Applied and Environmental Microbiology, Nov. 2000, p. 5083–5086 Vol. 66, No. 11
0099-2240/00/$04.00+0
Copyright © 2000, American Society for Microbiology. All Rights Reserved.

Low Sensitivity of *Listeria monocytogenes* to Quaternary Ammonium Compounds

L. Mereghetti,[1]* R. Quentin,[2] N. Marquet-van der Mee,[1] and A. Audurier[1]

Laboratoire de Microbiologie, Faculté de Médecine de Tours,[1] and Département de Microbiologie Médicale et Moléculaire, Unité de Bactériologie, Centre Hospitalier Universitaire Bretonneau,[2] 37032 Tours Cedex, France

Received 24 April 2000/Accepted 15 August 2000

Ninety-seven epidemiologically unrelated strains of *Listeria monocytogenes* were investigated for their sensitivities to quaternary ammonium compounds (benzalkonium chloride and cetrimide). The MICs for seven serogroup 1/2 strains were high. Three came from the environment and four came from food; none were isolated from human or animal samples. All 97 strains carried the *mdrL* gene, which encodes a multidrug efflux pump, and the *orfA* gene, a putative transcriptional repressor of *mdrL*. The absence of plasmids in four of the seven resistant strains and the conservation of resistance after plasmid curing suggested that the resistance genes are not plasmid borne. Moreover, PCR amplification and Southern blot hybridization experiments failed to find genes phylogenetically related to the *qacA* and *smr* genes, encoding multidrug efflux systems previously described for the genus *Staphylococcus*. The high association between nontypeability by phages and the loss of sensitivity to quaternary ammonium compounds are suggestive of an intrinsic resistance due to modifications in the cell wall.

Listeria monocytogenes is the agent of human listeriosis, which is characterized by a variety of severe syndromes, including meningitis, meningoencephalitis, and sepsis, which mostly affects old and immunosuppressed individuals and pregnant women (20). This bacterium is frequently present in soil and surface water samples, and it has been found in a wide range of dairy products, meats, and seafood (3, 18). It is generally believed that the consumption of contaminated food is the principal route of infection, especially since the increase in industrial food production (21).

Despite the application of rigorous procedures of cleaning and disinfection of the processing environment in the food industry, processed food has been contaminated by *L. monocytogenes* even when the raw ingredients were free of the pathogen (4, 23). *L. monocytogenes* can attach to various kinds of surfaces, and it has been found in biofilms in meat and dairy processing environments (9). Various types of dairy and other food plant sanitizers are widely used. Quaternary ammonium compounds (QACs) are employed both as disinfectants for manual processing lines and surfaces in the food industry and as antiseptics in human medicine. It is possible that some strains of *L. monocytogenes* may have acquired resistance to these disinfectants.

No mechanism of resistance to QACs has been described for *L. monocytogenes*, but one such mechanism is well known for the genus *Staphylococcus*. It is a multidrug efflux system encoded by the *qacA* and *smr* genes, found on both conjugative and nonconjugative plasmids (17). Moreover, the recent identification of a new locus in *L. monocytogenes* involved in cellobiose-dependent repression of *hly* expression led to the discovery of a gene named *mdrL*. This gene codes for a putative protein homologous (21 to 24% identity) to a member of the multidrug resistance efflux pump family of *Bacillus subtilis*. Another gene, named *orfA*, may produce a repressor of *mdrL* (8).

* Corresponding author. Mailing address: Laboratoire de Microbiologie, Faculté de Médecine de Tours, 2 bis Bd Tonnellé, 37032 Tours Cedex, France. Phone: 33 2 47478113. Fax: 33 2 47478530. E-mail: laurent.mereghetti@med.univ-tours.fr.

The aims of our study were the following: (i) to establish the levels of sensitivity to QACs of *L. monocytogenes* isolates from various ecosystems, (ii) to evaluate the distribution of the *orfA* and *mdrL* genes in the different listerial populations, and (iii) to examine whether *L. monocytogenes* strains contain plasmid genes closely related to *qacA* and *smr* as a possible cause of low sensitivity to QACs.

