^{-9}$	1.8×10^{-7}	2.8×10^{-8}	1.8×10^{-8}	6.7×10^{-7}	1.6×10^{-7}
L1325	3.7×10^{-6}	5.6×10^{-7}	$<1.0 \times 10^{-9}$	1.5×10^{-7}	2.0×10^{-8}	1.1×10^{-8}	6.7×10^{-7}	2.1×10^{-7}
L0918	2.6×10^{-6}	1.5×10^{-7}	$<1.0 \times 10^{-9}$	1.5×10^{-7}	1.4×10^{-8}	7.4×10^{-9}	5.5×10^{-8}	6.1×10^{-8}
L0926	3.3×10^{-7}	1.6×10^{-7}	$<1.0 \times 10^{-9}$	1.3×10^{-7}	5.5×10^{-8}	1.9×10^{-8}	4.0×10^{-7}	1.5×10^{-7}
L. innocua								
L0910	$<1.0 \times 10^{-9}$	$<1.0 \times 10^{-9}$	$<1.0 \times 10^{-9}$	$<2.8 \times 10^{-9}$	$<3.5 \times 10^{-9}$	$<2.4 \times 10^{-9}$	$<2.3 \times 10^{-9}$	$<2.4 \times 10^{-9}$
L1214B	$<1.0 \times 10^{-9}$	$<1.0 \times 10^{-9}$	$<1.0 \times 10^{-9}$	$<3.3 \times 10^{-9}$	$<4.6 \times 10^{-9}$	$<2.0 \times 10^{-9}$	$<1.8 \times 10^{-9}$	$<2.3 \times 10^{-9}$
L0921	$<1.0 \times 10^{-9}$	$<1.0 \times 10^{-9}$	$<1.0 \times 10^{-9}$	$<3.0 \times 10^{-9}$	$<3.7 \times 10^{-9}$	$<2.8 \times 10^{-9}$	$<1.8 \times 10^{-9}$	$<16 \times 10^{-9}$
L1306A	$<1.0 \times 10^{-9}$	$<1.0 \times 10^{-9}$	$<1.0 \times 10^{-9}$	$<4.6 \times 10^{-9}$	$<5.4 \times 10^{-9}$	$<3.1 \times 10^{-9}$	$<2.6 \times 10^{-9}$	$<3.5 \times 10^{-9}$
CLIP 11262	$<4.9 \times 10^{-10}$	$<2.3 \times 10^{-10}$	$<4.9 \times 10^{-10}$	5.1×10^{-9}	7.9×10^{-9}	5.6×10^{-9}	9.7×10^{-9}	4.1×10^{-8}
L1221	6.1×10^{-8}	2.7×10^{-7}	$<4.3 \times 10^{-9}$	1.5×10^{-7}	8.2×10^{-8}	1.1×10^{-8}	3.4×10^{-8}	6.6×10^{-9}
L1333a	1.3×10^{-8}	2.8×10^{-8}	$<4.1 \times 10^{-9}$	6.7×10^{-8}	1.3×10^{-8}	1.6×10^{-7}	3.8×10^{-7}	3.6×10^{-7}

[a] Results are from one representative experiment, and all experiments were performed in at least three independent trials.

BC MICs for the donor strains. In contrast, for strains used as recipients the MICs for both cadmium and BC were 10 μg/ml.

Nonpathogenic *Listeria* spp. can effectively mediate the conjugative cotransfer of BC and cadmium resistance to *L. monocytogenes*. To assess the ability of nonpathogenic *Listeria* spp. to serve as donors of BC resistance to *L. monocytogenes*, we used as potential recipients five *L. monocytogenes* strains of the three serotypes predominant in human listeriosis (1/2a, 1/2b, and 4b) (Table 1). All *L. monocytogenes* strains used as potential recipients were susceptible to cadmium and BC (MICs of 10 μg/ml for both compounds) (Table 1).

Regardless of the serotype of the *L. monocytogenes* strains used as recipients, conjugative transfer of resistance from nonpathogenic listeriae to *L. monocytogenes* generally exhibited the same dependence on donor strains that was observed with conjugations among nonpathogenic listeriae. Generally, the *L. innocua* strains that failed to donate cadmium resistance to other nonpathogenic listeriae also failed to transfer such resistance to *L. monocytogenes* (Table 2). On the other hand, the nonpathogenic *Listeria* spp. donor strains that efficiently transferred BC and cadmium resistance to other nonpathogenic listeriae also transferred these resistance determinants to *L. monocytogenes*. When these strains were used as donors, transconjugants were readily obtained from all *L. monocytogenes* strains in the panel, including the two strains associated with the recent cantaloupe outbreak (Table 2).

