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Antimicrobial Susceptibility of *Pediococcus* spp. and Genetic Basis of Macrolide Resistance in *Pediococcus acidilactici* HM3020

JACQUES TANKOVIC, ROLAND LECLERCQ,* AND JEAN DUVAL

Service de Bactériologie-Virologie-Hygiène, Hôpital Henri Mondor, Université Paris XII, 94010 Créteil, France

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We determined the MICs of 28 antimicrobial agents against 36 clinical strains of *Pediococcus* spp. (25 *P. acidilactici*, 9 *P. pentosaceus*, and 2 *P. urinaeequi* strains). Penicillin G, imipenem, gentamicin, netilmicin, erythromycin, clindamycin, rifampin, chloramphenicol, daptomycin, and ramoplanin were the most active. All strains of *P. acidilactici* were susceptible to novobiocin, whereas all isolates of *P. pentosaceus* were resistant. Novobiocin could therefore be helpful for differentiation of these two closely related species. *P. acidilactici* HM3020 was inducibly resistant to macrolide, lincosamide, and streptogramin B-type (MLS) antibiotics. Resistance was due to a determinant homologous to *ermAM* and carried by a nontransferable 46-kb plasmid, pVM20. This plasmid was structurally distinct from two enterococcal MLS resistance plasmids, pIP819 and pAMβ1. The 34 strains of *P. acidilactici* and *P. pentosaceus* were resistant to tetracycline, and total DNA of these strains did not hybridize to probes specific for *tetK*, *tetL*, *tetM*, and *tetO*.

Pediococci are facultative anaerobe homofermentative gram-positive cocci belonging to the lactic acid bacteria group (30). They are common on plants, in dairy products, and in alcoholic beverages (9) and are used in the food industry and as silage additives (29). They have also been found in human saliva and feces (31, 33). Although pediococci are classically considered as nonpathogenic for humans, two closely related species, *Pediococcus acidilactici* and *Pediococcus pentosaceus*, have been isolated with an increasing frequency from clinical specimens during the last few years (5, 26, 30). Two cases of septicemia caused by these species have been reported (6, 10). Pediococci are intrinsically resistant to high levels of glycopeptides (vancomycin and teicoplanin). They share this property with other gram-positive bacteria, leuconostocs, and certain species of lactobacilli (35). These glycopeptide-resistant bacteria are emerging as opportunistic pathogens in humans, possibly in relation to an increased use of vancomycin and teicoplanin in therapeutics (17).

Few studies on the antimicrobial susceptibility of pediococci have been performed (35, 39), and the mechanisms of antibiotic resistance in this genus have not yet been studied. In this work, we tested the activity of 28 antimicrobial agents against 36 strains of *Pediococcus* spp. and studied the mechanism and genetic basis of macrolide resistance in a clinical isolate, *P. acidilactici* HM3020.

MATERIALS AND METHODS

Bacterial strains and plasmids. A total of 32 clinical strains of *Pediococcus* spp. (23 *P. acidilactici*, 7 *P. pentosaceus*, and 2 *P. urinaeequi* strains) isolated from blood and stool cultures in France and Belgium between 1989 and 1991 and 4 type strains (*P. acidilactici* ATCC8042 and DSM20284 and *P. pentosaceus* ATCC8081 and ATCC33316) were studied. Erythromycin-resistant strain *P. acidilactici* HM3020 was isolated from a stool specimen in 1989. Strains were identified by the methods described by Facklam et al. (8) and Garvie (9). *Staphylococcus aureus* ATCC25923 was used as a control for susceptibility testing. Streptomycin-resistant strains *Lactococcus lactis* IL1419 (22) and *Enterococcus faecalis* BM4110 (7) and novobiocin-resistant strain *P. pentosaceus* HM3008 (from our collection) were used as recipient strains in mating experiments.