Sensitivity of *L. monocytogenes* strains to QACs. Ninety-seven epidemiologically unrelated strains were selected to represent various *L. monocytogenes* ecosystems: the environment ($n = 19$), food products ($n = 41$), and human ($n = 19$) and animal ($n = 18$) pathological samples. All isolates were biochemically characterized by conventional identification methods (1). Antisera 1/2 and 4 were used for serogrouping according to the instructions of the manufacturer (Difco, Detroit, Mich.). MICs were determined by a dilution method on Mueller-Hinton agar medium (bioMérieux). Aliquots of 0.3 μl of bacterial inoculum adjusted to a turbidity of 0.5 McFarland unit were spotted onto agar containing the disinfectants to be tested (5×10^4 bacteria per spot). The following disinfectants were tested: benzalkonium chloride (1 to 20 mg/liter), cetrimide (2 to 40 mg/liter), chlorhexidine digluconate (0.5 to 10 mg/liter), acriflavine (5 to 500 mg/liter), and ethidium bromide (5 to 125 mg/liter). Agar plates were incubated at 37°C for 18 h. For benzalkonium chloride and cetrimide, dilutions were at 1-mg/liter steps. *Staphylococcus aureus* A-83 (harboring the *qacA* gene), A-82 (harboring the *smr* gene), and A-84 (sensitive to QACs) were included as positive and negative controls (Centre National de Référence des Staphylocoques, Lyon, France).

Two distinct populations were identified (Fig. 1 and 2). Ninety strains were scored as susceptible: the MICs of benzalkonium chloride were under 4 mg/liter and the MICs of cetrimide were under 14 mg/liter. Seven strains were scored as less susceptible: the MICs of benzalkonium chloride were over 7 mg/liter and the MICs of cetrimide were over 18 mg/liter. For these seven strains, the MICs of QACs and of chlorhexidine were also high. No significant association was found between the high MICs of QACs and the MICs of ethidium bromide or

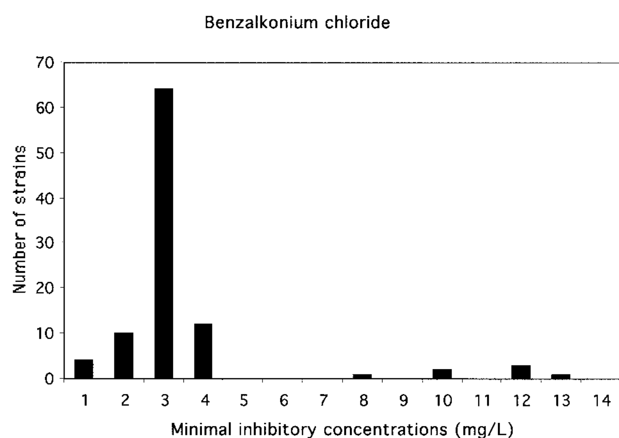

FIG. 1. Distribution of the 97 *L. monocytogenes* strains according to the MICs of benzalkonium chloride. Two populations were observed. One group, scored as susceptible, consisted of 90 strains for which the MICs were ≤4 mg/liter; the second group, scored as less susceptible, consisted of 7 strains for which the MICs were ≥8 mg/liter. These seven strains were also less susceptible to cetrimide.

acriflavine. Thus, the MICs of QACs in vitro were high for 7% of our *L. monocytogenes* strains. This poor sensitivity may explain the persistence of some *L. monocytogenes* strains on manual processing lines and surfaces in food industry plants despite strict application of cleaning and disinfecting procedures (15). Consequently, the use of two different sanitizers employed alternately for the cleaning of food plants and the food industry environment may be beneficial.

The seven resistant strains belong to serovars 1/2a and 1/2c. None of the 37 serogroup 4 strains were resistant to QACs (Table 1). Three of these seven resistant strains of *L. monocytogenes* were from environmental samples from food industry sites and 4 were from food products (Table 1). None came from animals, humans, or other environment samples. Therefore, the existence of strains poorly sensitive to QACs in food samples does not appear to be a major cause of human contamination, unless the resistance of the environmental and food isolates is lost upon infection of a human.

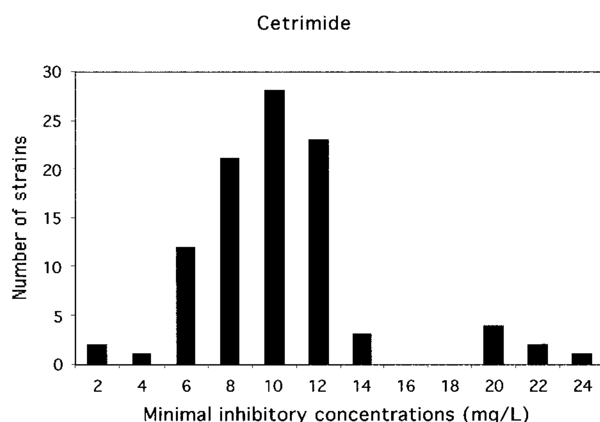

FIG. 2. Distribution of the 97 *L. monocytogenes* strains according to the MICs of cetrimide. Two populations were observed. One group scored as susceptible, consisted of 90 strains for which the MICs were ≤14 mg/liter. The second group, scored as less susceptible, consisted of 7 strains for which the MICs were ≥20 mg/liter. These seven strains were also less susceptible to benzalkonium chloride.