Acquisition of *cadA2* by *L. monocytogenes* was confirmed by PCR (Fig. 3A and B and data not shown). Furthermore, when the donors also harbored *bcrABC*, this cassette was detected in the cadmium-resistant transconjugants as well (Fig. 3C and data not shown). Transconjugants were also tested for hemolytic activity on blood agar plates and by PCR with primers specific for *hly*, encoding the *L. monocytogenes* virulence determinant listeriolysin O and absent from *L. innocua* or *L. welshimeri*. All tested transconjugants were hemolytic and produced the *hly* amplicon (Fig. 3D and data not shown), confirming that they were derived from the *L. monocytogenes* recipients and were not spontaneous streptomycin-resistant derivatives of the donors. As noted above with transconjugants from conjugations between nonpathogenic *Listeria* spp., the *L. monocytogenes* transconjugants exhibited elevated MICs for cadmium and BC (200 μg/ml and 35 to 40 μg/ml, respectively), similar to those of the donor strains.

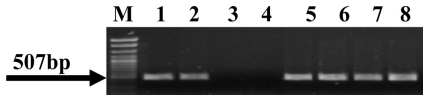

FIG 2 Identification of *L. welshimeri* transconjugants from conjugations between *L. welshimeri* as recipient and *L. innocua* as donor. PCR used the *L. welshimeri*-specific primers Lw_0908_F and Lw_0908_R. Lanes 1 and 2, *L. welshimeri* strains L0725 and L1316S, respectively; lanes 3 and 4, *L. innocua* strains L1221 and L1333a, respectively; lanes 5, 6, 7, and 8, transconjugants from conjugations of *L. welshimeri* L1316S as the recipient with the donors *L. innocua* 1221 (lanes 5 and 6) and *L. innocua* 1333a (lanes 7 and 8). Arrow points to the expected PCR product.

Temperature affects the transfer of BC and cadmium resistance to nonpathogenic listeriae, while the transfer to *L. monocytogenes* is equally efficient at 25 and 37°C. Nonpathogenic *Listeria* spp. donor-recipient combinations that failed to yield transconjugants at 25°C were similarly negative at 37°C (data not shown). However, for successful conjugations the frequency of transfer of BC and cadmium resistance among nonpathogenic listeriae was higher when conjugations were done at 25 than at 37°C. The impact of temperature was dependent on the recipient strain, being noticeably stronger for *L. welshimeri* L0927S than for *L. welshimeri* L1316S (Fig. 4). Interestingly, the efficiency of the transfer of resistance from these same donors to *L. monocytogenes* was not affected by temperature. Transfer was equally efficient at 25 and 37°C for all *L. monocytogenes* strains in the panel, regardless of serotype (data not shown).

Evidence of pLM80-like plasmids in BC- and cadmium-resistant nonpathogenic donors. To determine whether the *L. innocua* and *L. welshimeri* strains used as donors harbored plasmids similar to pLM80, these strains were tested by PCR with primers derived from a panel of ORFs at different sites of pLM80 (see Table S1 in the supplemental material). The findings suggested that strains positive for both *bcrABC* and *cadA2* (*L. innocua* 1221 and all four *L. welshimeri* donors) harbored plasmids highly similar to pLM80, with all primer pairs in the panel yielding the expected amplicons. In contrast, strains harboring *cadA2* but lacking *bcrABC* (*L. innocua* strains 1333a and CLIP

