ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2001, p. 905–912 Vol. 45, No. 3
0066-4804/01/$04.00+0 DOI: 10.1128/AAC.45.3.905–912.2001

Bacteriocin Production in Vancomycin-Resistant and Vancomycin-Susceptible *Enterococcus* Isolates of Different Origins

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Received 15 August 2000/Returned for modification 3 October 2000/Accepted 22 December 2000

Bacteriocin production was determined for 218 *Enterococcus* isolates (*Enterococcus faecalis* [93] and *E. faecium* [125]) obtained from different origins (human clinical samples [87], human fecal samples [78], sewage [28], and chicken samples [25]) and showing different vancomycin susceptibility patterns (vancomycin resistant, all of them *vanA* positive [56], and vancomycin susceptible [162]). All enterococcal isolates were randomly selected except for the vancomycin-resistant ones. A total of 33 isolates of eight different bacterial genera were used as indicators for bacteriocin production. Forty-seven percent of the analyzed enterococcal isolates were bacteriocin producers (80.6% of *E. faecalis* and 21.6% of *E. faecium* isolates). The percentage of bacteriocin producers was higher among human clinical isolates (63.2%, 81.8% of vancomycin-resistant isolates and 60.5% of vancomycin-susceptible ones) than among isolates from the other origins (28 to 39.3%). Only one out of the 15 vancomycin-resistant isolates from human fecal samples was a bacteriocin producer, while 44.4% of fecal vancomycin-susceptible isolates were. The bacteriocin produced by the *vanA*-containing *E. faecium* strain RC714, named bacteriocin RC714, was further characterized. This bacteriocin activity was cotransferred together with the *vanA* genetic determinant to *E. faecalis* strain JH2-2. Bacteriocin RC714 was purified to homogeneity and its primary structure was determined by amino acid sequencing, showing an identity of 88% and a similarity of 92% with the previously described bacteriocin 31 from *E. faecalis* YI717. The presence of five different amino acids in bacteriocin RC714 suggest that this could be a new bacteriocin. The results obtained suggest that the epidemiology of vancomycin resistance may be influenced by different factors, including bacteriocin production.

Organisms of the genus *Enterococcus*, and in particular *Enterococcus faecium*, have become a significant cause of nosocomial infections and usually show multiple drug resistance (45). Resistance to the most commonly used antibiotics for gram-positive bacteria provides these organisms with a selective advantage in the hospital environment (40, 43). In Europe, a number of studies have documented the spread of vancomycin-resistant (Van^r) enterococci in sewage, food, animals, and human fecal samples taken from healthy volunteers (1, 5, 8, 17, 37, 38, 49, 54, 56, 57). Paradoxically, in Europe there is a low incidence of Van^r enterococci among human clinical isolates compared with the United States (45, 55, 58). The factors involved in these epidemiological differences remain unknown.

Bacteriocins are peptides or proteins produced by different bacteria that inhibit the growth of strains and species usually related to bacteriocin-producing bacteria (31). The ability to produce bacteriocins has been shown to confer an ecological advantage (48). In the genus *Enterococcus*, bacteriocin production has been linked to the same genetic determinant as β-hemolysin synthesis (4, 7, 13, 27, 30), and its production is a pathogenic marker (30, 39). In *E. faecalis*, the best-characterized inhibitor substances are the pAD1-encoded bacteriocin-hemolysin (or cytolysin) (50, 52) and the peptide AS-48 (24, 26), both encoded by transferable plasmids (3). Other bacteriocins have been characterized in *E. faecalis* (bacteriocin 31, encoded by a conjugative plasmid [53]) or in *E. faecium* (enterocin A [2], enterocin I [22], enterocin P [11], enterocin L50A/L50B [12], and enterocin B [9]). A number of less-characterized bacteriocins in *Enterococcus* spp. have been reported (enterocin EFS2 [41], enterocin 1146 [47], and enterocin 900 [23], among others). The cotransference of bacteriocin production and the pheromone response, together with antibiotic resistance, have been described for *Enterococcus* strains (28, 39). The purpose of this study was to determine the relationship of bacteriocin production and vancomycin resistance in *Enterococcus* isolates of different species and origins.

(Part of this work was presented previously [R. del Campo et al., Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C95, 1998].)