Plasmid pIP819, carrying the *ermAM* and *vanA* genes, was isolated from *E. faecalis* BM4166 (22). Plasmid pAMβ1, carrying the *ermAM* gene, was isolated from *E. faecalis* DS5 (4).

Antibiotic susceptibility testing. Disk-agar diffusion tests were performed on Mueller-Hinton agar supplemented with 5% horse blood (Diagnostics Pasteur, Marnes-la-Coquette, France) with an inoculum of 10^7 CFU/ml. The disks of antibiotics, including that of novobiocin (5 μg), were provided by Diagnostics Pasteur. MICs were determined by the agar dilution technique with Mueller-Hinton agar with 5% horse serum and an inoculum of 10^4 CFU per spot. The antimicrobial agents tested were provided by the manufacturers as powders suitable for susceptibility testing. All cultures were incubated aerobically at 37°C for 24 h. Breakpoints used were those recommended by the Comité de l'Antibiogramme de la Société Française de Microbiologie (1).

Since β-lactams appear to be the primary treatment against infections due to pediococci (6, 10) and since tolerance to these drugs has been described for the closely related genus *Leuconostoc* (13, 16), we tested the bactericidal activity of penicillin G and imipenem by time-kill curves against two clinical isolates, *P. acidilactici* HM3021 and *P. pentosaceus* HM3031 (MICs of penicillin G and imipenem for both strains were 0.5 and 0.12 μg/ml, respectively). Overnight cultures in brain-heart infusion broth (Diagnostics Pasteur) yielded an inoculum of approximately 10^7 CFU/ml. Concentrations of penicillin G of 2 and 16 μg/ml (4× and 32× the MIC, respectively) were used. Imipenem was used at 0.5 and 4 μg/ml (4× and 32× the MIC, respectively). For each drug, the concentrations used were within clinically achiev-

* Corresponding author.

able ranges. After 0, 3, 6, and 24 h of incubation at 37°C, aliquots were plated on blood agar. Plates were incubated at 37°C for 24 h before CFU were counted. Bactericidal activity was defined as a ≥ 3 $\log_{10}$ decrease in the inoculum after 24 h of incubation. The results obtained are means of three independent experiments.

Induction of erythromycin resistance and MLS inactivation experiments. Strain HM3020 was grown overnight in MRS (De Man, Rogosa, and Sharpe) broth (Diagnostics Pasteur) with or without erythromycin at a subinhibitory concentration (0.5 μg/ml). Noninduced and induced cells were then grown in fresh medium, either antibiotic free or containing 32 μg of erythromycin per ml. Bacterial growth was monitored with a spectrophotometer at 600 nm. The results obtained are means of two sets of experiments. Inactivation of macrolide, lincosamide, and streptogramin (MLS) antibiotics was screened by the Gots test with *Micrococcus luteus* ATCC9341 as the indicator organism (12).

Mating and curing experiments. Mating experiments were performed as described previously (38). Antibiotic concentrations for selection were as follows: erythromycin, 5 μg/ml; novobiocin, 8 μg/ml; and streptomycin, 500 μg/ml. Curing of erythromycin resistance with novobiocin at 42°C was performed as described previously (27).

DNA preparation. Total DNA was isolated by phenol-chloroform extraction and plasmid DNA was isolated by ultracentrifugation in dye-buoyant density gradients, as described previously (20). DNA was digested with *Eco*RI and *Hin*dIII restriction endonucleases (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. Digested DNA was analyzed by electrophoresis on 0.8% agarose slab gels.

DNA-DNA hybridization. DNAs of pIP819 (22) and of recombinant pUC18 plasmids containing intragenic portions of *ermA* (pAT271) (24, 28), *ermAM* (pAT274) (24), *ermC* (pAT272) (24), *tetK* (pAT102) (19), *tetL* (pAT103) (15), *tetM* (pAT101) (25), and *tetO* (pAT122) (34) were used as probes. They were labeled with digoxigenin-dUTP in a random hexanucleotide-primed Klenow enzyme-mediated reaction, using a gene probe labeling and detection kit (Boehringer). Digested DNA was transferred after electrophoresis to a nylon sheet. DNA-DNA hybridization in stringent conditions was at 65°C in 0.1% sodium dodecyl sulfate (SDS)–0.7% nonfat dry milk–6× SSC overnight (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) (18). Washings were at 65°C in 2× SSC–0.1% SDS.