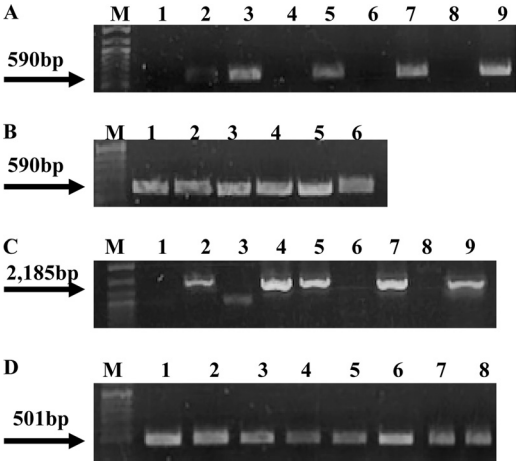

FIG 3 Presence of *cadA2* and *bcrABC* in *L. welshimeri* and *L. monocytogenes* transconjugants. (A) PCR-based detection of *cadA2* using the primers cadA-pLM80F and cadA-pLM80R. Lanes 1, 4, 6, and 8, strains used as recipients (*L. welshimeri* strains L0927S and L1316S [lanes 1 and 4, respectively] and *L. monocytogenes* 1/2a3 and *L. innocua* L1206 [lanes 6 and 8, respectively]); lanes 2, 3, and 5, transconjugants from conjugations between *L. welshimeri* L0927S and *L. innocua* L1221 (lane 2), *L. welshimeri* L1316S and *L. welshimeri* L0918 (lane 3), L1316S with *L. innocua* L1221 (lane 5); lane 7, transconjugant from conjugation between *L. monocytogenes* 1/2a3 (recipient) and *L. welshimeri* L1325 (donor). Lane 9, *L. monocytogenes* H7550, used as a positive control (12). Lane M, DNA molecular marker XIV (Roche). The arrow points to the expected *cadA2* PCR product. (B) PCR-based detection of *cadA2* in transconjugants from conjugations between *L. monocytogenes* 2381L (recipient) and *L. welshimeri* L1325 (donor). Lane M, same as in panel A. The arrow points to the expected *cadA2* PCR product. (C) PCR-based detection of *bcrABC* using BcF and BcR primers. Lanes 1, 3, 6, and 8, strains used as recipients (*L. welshimeri* strains L0927S and L1316S [lanes 1 and 3, respectively, with unspecific PCR product in lane 3] and *L. monocytogenes* strains 1/2a3 and *L. innocua* L1206 [lanes 6 and 8, respectively]); lanes 2, 4, and 5, transconjugants from conjugations between *L. welshimeri* L1316S (recipient) and *L. welshimeri* L1325 (donor); lane 7, transconjugant from conjugation between *L. monocytogenes* 1/2a3 (recipient) and *L. welshimeri* L1325 (donor); lane 9, *L. monocytogenes* H7550, used as a positive control (12). Lane M, same as in panel A. The arrow points to the expected *bcrABC* PCR product. Weak band in lane 3 is unspecific PCR product. (D) PCR-based detection of *hly* in *L. monocytogenes* transconjugants using the primers hlyAF and hlyAR. Lanes 1 and 2, transconjugants from conjugations between *L. monocytogenes* 1/2a3 with *L. innocua* L1221 (lane 1) and *L. innocua* L1333a (lane 2); lanes 3 to 7, transconjugants from conjugations between *L. monocytogenes* 2381L with *L. welshimeri strains* L0725 (lane 3), L1325 (lane 4), and *L0926* (lane 5) and with *L. innocua* strains L1221 (lane 6) and L1333a (lane 7); lane 8 is *L. monocytogenes* 2381L. Lane M, same as in panel A. The arrow points to the expected *hly* PCR product.

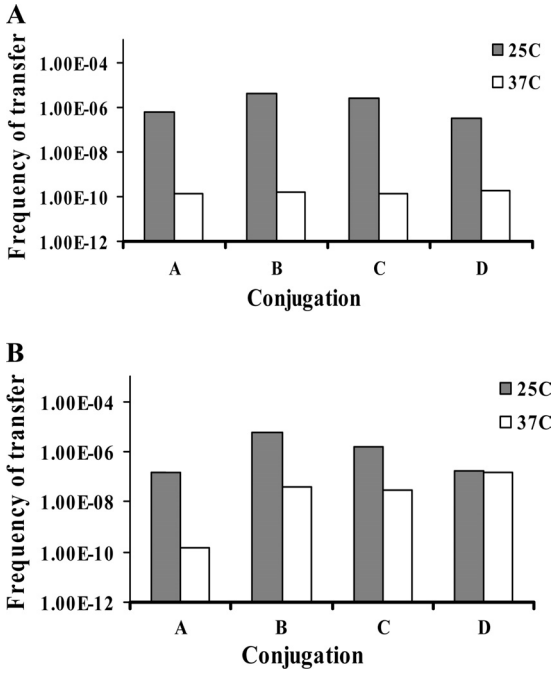

FIG 4 Impact of conjugation temperature (25°C versus 37°C) on the frequency of transfer of cadmium resistance in conjugations among nonpathogenic *Listeria* spp. (A) A, B, C, and D, transfer frequency from conjugations between *L. welshimeri* L0927S as recipient and *L. welshimeri* strains L0725, L1325, L0918, and L0926, respectively, as donors. (B) A, B, C, and D, transfer frequency from conjugations between *L. welshimeri* L1316S as recipient and *L. welshimeri* strains L0725, L1325, L0918, and L0926, respectively, as donors. Conjugations were done on agar plates at 25°C (gray bars) or 37°C (white bars), and the transfer frequency was determined as described in Materials and Methods. The results are from one representative experiment, and experiments were performed in at least three independent trials.

