Applied and Environmental Microbiology, Jan. 2010, p. 627–630 Vol. 76, No. 2
0099-2240/10/$12.00 doi:10.1128/AEM.01751-09

Diverse Cadmium Resistance Determinants in *Listeria monocytogenes* Isolates from the Turkey Processing Plant Environment▽

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Received 23 July 2009/Accepted 15 November 2009

Two different *cadA* cadmium resistance determinants (*cadA1*, first identified in Tn*5422*, and *cadA2*, associated with pLM80) were detected among cadmium-resistant *Listeria monocytogenes* strains from turkey processing plants. Prevalence of *cadA1* versus *cadA2* was serotype associated. Cadmium-resistant isolates that were also resistant to benzalkonium chloride (BC) were more likely to harbor *cadA2* alone or together with *cadA1* than isolates that were cadmium resistant but BC susceptible.

Listeria monocytogenes is a major cause of death due to food-borne illness in the United States and other industrialized nations. Frequently implicated vehicles include foods that are ready to eat, highly processed, and cold stored; environmental contamination of food processing plants is critical to contamination of such foods (9, 10, 19).

Resistance to disinfectants may be an important factor contributing to *Listeria*'s ecology in food processing plants. Recently, we showed that resistance to the quaternary ammonium disinfectant benzalkonium chloride (BC) was encountered in 60% and 51% of strains of serotype 1/2a (or 3a) and serotype 1/2b (or 3b), respectively, isolated from turkey processing plant environments in the United States (17). Furthermore, all BC-resistant strains were also resistant to the heavy metal cadmium, although the opposite did not hold true; 23% of the cadmium-resistant strains were susceptible to BC (17).

The three most recent multistate outbreaks of listeriosis in the United States (the 1998-1999 hot dog outbreak [2] and outbreaks in 2000 [3] and 2002 [4], both involving turkey deli meats) involved cadmium-resistant strains. Strains from the 1998-1999 and 2000 outbreaks were resistant to BC as well (6, 17; S. Kathariou and R. M. Siletzky, unpublished). These findings, along with earlier reports of association between cadmium resistance and persistence of *L. monocytogenes* in food processing plants (8), suggest the need to further characterize cadmium resistance among environmental strains of *L. monocytogenes*.

Three distinct cadmium resistance determinants associated with mobile elements have been identified in *L. monocytogenes* with high-level resistance to cadmium. The plasmid-borne, transposon Tn*5422*-associated cadmium resistance cassette (*cadAC*) was found earlier in ca. 95% of cadmium-resistant strains (12, 13). However, genome sequencing of a *L. monocytogenes* strain from the 1998-1999 hot dog outbreak revealed that a large plasmid (pLM80, ca. 80 kb) harbored a *cadAC* cadmium resistance cassette distinct at the nucleotide sequence level from that harbored by Tn*5422* (nucleotide sequence identity, <50%; protein sequence identity, ca. 68%); pLM80 also harbored genes for resistance to BC, and outbreak-derived *L. monocytogenes* isolates harboring pLM80 were resistant to both cadmium and BC (6, 17, 18). A third type of *cadAC* was identified as a component of an integrative conjugative element (ICE) in the chromosome of *L. monocytogenes* EGDe (serotype 1/2a) (7); this strain is resistant to cadmium but not to BC (R. Siletzky and S. Kathariou, unpublished). However, no information is currently available on the relative prevalences of these three types of cadmium resistance cassettes among strains of *L. monocytogenes* from food processing plant environments.