MATERIALS AND METHODS

Bacterial isolates and media. This study included 218 *Enterococcus* isolates (93 *E. faecalis* and 125 *E. faecium*) with different vancomycin susceptibility patterns (162 vancomycin susceptible [Van^s] and 56 Van^r) and from different origins (87 human clinical samples, 78 human fecal samples, 28 sewage samples, and 25 chicken samples) (Table 1). Enterococcal isolates from human clinical samples (blood, urine, wounds, etc.) corresponded to consecutive *E. faecalis* and *E. faecium* isolates obtained from different patients from San Millán Hospital, La Rioja, Spain (1996). Enterococcal isolates from human fecal samples were recovered from consecutive samples from in- and out-patients in Hospital Clínico, Zaragoza, Spain (1996). Fecal samples were seeded on M-*Enterococcus* agar (Biomérieux, La Balme, France), and one colony per plate was studied and retained if it belonged to the species *E. faecalis* or *E. faecium*. Identification was carried out by the API-20 Strept System (Biomérieux) and by PCR, using specific primers for *E. faecium* (16) and *E. faecalis* (18). Enterococcal isolates from chicken samples corresponded to those recovered from chicken feces or chicken

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TABLE 1. *Enterococcus* isolates used for the screening of bacteriocin production

Species (no.)	Origin (no.)	Phenotype (no.)
E. faecalis (93)	Human clinical samples (57)	Van^r (8)
		Van^s (49)
	Human fecal samples (19)	Van^r (2)
		Van^s (17)
	Sewage (10)	Van^s (10)
	Chicken samples (7)	Van^r (7)
E. faecium (125)	Human clinical samples (30)	Van^r (3)
		Van^s (27)
	Human fecal samples (59)	Van^r (13)
		Van^s (46)
	Sewage (18)	Van^r (5)
		Van^s (13)
	Chicken samples (18)	Van^r (18)

products in the La Rioja area (Spain). All Van^r isolates were characterized as having a *vanA* genotype by PCRs (59) and were included in this study on the basis of vancomycin resistance. A total of 33 isolates of eight different bacterial genera (*Enterococcus, Listeria, Staphylococcus*, *Lactobacillus, Leuconostoc, Pediococcus, Escherichia*, and *Bacillus*) were used as bacteriocin production indicators (Table 2). These isolates were maintained as frozen stocks at −80°C in skim milk (Difco, Detroit, Mich.) and propagated twice in brain heart infusion agar (Difco), with the exception of the strains of the genera *Lactobacillus, Leuconostoc*, and *Pediococcus*, which were grown in Man, Rogosa, & Sharpe (MRS) agar (Biomérieux).

Bacteriocin and β-hemolysin assays. For qualitative bacteriocin detection, 50 μl of an overnight culture of the indicator isolate was added to 5 ml of molten soft tryptic soy broth (Difco) supplemented with 0.5% yeast extract and 0.7% agar (Difco), mixed, and poured onto a yeast extract-supplemented tryptic soy agar plate (Difco). A single colony of each *Enterococcus* isolate to be tested for bacteriocin production was transferred with a sterile toothpick to the agar plate seeded with the indicator. Plates were incubated at 37°C for 48 h and then observed for zones of inhibition around the strains. Isolates were considered bacteriocin producers (BAC$^+$) when they showed activity (inhibition zone) against at least 1 of the 33 indicator isolates. This assay does not discriminate between single or multiple bacteriocin production. β-hemolysin detection was performed in tryptic soy agar medium containing 5% horse blood (Biomérieux). A clear zone of β-hemolysis around the isolate growth was considered a positive reaction.

Susceptibility testing and PCR determinations. The antibiotic resistance phenotype of the enterococcal isolates was determined by agar dilution following the NCCLS standard method (46). For AS-48 bacteriocin and enterocin I detection, PCRs were performed using primers and conditions as described in other studies (22, 36). The pAM401-61 plasmid containing an *Sph*I-*Bgl*II fragment of the AS-48 genetic determinant was used as a positive control for AS-48 bacteriocin detection (kindly supplied by M. Martínez-Bueno); *E. faecium* 6T1a was used as a positive control for enterocin I (22).