11262) failed to produce several of the expected amplicons (Fig. 5). Strains harboring *cadA1* yielded amplicons with only three primer pairs in the panel, two of which were derived from plasmid replication-associated genes, whereas no amplicons were obtained using *L. innocua* L0910 (cadmium-resistant but lacking *cadA1* or *cadA2*) (Fig. 5). It is noteworthy that all donors consistently involved in high-efficiency transfer (*L. welshimeri* L0725, L1225, L0918, and L0926 and *L. innocua* L1333a and L1221) harbored LMOh7858_pLM80_0022, encoding a putative TraG/TraD family protein that may be involved in facilitating plasmid transfer (Fig. 5).

DISCUSSION

In this study we provide evidence that BC resistance mediated by *bcrABC* can be effectively transferred among certain strains of nonpathogenic *Listeria* spp. that harbored both *bcrABC* and the cadmium resistance determinant *cadA2*. We also showed that resistance could be transferred from nonpathogenic *Listeria* spp. to *L. monocytogenes* strains of all three serotypes primarily associated with human listeriosis (1/2a, 1/2b, and 4b). The findings indicated that cadmium resistance transfer can be effectively used as a surrogate for transfer of resistance to BC, since transconjugants selected on cadmium were resistant to both cadmium and to BC.

Transfer of resistance was not indiscriminate: even though all tested *L. monocytogenes* strains yielded transconjugants with at least some of the nonpathogenic donors, one of the nonpathogenic strains used as recipient failed to yield any transconjugants. Furthermore, transconjugants were produced with markedly higher frequency when nonpathogenic donors harbored *cadA2* (often while also harboring *bcrABC*) than when they harbored the alternative cadmium resistance determinant *cadA1*.

Nonpathogenic *Listeria* spp. have potential to serve as reservoirs for resistance genes and transfer them among themselves as well as to *L. monocytogenes* inhabiting the same environments, but data on such transfers remain scarce. Thus, far only one study reported conjugative transfer of disinfectant resistance attributes among *Listeria* spp. (29). That study used only two strains of nonpathogenic listeriae (*L. innocua* in both cases), and these exhibited resistance to the dye ethidium bromide but not to cadmium (29). In contrast, strains used as donors in the present study were resistant to cadmium but susceptible to ethidium bromide (M. Rakic-

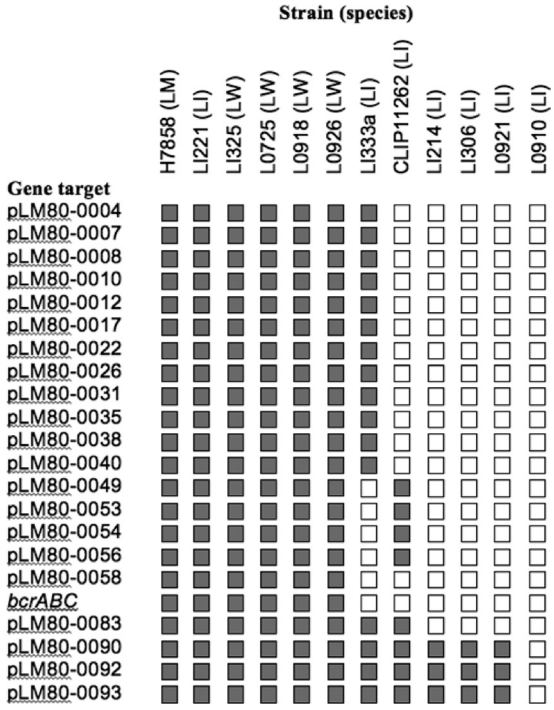

FIG 5 Detection of pLM80 genes in nonpathogenic *Listeria* spp. used as donors. Shaded or white boxes indicate the presence or absence, respectively, of the expected PCR product. LW, LI, and LM in strain (species) designations refers to *L. welshimeri*, *L. innocua*, and *L. monocytogenes*, respectively. *L. monocytogenes* H7858 harbors pLM80 (33) and is included as a positive control. PCR-based detection of the indicated genes was performed using the pLM80-derived primers listed in Table S1 in the supplemental material as described in Materials and Methods.