In this study, DNA probes specific to *cadA* associated with Tn*5422* (*cadA1*), pLM80 (*cadA2*), and ICE in *L. monocytogenes* EGDe (*cadA3*) were employed in Southern blot hybridizations of EcoRI-digested genomic DNA of 81 cadmium-resistant *L. monocytogenes* strains, 57 of which were also resistant to BC. The strains were isolated between 2002 and 2005 from environmental samples of turkey processing plants in the United States, and their levels of resistance to cadmium and BC were previously described (17). The strain panel included 44 strains of serotype 1/2a (or 3a), 29 of 1/2b (or 3b), 5 of the serotype 4b complex (4b and the closely related serotypes 4d and 4e), and 3 of serotype 1/2c (or 3c) (Table 1). DNA probes for *cadA1*, *cadA2*, and *cadA3* were prepared by PCR using, as a template, genomic DNA from *L. monocytogenes* strains BUG 912 (Lm 106) (13), H7550 (1998-1999 hot dog outbreak) (2), and EGDe (7) and the primers listed in Table 2. Probe labeling with digoxigenin and Southern blot analyses were done as described previously (21).

Hybridizations of EcoRI-digested genomic DNAs with *cadA1* and *cadA2* showed that 37 (46%) of the 81 cadmium-resistant strains harbored only *cadA1*, 16 (20%) harbored only *cadA2*, and 24 (30%) harbored both *cadA1* and *cadA2* (Table 1 and Fig. 1). The simultaneous presence of *cadA1* and *cadA2* in these strains was confirmed in repeated Southern blot analyses (data not shown). Furthermore, findings from Southern blot analyses of selected strains with probes derived from *cadC* of the corresponding cassettes (*cadC1* and *cadC2*; primers for probe construction are listed in Table 2) agreed completely

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▽ Published ahead of print on 30 November 2009.

TABLE 1. Cadmium resistance determinants among cadmium-resistant *L. monocytogenes* strains from turkey processing plants

Serotype and no. of strains in group	Strain(s)	Resistance[a] to:		Presence of *cadA* determinant[b]		
		Cd	BC	*cadA1*	*cadA2*	*cadA3*
1/2a (3a) (n = 44)						
12	1559, 1566, 1732, 2627, 175, 538, 777, 82, 90, 905, 1747, 1845	+	−	+	−	−
3	170, 171, 1907	+	+	−	+	−
18	869, 273, 408, 513, 600, 627, 653, 686, 754, 180, 181, 884, 933, 1096, 1171, 1178, 2622, 2647	+	+	+	−	−
11	86, 432, 84, 459, 483, 521, 93, 162, 165, 720, 740	+	+	+	+	−
1/2b (3b) (n = 29)						
3	174, 1281, 1282	+	−	−	+	−
5	1499, 27, 1507, 731, 1830	+	−	+	−	−
8	78, 463, 95, 172, 554, 854, 197, 1181	+	+	−	+	−
13	1104, 1308, 378, 83, 163, 523, 597, 677, 715, 1077, 1134, 2405, 2629	+	+	+	+	−
1/2c (3c) (n = 3)						
1	130	+	+	−	+	−
2	961, 2642	+	+	+	−	−
4b complex (n = 5)						
3	1501, 1494, 1500	+	−	−	−	−
1	1117	+	+	−	+	−
1	1497	+	+	−	−	−

[a] +, resistance; −, susceptibility. For cadmium and BC, resistance and susceptibility were assessed as described previously (17), on the basis of growth (or lack thereof) on 70 μg/ml $CdCl_2$ and 10 μg/ml BC, respectively.
[b] *cadA*, *cadA2*, and *cadA3* were derived from *cadA* determinants on Tn*5422*, pLM80, and the ICE of strain EGDe, respectively.

with those obtained with the *cadA1* and *cadA2* probes (data not shown), suggesting that *cadA1* and *cadA2* were always found together with their cognate *cadC* determinants (*cadC1* and *cadC2*, respectively). Of the 81 strains, 4 (5%) failed to hybridize with either *cadA1* or *cadA2* (Table 1 and Fig. 1). None of the environmental strains hybridized with *cadA3* (Table 1) or *cadC3* (data not shown).