Mating experiments. The transferability of bacteriocin production, as well as Van^r and erythromycin resistance (Ery^r) determinants was tested by conjugation using a filter method (19), with *E. faecalis* strain JH2-2 as recipient (rifampin and fusidic acid resistant, vancomycin and erythromycin susceptible, nonbacteriocin producer [Rif^r, Fus^r, Van^s, Ery^s, BAC$^-$]). All donor strains were Rif^s and Fus^s. Vancomycin-resistant transconjugants were first selected onto brain heart infusion agar plates supplemented with rifampin (100 μg/ml), fusidic acid (20 μg/ml), and vancomycin (20 μg/ml); bacteriocin production and Ery^r were then analyzed in the Van^r transconjugants obtained.

Pulsed-field gel electrophoresis (PFGE). Genomic DNA was prepared as previously described (44). A third part of the plug was digested with 10 U of *Sma*I (Amersham Life Science) for 18 h, and then an additional 10 U was added and the sample was left for another 4 h. Electrophoresis was then carried out (CHEF DR-II; Bio-Rad) in a 1.2% agarose gel with 0.5% Tris-borate-EDTA, and the following settings were applied: 5 to 35 s, 6 V/cm^2, and 30 h. The gel was stained with ethidium bromide for UV observation. Isolates were classified as indistinguishable, closely related, possibly related, or different according to previously published criteria for bacterial strain typing (51).

Characterization of the bacteriocin produced by *E. faecium* RC714. To perform a preliminary characterization of the bacteriocin activity from *vanA*-containing *E. faecium* RC714, a cell-free, filter-sterilized (0.22-μm-pore-size Millex-GV filter; Millipore SA, Molsheim, France), stationary-phase MRS culture supernatant was tested for stability to heat, pH, and proteolytic enzymes. To test for heat sensitivity, 1-ml samples were heated to 80, 90, and 100°C for 5, 10, and 20 min each. To test for pH sensitivity, 1-ml aliquots of active supernatants were adjusted to different pH values (3, 4, 5, 6, 7, 8, 9, 10, and 11) with 1 M NaOH or 0.6 M HCl. After the different treatments, the remaining bacteriocin activity was then tested by spotting a 25-μl aliquot on a plate seeded with *E. faecium* AR9 as the indicator strain. Plates were incubated at 37°C for 24 h and then observed for inhibition zones.

Active supernatants from *E. faecium* RC714 were tested for their susceptibility to the following proteolytic enzymes: trypsin, α-chemotrypsin, alkaline protease type XXI, proteinase K, papain, and lysozyme (Sigma, St. Louis, Mo.). All the enzymes (4 mg/ml) were prepared according to the manufacturer's instructions, and an aliquot of 750 μl of this solution was added to 250 μl of the active supernatants. The mixture was then incubated for 24 h, at 37°C for proteinase K, alkaline protease, or lysozyme and at 25°C for the other enzymes. In all cases, the remaining bacteriocin activity was determined by spotting 25 μl onto a plate seeded with the indicator *E. faecium* strain AR9, and then plates were incubated at 37°C for 24 h. Positive and negative controls were included.

Purification of the bacteriocin produced by *E. faecium* RC714. All the purification steps were carried out at room temperature, and all of the chromatographic equipment and media were purchased from Pharmacia Biotech. Bacteriocin was purified from a 2-liter MRS broth culture of the *vanA*-containing *E. faecium* RC714 strain, following the method previously described for the bacteriocin plantaricin S (35). Briefly, the supernantant was ammonium sulfate

TABLE 2. Bacterial isolates used as indicators for the screening of bacteriocin production