TABLE 1. Origins and serogroups of the 97 epidemiologically unrelated strains of *L. monocytogenes* studied

Serogroup	No. of strains from:				Total
	Environment	Animals	Food products	Humans	
1/2	16[a]	9	27[b]	8	60
4	3	9	14	11	37
All	19	18	41	19	97

[a] Including three strains resistant to QACs.
[b] Including four strains resistant to QACs.

Identification of the *mdrL* and *orfA* genes by PCR. Recently, a multidrug resistance efflux pump, MdrL, was identified in *L. monocytogenes*. Its amino acid sequence presents 21 to 24% identity with the Bmr and Blt efflux pumps of *B. subtilis* (8). Insertion mutagenesis of the reference strain L028 demonstrated that this efflux pump is responsible for substrate extrusion from *L. monocytogenes*. MICs of macrolides and heavy metals for the mutant strain were lower than those for the wild type (J. C. Perez-Diaz, M. T. Mata, M. C. Negri, and F. Baquero, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 671, 1999). This mechanism is a possible cause of the reduced sensitivity to QACs observed in our resistant strains. PCR amplification with primers lltb1 and lltb2 (Table 2) was used to identify the *mdrL* gene in our population of listeriae. The PCR mixture consisted of a buffer of 10 mM Tris-HCl–50 mM KCl–2.5 mM $MgCl_2$ (pH 8.3) (Perkin-Elmer), a 100 μM concentration of each of the four deoxyribonucleoside triphosphates (Boehringer, Mannheim, Germany), 20 pmol of each of the two primers, 25 ng of DNA, and 0.5 U of AmpliTaq DNA polymerase (Perkin-Elmer) in a total volume of 25 μl. The reaction procedure consisted of an initial denaturation step at 94°C for 120 s followed by 30 cycles of denaturation at 94°C for 60 s, primer annealing at 50°C for 60 s, and extension at 72°C for 90 s (10 min for the last extension). Amplicons of the expected size (1,136 bp) were obtained with all 97 strains, suggesting that the *mdrL* gene is present in all isolates. It is thus unlikely that the QAC resistance of some *L. monocytogenes* strains is due to the acquisition of *mdrL* gene, which appears to be ubiquitous.

The modulation of the efflux rate by regulation of multidrug efflux genes involving specific *trans*-acting repressor proteins may be the basis for the high MICs of QACs (6). No specific regulator has been described for the MdrL pump. Neverthe-

TABLE 2. Nucleotide sequences of primers used for PCR amplification of the genes *orfA*, *mdrL*, *qacA*, and *smr*

Gene	Primer[a]	Sequence (5′ to 3′)	Amplicon size (kb)
orfA	orf1	AAATGATTGCTCGTGAAGCT	0.467
	orf2	CGCACACCATTTTAATTCTG	
mdrL	lltb1	AAATGGATAACAGCGGCAG	1.136
	lltb2	TGTAAGGTAAAATGTGCTGG	
qacA	qac3	ACTACTGATATGATGACATCA	1.512
	qac4	AGTTATATCAAGTGATTTGGG	
smr	smr1	ATAGCCATAAGTACTGAAGTT	0.291
	smr2	ACCGAAAATGTTTAACGAAAC	

[a] These primers were formulated from sequences deposited in the EMBL/GenBank databases under accession no. X56628 for the *qacA* gene (18), M37889 for the *smr* gene (12), and AJ009627 for the *orfA* and *mdrL* genes (8).

less, a second putative protein, OrfA, which was identified at the same time as MdrL, may be a transcriptional repressor of *mdrL* gene expression (8). PCR amplification with primers orf1 and orf2 (Table 2) was used under the conditions described above to identify the *orfA* gene in our *L. monocytogenes* population. A fragment of the expected size (467 bp) was obtained for all 97 strains, suggesting that *orfA* is also ubiquitous.