The prevalences of *cadA1* and *cadA2* varied among strains of different serotypes (Table 1). Strains possessing only *cadA1* were noticeably more frequent among those of serotype 1/2a (or 3a) than among those of serotype 1/2b (or 3b) (30/44 [68%] versus 5/29 [17%]; $P < 0.01$). In contrast, strains harboring only *cadA2* were more prevalent among those of serotype 1/2b (or 3b) than among those of serotype 1/2a (or 3a) (11/29 [38%] versus 3/44 [7%]; $P < 0.01$) (Fig. 1). The prevalence of strains with both *cadA1* and *cadA2* was also higher among serotype 1/2b (or 3b) strains than among serotype 1/2a (or 3a) strains (13/29 [45%] versus 11/44 [25%]), although the difference was not statistically significant ($P = 0.08$). Despite the fact that cadmium-resistant strains of serotype 1/2c (or 3c) and of the serotype 4b complex were too few (three and five, respectively) for serotype-associated trends in the prevalences of *cadA1* and *cadA2* to be determined, the preliminary results are intriguing. Resembling serotype 1/2a (or 3a), two of the three serotype 1/2c (or 3c) strains harbored only *cadA1* (Table 1). On the other hand, *cadA2* alone was detected in one strain of the

TABLE 2. List of primers for probe preparation

Primer	Sequence 5′–3′	Target gene	GenBank accession no.
cadA-Tn*5422*F	CAGAGCACTTTACTGACCATCAATCGTT	Tn*5422*-associated *cadA* (*cadA1*)	L28104
cadA-Tn*5422*R	CTTCTTCATTTAACGTTCCAGCAAAAA		
cadC-Tn*5422*F	CGCAATCCCTTGCTTCTTTA	Tn*5422*-associated *cadC* (*cadC1*)	
cadC-Tn*5422*R	GGGTGAAGACTGGACTGGAA		
cadA-pLM80F	ACAAGTTAGATCAAAAGAGTCTTTTATT	*cadA* on pLM80 (*cadA2*)	AADR01000058
cadA-pLM80R	ATCTTCTTCATTTAGTGTTCCTGCAAAT		
cadC-pLM80F	TGAAGATGAGCTATGTGTTTGTGA	*cadC* on pLM80 (*cadC2*)	
cadC-pLM80R	AAAATTTACGCCAAGCTCCA		
cadA-EGDeF	TGGTAATTTCTTTAAGTCATCTCCCATT	*cadA* in ICE of strain EGD-e (*cadA3*)	AL591824
cadA-EGDeR	GCGATGATTGATAATGTCGATTACAAAT		
cadC-EGDeF	TCGCTACTGTTGATTTCACAATG	*cadC* in ICE of strain EGD-e (*cadC3*)	
cadC-EGDeR	CCTGTTTTGATGAGGAAAAGGT		

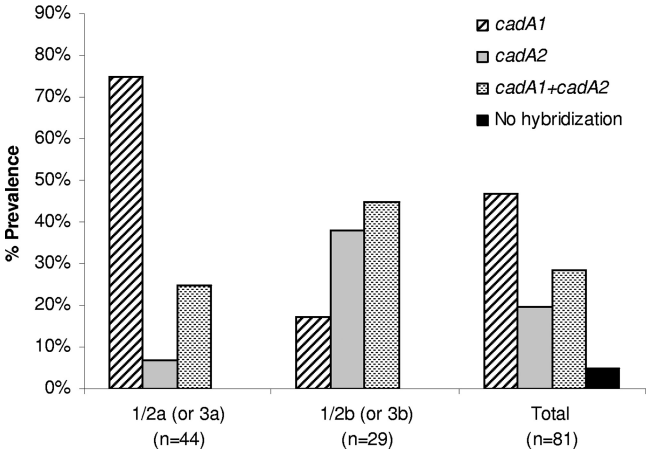

FIG. 1. Prevalence of cadmium resistance determinants among *L. monocytogenes* isolates of different serotypes from turkey processing plant environments. Cadmium resistance determinants *cadA1* and *cadA2* were detected by Southern blot analyses using probes derived from *cadA* of Tn*5422* and pLM80, respectively. The total includes three strains of serotype 1/2c (or 3c) and five of the serotype 4b complex.

serotype 4b complex (Table 1). Interestingly, the four cadmium-resistant strains that did not hybridize with either *cadA1* or *cadA2* were all of the serotype 4b complex (Fig. 1 and Table 1), suggesting the presence of novel, unidentified determinants for high-level cadmium resistance in these strains.