Isolate	Resistance phenotype[a]	Origin/source or reference[b]
E. faecium AR1	Van (*vanA*), Ery, Str, Kan	Sewage/LC
E. hirae P9	Van (*vanA*), Ery	Chicken feces/LC
E. faecalis H1	Van (*vanA*), Ery	Human feces/LC
E. faecium 517	Van (*vanA*), Ery, Str, Kan	Human feces/LC
E. gallinarum CECT970	Van (*vanC1*)	Chicken feces/CECT
E. faecalis SF-299	Van (*vanB*)	Gold et al. (25)
E. faecalis AR4	Str	Sewage/LC
E. faecalis AR6	Ery, Str, Kan	Sewage/LC
E. faecalis AR8		Sewage/LC
E. faecium AR9	Ery, Str	Sewage/LC
E. faecalis AR13		Sewage/LC
E. faecium AR18	Ery, Str, Kan	Sewage/LC
E. faecium AR24		Sewage/LC
E. faecalis AR30	Ery, Gen, Tob, Kan	Sewage/LC
E. faecium AR39	Str, Kan	Sewage/LC
E. faecalis AR42	Ery, Str, Kan	Sewage/LC
E. faecalis AR43	Ery, Gen, Tob, Kan, Str	Sewage/LC
E. faecium AR50	Ery	Sewage/LC
E. faecium AR58	Ery, Str, Kan	Sewage/LC
E. faecalis AR69	Str	Sewage/LC
S. epidermidis S3		Human clinical sample/LC
S. aureus M892		Human clinical sample/LC
S. haemolyticus S13		Human clinical sample/LC
E. coli C228		Human clinical sample/LC
L. monocytogenes CECT4032		Food/CECT
L. innocua CECT910		Animal clinical sample/CECT
L. murrayi CECT942		Food/CECT
L. grayi CECT931		Animal feces/CECT
B. subtilis CECT356		Unknown/CECT
L. paracasei C162	Van	Human clinical sample/LC
L. plantarum C193	Van	Human clinical sample/LC
Leuconostoc sp. C214	Van	Human clinical sample/LC
P. pentosaceus AR63	Van	Sewage/LC

[a] Van, vancomycin; Ery, erythromycin; Gen, gentamicin; Tob, tobramycin; Kan, kanamycin; Str, streptomycin. Isolates showed resistance to all the antibiotics listed.

[b] LC, Laboratory collection of the University of La Rioja (Spain); CECT, Spanish Culture Type Collection.

TABLE 3. Bacteriocin production in 218 *Enterococcus* isolates from different origins

Origin	No. of isolates	No. of bacteriocin producers (%)
Human clinical samples	87	55 (63.2)
Human fecal samples	78	29 (37.2)
Sewage	28	11 (39.3)
Chicken fecal samples	25	7 (28)
Total	218	102 (46.8)

precipitated, desalted through a PD10 column, consecutively applied to a cation-exchange and a hydrophobic interaction column, and finally subjected to C_2/C_{18} reverse-phase chromatography. Fractions showing activity after the C_2/C_{18} reverse-phase column step were pooled and subjected to a second run. Fractions from this second run showing inhibitory activity were stored at −80°C in 30% 2-propanol containing 0.1% trifluoroacetic acid until use.

SDS-PAGE. C_2/C_{18} reverse-phase column-purified bacteriocin RC714 was analyzed by sodium dodocyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (34) with an 18.5% acrylamide resolving gel. A molecular mass marker (range, 2,512 to 16,946 Da) kit (Pharmacia Biotech) was used for size standards. After electrophoresis, a gel was silver stained (42), and a similar gel was used for detection of antimicrobial activity (6) with *E. faecium* AR9 as the indicator strain.

N-terminal amino acid sequencing of bacteriocin RC714. Amino acid sequencing was performed by automated Edman degradation with a Beckman LF3000 sequencer-phenylthiohydantoin amino acid analyzer (System Gold) by F. Canals, Institut de Biologia Fonamental "Vicent Villar Palasí", Barcelona University, Barcelona, Spain.

RESULTS

Bacteriocin production in *Enterococcus* isolates. One hundred and two out of 218 (46.8%) *E. faecalis* or *E. faecium* isolates were found to produce an antibacterial substance active against at least 1 of the 33 indicator isolates, thus being considered BAC^+. Eighty percent of the *E. faecalis* isolates were BAC^+, whereas only 21.6% of the *E. faecium* isolates were. The proportion of BAC^+ isolates was significantly higher among isolates from human clinical samples (55 of 87 [63.2%]) than from those of human fecal samples (29 of 78 [37.2%]) ($P = 0.00041$) (Table 3). The frequencies of BAC^+ isolates from other origins were as follows: chicken (7 of 25 [28%]) and sewage (11 of 28 [39.3%]). Among the isolates obtained from human clinical samples, Van^r *Enterococcus* isolates showed a higher proportion of BAC^+ isolates (9 of 11 [81.8%]) than did Van^s isolates (46 of 76 [60.5%]). This trend to higher bacteriocin production among Van^r isolates from human clinical samples was observed for both *E. faecalis* and *E. faecium* isolates (Table 4). However, only 1 out of the 15 (6.7%) *vanA*-containing *Enterococcus* isolates from human fecal samples (13 *E. faecium* and 2 *E. faecalis*) was BAC^+ (*E. faecalis* H1), while 44.4% of human fecal Van^s *Enterococcus* isolates were BAC^+ (58.8% in *E. faecalis* and 39.1% in *E. faecium*).