Detection of plasmid DNA and plasmid curing. To assess whether QAC resistance is plasmid associated, we studied the plasmid contents of all strains and cured them by heat treatment. Strains were cultured on heart brain broth (Oxoïd, Dardilly, France) at 30°C. Plasmids were extracted by the alkaline method as described by Birnboim and Doly (2). *Escherichia coli* V517 was used as a standard plasmid-containing strain (plasmid DNA of 55, 7.4, 5.7, 4, 3.1, 2.8, and 2.2 kb) and was included in parallel with each extraction. Plasmid DNA was detected in only three of the seven QAC-resistant strains. These three QAC-resistant strains were cured of plasmids by heat treatment as previously described (11). *L. monocytogenes* 88-1710 and 89-367 containing cadmium resistance plasmids were included to confirm that the method used allowed plasmid curing by the observation of the loss of cadmium resistance. No differences were found between the MICs of QACs before and after plasmid curing. This, and the observation that four resistant strains were plasmid free, suggests that the genes responsible for the QAC resistance of our seven strains can be easily transferred among *L. monocytogenes* strains. Therefore, the spread of this resistance may be limited in the environment and in food products.

Detection of the *qacA* and *smr* genes. Multidrug resistance efflux pumps, QacA and Smr, have been found in the genus *Staphylococcus*. They confer resistance to a number of classes of antimicrobial organic cations, including QACs (13, 16). Around 13% of *Staphylococcus* strains isolated from the food industry show resistance to QACs, a finding similar to the 7% observed in our population of listeriae (7). To assess whether any genes related to the *qacA* and *smr* genes are implicated in the QAC resistance of our strains, PCR and Southern blot hybridization were used. PCR was performed as described above with primers qac3 and qac4, flanking the *qacA* gene, and with primers smr1 and smr2, flanking the *smr* gene (Table 2). DNA from six of the seven resistant strains gave a 2.7-kb fragment with the qac3 and qac4 primers, although a 1.4-kb fragment was expected. These PCR products were sequenced using the ThermoSequenase dye terminator cycle sequencing premix kit (Amersham Life Sciences, Cleveland, Ohio) and the Abi Prism 377 DNA sequencer (Perkin-Elmer). The 500 bases at the 5′ and 3′ ends of the amplified 2.7-kb fragment were sequenced. The sequences were dissimilar to any sequences in the *qacA* gene and also to any other known bacterial nucleotide sequence. PCRs with primers smr1 and smr2 flanking the *smr* gene did not amplify any fragment. Southern blot hybridization was performed as previously described (5). *qac* and *smr* probes were produced with DNA-amplified products of *S. aureus* A-83 DNA and *S. aureus* A-82 labeled with alkaline phosphatase-conjugated antibody of the AlkPhos direct labeling kit (Amersham Life Sciences). No hybridization was observed in Southern blotting experiments with the DNA of the seven strains. Therefore, the QAC resistance of *L. monocytogenes* does not appear to be due to a multidrug efflux system encoded by genes phylogenetically related to *qac* or *smr* genes.

QAC resistance and phage typing. Phage typing was done as previously described using the international set of phages and experimental phages (1). Of the 97 strains studied, 15 were nontypeable by the entire set of phages used. Five of the seven QAC-resistant strains were nontypeable. Therefore, QAC resistance was significantly associated with nontypeability ($P = 0.0008$, Student's t test). This is suggestive of structural changes in the walls of the resistant strains. Such a phage-resistant phenotype in experimentally mutated strains of *L. monocytogenes* has already been obtained and was the result of the lack of *N*-acetylglucosamine in the teichoic acid of the cell wall (22). Therefore, an explanation for the poor sensitivity of some *L. monocytogenes* strains may be an intrinsic resistance arising from modifications of the thickness and the degree of cross-linking of peptidoglycan in the cell wall (14). This type of phenomenon has already been described for the genus *Bacillus* and for mucoid strains of *S. aureus* (10, 14).

In conclusion, high MICs of QACs were observed for some *L. monocytogenes* strains isolated from the environment and from food products. This resistance is not plasmid associated and is not due to the *qacA* and *smr* genes or to closely related genes. Overexpression of the MdrL protein encoded by the *mdrL* gene, ubiquitous in *L. monocytogenes*, or an intrinsic resistance may explain the high MICs of QACs displayed in vitro by 7% of the strains studied.

We thank M. E. Reverdy (Centre National de Référence des Staphylocoques) for kindly providing *S. aureus* strains A-82, A-83, and A-84. We also thank A. Fenneteau for technical assistance in phage typing.