RESULTS

Antibiotic susceptibility testing. The results of MIC tests are shown in Table 1. Imipenem was the most active β-lactam tested (MIC for 90% of the strains [MIC_{90}] = 0.12 μg/ml). Penicillin G (MIC_{90} = 0.5 μg/ml), amoxicillin (MIC_{90} = 1 μg/ml), and piperacillin (MIC_{90} = 2 μg/ml) were moderately active. Ticarcillin (MIC_{90} = 16 μg/ml) and cefotaxime (MIC_{90} = 8 μg/ml) were poorly active. This is in contrast with the large zone diameters (33 and 27 mm, respectively) obtained with the two antibiotics.

Netilmicin and gentamicin (MIC_{90} = 1 μg/ml for both) were the most active of the four aminoglycosides tested.

All the strains tested were resistant to pristinamycin factor II, a streptogramin A-type antibiotic. Nearly all strains were susceptible to erythromycin, spiramycin, lincomycin, clindamycin, pristinamycin factor I (a streptogramin B-type antibiotic), and pristinamycin complex (combination of pristinamycin factors I and II). Clindamycin and erythromycin were the most active drugs (MIC_{90}s = 0.015 and 0.12 μg/ml, respectively). Three strains were resistant to macrolides or lincosamides. The two *P. urinaeequi* strains tested showed decreased susceptibility to lincosamides only (MICs of lincomycin = 4 and 8 μg/ml; MICs of clindamycin = 0.5 and 1 μg/ml); one *P. acidilactici* strain, HM3020, had high-level resistance to macrolides and lincosamides and was studied further.

TABLE 1. MICs of 28 antimicrobial agents for 36 strains of *Pediococcus* spp.

Drug	MIC (μg/ml)[a]		
	Range	50%	90%
Penicillin G	0.25–1	0.5	0.5
Amoxicillin	0.25–2	1	1
Ticarcillin	4–32	16	16
Piperacillin	0.5–2	2	2
Imipenem	0.03–0.12	0.12	0.12
Cefaclor	32–128	64	128
Cefotaxime	1–8	4	8
Gentamicin	0.25–2	1	1
Netilmicin	0.25–2	0.5	1
Tobramycin	2–32	4	32
Amikacin	2–32	4	16
Erythromycin	0.06–>128	0.12	0.12
Spiramycin	0.12–>128	0.5	0.5
Lincomycin	0.06–>128	0.5	1
Clindamycin	≤0.008–16	0.015	0.015
Pristinamycin factor I	1–16	4	8
Pristinamycin factor II	16–>128	64	>128
Pristinamycin	0.25–2	0.25	1
Tetracycline	4–128	32	64
Minocycline	1–32	8	16
Novobiocin	0.5–128	1	64
Vancomycin	512–>1,024	>1,024	>1,024
Teicoplanin	32–>1,024	>1,024	>1,024
Daptomycin	0.12–0.5	0.25	0.5
Ramoplanin	0.25–2	0.5	1
Rifampin	0.5–8	2	4
Chloramphenicol	1–8	2	4
Ciprofloxacin	1–32	16	32

[a] 50% and 90%, MICs for 50 and 90% of isolates tested, respectively.

The 34 strains of *P. acidilactici* and *P. pentosaceus* tested were resistant to tetracycline (MIC_{90} = 64 μg/ml). Minocycline was only slightly more active (MIC_{90} = 16 μg/ml). The two *P. urinaeequi* strains were susceptible to tetracycline (MICs = 4 μg/ml) and minocycline (MICs = 1 μg/ml).