Martinez, unpublished findings). Thus, it is hard to compare data from this earlier study with the current findings.

In the case of nonpathogenic recipients, transfer was found to be more efficient at 25°C than at 37°C, suggesting that it may represent an environmental adaptation. In contrast, no difference in transfer frequency between 25 and 37°C was noted for *L. monocytogenes*, regardless of serotype, suggesting the potential for such transfers to be taking place not only in the environment but *in vivo* as well, e.g., in the mammalian gastrointestinal tract.

In the present study conjugations were on agar or on membrane filters overlaid on solid media. Only limited information is currently available on horizontal gene transfer in listeriae in foods and other complex systems such as the gastrointestinal tract (6, 11). Further studies are needed to determine whether conjugative transfer frequency is impacted by the presence of the conjugation partners on surfaces relevant to food processing environments (e.g., stainless steel and food) and within polymicrobial biofilms.

Although direct evidence of nonpathogenic *Listeria* spp. behaving as resistance gene reservoirs for *L. monocytogenes* is still lacking, coselection and disinfectant resistance gene distribution in other pathogens perhaps illustrate analogous processes at work. For instance, Ciric et al. have shown the presence of a *Streptococcus oralis* Tn*916*-like conjugative transposon, Tn*6087*, that confers resistance to cetrimide bromide, a disinfectant in the QAC family, as well as tetracyclines, potentially providing a mechanism for coselection of disinfectant and antibiotic resistance (7). In food-derived staphylococci, the presence of BC resistance genes linked to antibiotic resistance genes suggested the potential for similar coselection (48). Bjorland et al. demonstrated the widespread distribution of QAC resistance genes in bovine and equine coagulase-negative staphylococci (3). The wide distribution of such genes and the potential of increased fitness through coselection have important public health implications (45).

L. monocytogenes plasmids (e.g., pLM80) harboring both *bcrABC* and *cadA2* have been characterized in the course of genome sequencing investigations (12, 24, 33). Genome sequence data of the nonpathogenic listeriae used as donors in the present study are currently not available. Nonetheless, when donors harbored both *bcrABC* and *cadA2*, the transconjugants acquired both of these determinants, even though selection was only for cadmium resistance, suggesting plasmid-associated resistance genes similar to those harbored by pLM80. Further evidence for pLM80-like plasmids was provided by detection of all other tested pLM80-associated genes in these donor strains. Such data provide compelling reasons to further elucidate the sequence content of the plasmids of these *L. welshimeri* and *L. innocua* donor strains harboring *bcrABC* and *cadA2*.

Our PCR data suggest that different plasmids were harbored by strains containing *cadA1*. However, it was intriguing that such strains failed to serve as efficient donors to other listeriae. Plasmids harboring *cadA1* have been extensively described in *L. monocytogenes* (4, 24, 26, 27), and the conjugative transfer of one such plasmid was demonstrated in an earlier study. A single donor-recipient strain combination was examined in that study, and nonpathogenic strains were not included (28). Further studies are needed to determine whether our findings reflect differences in the plasmids or in the types of strains used in the conjugations.

In conclusion, we have demonstrated the potential for resistance to BC and to cadmium to be conjugatively transferred among nonpathogenic listeriae and from these strains to *L. monocytogenes* of diverse serotypes. Our findings also demonstrate coselection of resistance to BC and cadmium when the nonpathogenic *Listeria* spp. donors were resistant to both of these agents: transconjugants selected in the presence of cadmium were also resistant to BC. Coselection between cadmium resistance and BC resistance has not been documented before in *Listeria* or other bacteria. Future work should examine additional factors in food processing and other dynamic environments that may affect the efficacy of resistance transfer, such as varied growth surfaces and the presence of mixed and single-species biofilms. Further study of conjugative dissemination of BC and cadmium resistance in *Listeria* spp. would provide the opportunity to assess impact on fitness in disinfectant-abundant environments such as food processing plants and health care settings. The data from such studies would be needed to characterize potential impacts of such resistance determinant acquisitions on additional attributes, including those associated with virulence.

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