The prevalences of BC resistance were similar among serotype 1/2a (or 3a) and serotype 1/2b (or 3b) cadmium-resistant strains (73 and 72%, respectively) (17). Strains that were resistant to both cadmium and BC (Cd^r BC^r) harbored *cadA1* alone (20/58 [34%]), *cadA2* alone (13/58 [22%]), or both determinants (24/58 [41%]). Thus, 64% of Cd^r BC^r strains harbored *cadA2*, either alone or together with *cadA1* (Fig. 2). In contrast, most (17/23 [74%]) strains resistant to cadmium but susceptible to BC (Cd^r BC^s) possessed *cadA1* alone, whereas 13% (3/23) harbored *cadA2* alone. The remaining three strains were serotype 4b strains that failed to hybridize with any of the

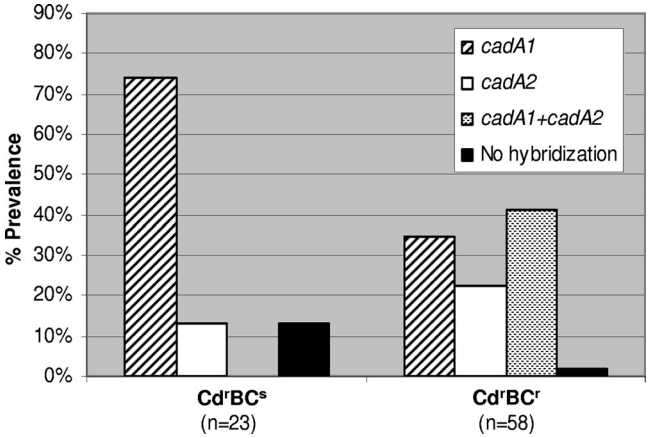

FIG. 2. Prevalence of cadmium resistance determinants among *L. monocytogenes* strains differing in susceptibility to BC. Cd^r BC^s, resistant to cadmium but susceptible to BC; Cd^r BC^r, resistant to both cadmium and BC.

TABLE 3. Cadmium resistance determinants among *L. monocytogenes* Cd^r BC^r strains of serotypes 1/2a (or 3a) and 1/2b (or 3b)

Cd^r BC^r strain	No. (%) of strains hybridizing with:		
	cadA1 only	*cadA2* only	*cadA1* and *cadA2*
1/2a (or 3a) (n = 32)	18 (56)	3 (9)	11 (34)
1/2b (or 3b) (n = 21)	0 (0)	8 (38)	12 (62)

cadA probes (Table 1). Remarkably, all strains harboring both *cadA1* and *cadA2* were resistant both to cadmium and to BC, and conversely, none of the Cd^r BC^s strains hybridized with both *cadA1* and *cadA2* (Fig. 2 and Table 1).

The higher prevalences of *cadA1* among serotype 1/2a (or 3a) strains and of *cadA2* among serotype 1/2b (or 3b) noted above were found to be even more pronounced when only Cd^r BC^r strains of these serotypes were examined (Table 3). A large fraction (18/32 [56%]) of the serotype 1/2a (or 3a) Cd^r BC^r strains harbored *cadA1* alone; in contrast, none of the 21 Cd^r BC^r strains of serotype 1/2b (or 3b) harbored *cadA1* alone; 38% of these strains harbored only *cadA2*, while the remaining 62% harbored both *cadA2* and *cadA1* (Table 3).