REFERENCES

1. **Audurier, A., and C. Martin.** 1989. Phage typing of *Listeria monocytogenes*. Int. J. Food Microbiol. **8:**251–257.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
3. **Farber, J. M., and P. I. Peterkin.** 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. **55:**476–511.
4. **Genigeorgis, C. A., D. Dutulescu, and J. Fernandez.** 1989. Prevalence of *Listeria* spp. in poultry meat at the supermarket and slaughterhouse level. J. Food Prot. **52:**618–624.
5. **Gousset, N., A. Rosenau, P.-Y. Sizaret, and R. Quentin.** 1999. Nucleotide sequences of genes coding for fimbrial proteins in a cryptic genospecies of *Haemophilus* spp. isolated from neonatal and genital tract infections. Infect. Immun. **67:**8–15.
6. **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.
7. **Heir, E., G. Sundheim, and A. L. Holck.** 1995. Resistance to quaternary ammonium compounds in *Staphylococcus* spp. isolated from the food industry and nucleotide sequence of the resistance plasmid pST827. J. Appl. Bacteriol. **79:**149–156.
8. **Huillet, E., S. Larin, P. Pardon, and P. Berche.** 1999. Identification of a new locus in *Listeria monocytogenes* involved in cellobiose-dependent repression of *hly* expression. FEMS Microbiol. Lett. **174:**265–272.
9. **Jeong, D. K., and J. F. Frank.** 1994. Growth of *Listeria monocytogenes* at 10°C in biofilms with microorganisms isolated from meat and dairy processing environments. J. Food Prot. **57:**576–586.
10. **Kolawole, D. O.** 1984. Resistance mechanisms of mucoid-grown *Staphylococcus aureus* to the antimicrobial action of some disinfectants and antiseptics. FEMS Microbiol. Lett. **25:**205–209.
11. **Lebrun, M., J. Loulergue, E. Chaslus-Dancla, and A. Audurier.** 1992. Plasmids in *Listeria monocytogenes* in relation to cadmium resistance. Appl. Environ. Microbiol. **58:**3183–3186.
12. **Littlejohn, T. G., D. Diberardino, L. J. Messerotti, S. J. Spiers, and R. A. Skurray.** 1991. Structure and evolution of a family of genes encoding antiseptic and disinfectant resistance in *Staphylococcus aureus*. Gene **101:**59–66.
13. **Littlejohn, T. G., I. T. Paulsen, M. T. Gillepsie, J. M. Tennent, M. Midgley, I. G. Jones, A. S. Purewal, and R. A. Skurray.** 1992. Substrate specificity and energetics of antiseptic and disinfectant resistance in *Staphylococcus aureus*. FEMS Microbiol. Lett. **74:**259–265.
14. **McDonnell, G., and A. D. Russell.** 1999. Antiseptics and disinfectants: activity, action, and resistance. Clin. Microbiol. Rev. **12:**147–179.
15. **Mustapha, A., and M. B. Liewen.** 1989. Destruction of *Listeria monocytogenes* by sodium hypochlorite and quaternary ammonium sanitizers. J. Food Prot. **52:**306–311.
16. **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. USA **93:**3630–3635.
17. **Paulsen, I. T., M. H. Brown, and R. A. Skurray.** 1996. Proton-dependent

multidrug efflux system. Microbiol. Rev. **60:**575–608.
18. **Rocourt, J., and P. Cossart.** 1997. *Listeria monocytogenes*, p. 337–352. *In* M. P. Doyle, L. R. Beuchat, and T. J. Montville (ed.), Food microbiology. American Society for Microbiology, Washington, D.C.
19. **Rouch, D. A., D. S. Cram, D. DiBerardino, T. G. Littlejohn, and R. A. Skurray.** 1990. Efflux-mediated antiseptic resistance gene *qacA* from *Staphylococcus aureus*: common ancestry with tetracycline- and sugar-transport proteins. Mol. Microbiol. **4:**2051–2062.
20. **Schuchat, A., B. Swaminathan, and C. V. Broome.** 1991. Epidemiology of human listeriosis. Clin. Microbiol. Rev. **4:**169–183.
21. **Tappero, J. W., A. Schuchat, K. A. Deaver, L. Mascola, and J. D. Wenger.** 1995. Reduction in the incidence of human listeriosis in the United States. Effectiveness of prevention efforts. JAMA **273:**1118–1122.
22. **Tran, H. L., F. Fiedler, D. A. Hodgson, and S. Kathariou.** 1999. Transposon-induced mutations in two loci of *Listeria monocytogenes* serotype 1/2a result in phage resistance and lack of *N*-acetylglucosamine in the teichoic acid of the cell wall. Appl. Environ. Microbiol. **65:**4793–4798.
23. **Walker, R. L., L. H. Jensen, H. Kinde, A. V. Alexander, and L. S. Owen.** 1991. Environment survey for *Listeria* species in frozen milk plants in California. J. Food Prot. **54:**178–182.