The 25 *P. acidilactici* strains tested were susceptible to novobiocin (zone diameters ≥16 mm, MICs ≤2 μg/ml) whereas the 9 *P. pentosaceus* and the two *P. urinaeequi* strains studied were resistant (zone diameters ≤10 mm, MICs ≥32 μg/ml).

All the strains tested were highly resistant to glycopeptides (MIC_{90} > 1,024 μg/ml) and susceptible to daptomycin (MIC_{90} = 0.5 μg/ml) and ramoplanin (MIC_{90} = 1 μg/ml). They were also moderately susceptible to rifampin and chloramphenicol (MIC_{90} of the two drugs = 4 μg/ml). Ciprofloxacin was poorly active (MIC_{90} = 32 μg/ml).

Time-kill curves. The results obtained were similar for the two strains tested, *P. acidilactici* HM3021 and *P. pentosaceus* HM3031. Penicillin G at 2 and 16 μg/ml and imipenem

at 0.5 and 4 μg/ml reduced bacterial counts after 24 h only from 1 $\log_{10}$ to 2 $\log_{10}$ and so were not bactericidal (data not shown). The maximum bacterial count decrease (2 $\log_{10}$) was with imipenem at 4 μg/ml for both strains (data not shown).

Expression of MLS resistance in *P. acidilactici* HM3020. The MICs of erythromycin, spiramycin, and lincomycin were greater than 128 μg/ml, and that of clindamycin was 16 μg/ml. The MIC of pristinamycin factor I was in the susceptible range (MIC = 8 μg/ml). However, by disk-agar diffusion test, the strain appeared susceptible after 24 h of incubation but a marked reduction of the inhibition zone was observed after 36 h of incubation. This was not observed for MLS-susceptible strains. When disks of pristinamycin factor I and erythromycin were placed side by side, an antagonism phenomenon (14) was observed (data not shown). Resistance to erythromycin appears to be inducible since growth in erythromycin-containing broth was more rapid in cells grown in the presence of subinhibitory concentrations of the antibiotic (data not shown). Inactivation of erythromycin and clindamycin was not detected by the microbiological technique used.

Genetic basis of MLS resistance in *P. acidilactici* HM3020. Resistance to MLS antibiotics could not be transferred by conjugation from HM3020 to *L. lactis* IL1419, *E. faecalis* BM4110, or *P. pentosaceus* HM3008. In curing experiments with novobiocin, resistance to erythromycin and lincomycin of HM3020 was lost at a low rate (approximately 1%). Plasmid DNAs from HM3020 and from a cured derivative, HM3020-1, were analyzed by agarose gel electrophoresis before and after digestion with *Hin*dIII or *Eco*RI endonuclease (data not shown). HM3020 carried two plasmids, designated pVM20 and pVM21, 46 and 4 kb in size, respectively. pVM20 was lacking in the MLS-susceptible HM3020-1. The probe specific for *ermAM* hybridized under stringent conditions to a 8-kb *Hin*dIII fragment of pVM20.

We compared pVM20 with two enterococcal MLS resistance plasmids carrying also the *ermAM* determinant, pIP819 and pAMβ1, 34 and 25.5 kb in size, respectively. The *Hin*dIII restriction enzyme pattern of pVM20 differed markedly from those of pIP819 and of pAMβ1, and only two of the six *Hin*dIII fragments of pVM20 hybridized with pIP819 DNA used as a probe (data not shown).

Hybridization with *tet* probes. Total DNA of 24 tetracycline-resistant *P. acidilactici* or *P. pentosaceus* strains, analyzed by Southern blot, did not hybridize under stringent conditions with any of the probes specific for *tetK*, *tetL*, *tetM*, and *tetO* (data not shown). These probes hybridized to total DNA of positive control strains harboring plasmids pAT102 (19), pAT103 (15), pAT101 (25), and pAT122 (34).