Our findings revealed that, in addition to the cadmium resistance determinant *cadA1* described earlier for *L. monocytogenes* (13), cadmium-resistant strains from the turkey processing plant environment frequently harbored an alternative cadmium resistance determinant (*cadA2*), either alone or together with *cadA1*. It is noteworthy that strains harboring both *cadA1* and *cadA2* were always resistant to both cadmium and BC (Cd^r BC^r). Thus, detection of both determinants in the same organism can be useful in predicting BC resistance. Preliminary data from our laboratory with strain 163 (harboring both *cadA1* and *cadA2* and resistant to both cadmium and BC) indicated that a BC-susceptible, cadmium-resistant derivative of this strain hybridized only with *cadA1* but that a derivative susceptible both to cadmium and to BC lacked hybridization with either probe (S. Mullapudi, R. M. Siletzky, and S. Kathariou, unpublished findings); such findings suggest that *cadA2* and the BC resistance determinant were harbored by one plasmid and that *cadA1* was harbored by another. Plasmids of strain 163 and other strains and their derivatives are being further characterized in our laboratory.

In this study, *cadA2*, alone or together with *cadA1*, was significantly more prevalent among strains that were resistant to both cadmium and BC than among cadmium-resistant but BC-susceptible strains ($P < 0.01$). BC and other quaternary ammonium disinfectants are extensively used in food processing plants (14, 16), and selection for resistance to these compounds may at least partially drive the observed higher prevalence of *cadA2* among Cd^r BC^r strains than among Cd^r BC^s strains. It is tempting to speculate that acquisition and establishment of plasmids such as pLM80 (harboring both *cadA2* and BC resistance genes) may be one of the mechanisms that the bacteria use to respond to such selective pressure. Plasmids harboring *cadA2* and BC resistance genes may have been relatively recently established for *L. monocytogenes*, possibly explaining the failure to identify *cadA2* in earlier studies which involved *L. monocytogenes* strains from earlier periods and

from sources other than the processing plant environment (12, 13).

Another unexpected finding of this study was that the relative prevalences of *cadA1* and *cadA2* varied significantly by serotype, with *cadA1* being more prevalent among serotype 1/2a (or 3a) strains and *cadA2* being more prevalent among strains of serotype 1/2b (or 3b). The reasons for these differences are currently unknown but may reflect serotype-associated differences in prevalence between plasmids with *cadA1* and those harboring *cadA2*. Similarly, the lack of hybridization of four serotype 4b strains with either *cadA1* or *cadA2* may suggest an absence of plasmids harboring the determinants. Previous data indicate that plasmids were less common among serotype 4b strains than among those of serogroup 1/2 (8, 11, 15).

None of the cadmium-resistant strains in this study appeared to harbor *cadA3*, detected in the ICE of *L. monocytogenes* EGDe (7). ICE-associated *cadA* determinants similar to *cadA3* have been detected in other Gram-positive bacteria and were hypothesized to result from lateral gene transfer (5, 20). Interestingly, transcription of *cadC3* (*lmo1102*) in the ICE of EGDe was found to be strongly induced in the spleens of mice infected intravenously with this strain, and the gene was implicated in virulence in this model (1). Our findings and the fact that *cadA3* has not yet been detected among other sequenced *Listeria* genomes suggest that this chromosomal cadmium resistance determinant is rare in *L. monocytogenes*, possibly due to limits in the distribution and dissemination of the ICE that harbors it. Previous studies also indicated that cadmium resistance was largely plasmid associated in *L. monocytogenes* (8, 11, 15). Further studies are needed to elucidate the mechanisms underlying the prevalences of the different cadmium resistance determinants and the possible role of these determinants in the ecology and evolution of *L. monocytogenes* in food processing plants and other environments.

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

We thank P. Cossart for the gift of *L. monocytogenes* BUG 912 (Lm 106). We thank all members of our laboratory for discussions, encouragement, and support in the course of the project.

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