Among the *vanA*-containing *E. faecium* isolates obtained from sewage, two of five were BAC^+, whereas only 1 of 13 Van^s *E. faecium* isolates was found to be BAC^+. All 10 *E. faecalis* isolates from sewage were Van^s, and 8 of them were BAC^+. All 25 *Enterococcus* isolates studied from chicken samples were Van^r, and 7 of them were BAC^+ (*E. faecalis*, 6 of 7; *E. faecium*, 1 of 18) (Table 4). β-hemolytic activity was detected in 9 of 32 Van^s BAC^+ clinical isolates. Interestingly, this activity was not observed in any of the BAC^+ *vanA*-containing *Enterococcus* isolates.

These data show that there is a high prevalence of BAC^+ isolates among *vanA*-containing *E. faecalis* or *E. faecium* from human clinical samples. To explore the possibility that a number of these isolates could correspond to widely disseminated clones, the PFGE patterns of all the BAC^+ and *vanA*-containing *E. faecalis* (15 isolates) or *E. faecium* (4 isolates) strains from different origins included in this study were analyzed. Among the 15 *E. faecalis* isolates, seven different PFGE patterns were found, and five different patterns were detected among the 8 isolates from human clinical samples. A single pattern was found in four *E. faecalis* strains obtained from human clinical samples in distant geographic sites. All four

TABLE 4. Bacteriocin production in *Enterococcus* isolates from different origins and with different vancomycin susceptibility patterns

Origin (no. of isolates)	Resistance phenotype (no. of isolates)	No. of bacteriocin producers (%)	Species (no.)	No. of bacteriocin producers (%)
Human clinical samples (87)	Van^r (11)	9 (81.8)	*E. faecalis* (8)	8 (100)
			E. faecium (3)	1 (33.3)
	Van^s (76)	46 (60.5)	*E. faecalis* (49)	42 (85.7)
			E. faecium (27)	4 (14.8)
Human fecal samples (78)	Van^r (15)	1 (6.7)	*E. faecalis* (2)	1 (50)
			E. faecium (13)	0
	Van^s (63)	28 (44.4)	*E. faecalis* (17)	10 (58.8)
			E. faecium (46)	18 (39.1)
Sewage (28)	Van^r (5)	2 (40)	*E. faecium* (5)	2 (40)
	Van^s (23)	9 (39.1)	*E. faecalis* (10)	8 (80)
			E. faecium (13)	1 (7.7)
Chicken samples (25)	Van^r (25)	7 (28)	*E. faecalis* (7)	6 (85.7)
			E. faecium (18)	1 (5.5)
Total (218)	Van^r (56)	19 (33.9)	*E. faecalis* (17)	15 (88.2)
			E. faecium (39)	4 (10.2)
	Van^s (162)	83 (51.2)	*E. faecalis* (76)	60 (78.9)
			E. faecium (86)	23 (26.7)

TABLE 5. Inhibitory activity of BAC$^+$ enterococcal isolates from different origins against each of the bacterial indicators used