DISCUSSION

All strains of *Pediococcus* spp. studied were moderately susceptible to penicillin G, imipenem, gentamicin, and netilmicin, the other β-lactams and aminoglycosides being less active (Table 1). The discrepancies obtained for ticarcillin and cefotaxime between the high MICs and the large zone diameters by disk diffusion can be due either to the low growth rate of the organism or to the presence of blood in the medium used for disk diffusion tests, as previously shown for cefotaxime against enterococci (32). Among the other drugs tested, the most active were clindamycin, erythromycin, daptomycin, ramoplanin, rifampin, and chloramphenicol. These results are in agreement with those obtained in other studies (35, 39). We have shown that pediococci are intrinsically resistant to pristinamycin factor II, a streptogramin A-type antibiotic. However, synergy between pristinamycin factors I and II was conserved when tested by the double-disk synergy test (data not shown). This probably explains the low MICs observed for pristinamycin (MIC_{90} = 1 μg/ml).

Penicillin G and imipenem were not bactericidal against the two strains tested by time-kill curves. Tolerance to the bactericidal effect of penicillin G has also been observed for the closely related genus *Leuconostoc* (13, 16). However, β-lactams seem effective in vivo against pediococci: two reported cases of septicemia due to *Pediococcus* spp. were cured by penicillin G or ampicillin (6, 10).

Susceptibility testing of novobiocin by disk diffusion could be used to differentiate the two clinically important species, *P. acidilactici* (susceptible) and *P. pentosaceus* (resistant), which are difficult to distinguish by biochemical tests, the only reliable one being maltose fermentation (negative for *P. acidilactici* and positive for *P. pentosaceus*) (8).

P. acidilactici HM3020 was inducibly resistant to MLS antibiotics. Macrolides (erythromycin and spiramycin) and lincosamides (lincomycin and clindamycin) were strong inducers; by contrast, pristinamycin factor I was a weak inducer since the MIC of the drug was low (8 μg/ml) and since resistance was expressed in disk diffusion test after 36 h of incubation only. This type of expression resembles those described for streptococci (14).

MLS resistance of HM3020 was mediated by a 46-kb nonconjugative plasmid, pVM20, which carried a determinant homologous to *ermAM* (data not shown). Numerous plasmids, ranging from 7 to 190 kb in size, have been described for *Pediococcus* spp. (11, 29). These plasmids encode bacteriocin production, immunity, and carbohydrate fermentation, but in only one instance antibiotic resistance in a strain of *P. acidilactici* isolated from maize silage (37). Like pVM20, this plasmid codes for MLS resistance only and is nonconjugative; however, its size (60 kb) is much larger and the resistance determinant has not been characterized.

ermAM determinants are widespread in streptococci and enterococci (21). Comparison by restriction and hybridization of pVM20 with two enterococcal MLS resistance plasmids carrying also the *ermAM* determinant, pIP819 and pAMβ1, does not favor an enterococcal or streptococcal origin for pVM20, and its size differs also markedly from that of the small MLS resistance plasmids (less than 7 kb) carrying the *ermAM* determinant isolated from strains of *Lactobacillus* and *Aerococcus* spp. (2, 3). The possibility that the *ermAM* gene of pVM20 was borne by a transposon, as reported for streptococci and enterococci (7, 20, 36), has not been tested in this study.

The 34 strains of *P. acidilactici* and *P. pentosaceus* tested were resistant to tetracycline, minocycline being only slightly more active. All but two of the 42 strains tested by other authors were also classified as resistant or intermediary to tetracycline (MIC >4 μg/ml) (10, 30, 35). Hybridizations under conditions of high stringency showed that the gene conferring tetracycline resistance in pediococci was not homologous to determinants frequently responsible for acquired resistance to this antibiotic in gram-positive cocci (*tetK*, *tetL*, *tetM*, and *tetO*) (23).

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