Indicator isolate[a]	No. (%) of BAC$^+$ isolates with inhibitory activity against indicators				
	Human clinical samples (55 BAC$^+$)	Human fecal samples (29 BAC$^+$)	Sewage (11 BAC$^+$)	Chicken samples (7 BAC$^+$)	All isolates (102 BAC$^+$)
E. faecalis H1 (*vanA*)	17 (30.9)	2 (6.9)	2 (18.2)	0	21 (20.6)
E. faecium (*vanA*)[b]	21.5 (39.1)	17.5 (60.3)	4.5 (41)	4 (57.1)	47.5 (46.6)
E. hirae P9 (*vanA*)	15 (27.7)	4 (13.8)	2 (18.2)	0	21 (20.6)
E. faecalis SF-299 (*vanB*)	16 (29)	8 (27.6)	6 (54.5)	3 (42.8)	33 (32.3)
E. gallinarum CECT970 (*vanC-1*)	17 (30.9)	11 (37.9)	3 (27.3)	0	31 (30.4)
E. faecalis Van^{sb}	20.5 (37.2)	4.7 (16.3)	4.2 (38.6)	3.2 (46.4)	32.7 (32.1)
E. faecium Van^{sb}	20 (36.3)	10.8 (37.3)	3 (27.3)	4.2 (59.5)	37.3 (36.6)
L. monocytogenes CECT4032	20 (36.3)	16 (55.2)	6 (54.5)	7 (100)	49 (48)
L. innocua CECT910	14 (25.4)	4 (13.8)	5 (45.4)	6 (85.7)	29 (28.4)
L. murrayi CECT942	5 (9)	11 (37.9)	1 (9.9)	1 (14.3)	18 (17.6)
L. grayi CECT931	12 (21.8)	12 (41.3)	3 (27.3)	1 (14.3)	28 (27.4)
L. paracasei C162	12 (21.8)	0	0	0	12 (11.7)
L. plantarum C193	4 (7.2)	0	1 (2.5)	0	5 (4.9)
Leuconostoc sp. C214	1 (1.8)	0	0	0	1 (0.9)
P. pentosaceus AR63	18 (32.7)	10 (34.5)	2 (18.2)	0	30 (29.4)

[a] Indicator isolates correspond to those referred to in Table 2. Results of inhibitory activity against *B. subtilis, Staphylococcus* spp., or *E. coli* are not included, because negative results were obtained with all the BAC$^+$ enterococcal isolates tested.
[b] There is more than one isolate in these indicator categories. The results represent the average values.

BAC$^+$ and *vanA*-containing *E. faecium* isolates corresponded to different PFGE patterns.

Spectrum of activity of bacteriocinogenic isolates. The results of the assay of the inhibitory activity of BAC$^+$ enterococcal isolates against the indicators are summarized in Table 5. In the case of the Van^s *E. faecalis*, Van^s *E. faecium*, and Van^r *E. faecium* isolates, more than one isolate was used as the indicator, and the results express the average values. In general, the most frequently inhibited indicators were *Listeria monocytogenes* (48%), *vanA*-containing *E. faecium* (46.6%), Van^s *E. faecium* (36.6%), and Van^s *E. faecalis* (32.1%). *Pediococcus pentosaceus* was the most inhibited of the lactic acid bacteria studied (29.4%). Among *vanA*-containing *Enterococcus* indicator strains, *E. faecalis* and *E. hirae* were similarly inhibited (20.6%), while *vanA*-containing *E. faecium* was inhibited by 46.6% of the BAC$^+$ isolates.

Interestingly, *vanA*-containing *E. faecalis* was inhibited by 30.9% of the BAC$^+$ isolates from human clinical samples but only by 6.9% of human fecal isolates ($P = 0.0061$). A similar trend was found for *vanA*-containing *E. hirae*, which was also more frequently inhibited by human clinical isolates than by human fecal isolates (27.7 and 13.8%, respectively). However, *vanA*-containing *E. faecium* was inhibited to a higher degree by human fecal isolates than by human clinical ones. *L. monocytogenes* was inhibited by all the BAC$^+$ isolates from chicken samples. *L. monocytogenes* was also more frequently inhibited by BAC$^+$ enterococcal isolates from human fecal samples (55.2%) than by those obtained from human clinical samples (36.3%). None of the BAC$^+$ enterococcal isolates showed inhibitory activity against *Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus haemolyticus,* or *Escherichia coli*.

The 10 BAC$^+$ *vanA*-containing *Enterococcus* isolates obtained from human samples were tested for antimicrobial activity using all of them as producers and indicators. A group of five isolates (*E. faecalis* RC715, RC716, RC719, C215, and E337, corresponding to two different PFGE patterns) showed an identical bacteriocin inhibition pattern that was different from the bacteriocin inhibition patterns of the other five BAC$^+$ *vanA* isolates. A second group of three isolates (*E. faecalis* RC718, C237, and H1, all three with different PFGE patterns) also had a common pattern of bacteriocin inhibition which was quite different from that in the first group. The remaining two isolates (*E. faecalis* RC721 and *E. faecium* RC714) showed two different patterns of bacteriocin inhibition.

Among bacteriocin producers, two types of isolates were considered: (i) isolates producing bacteriocin with a broad interspecific activity, considered as such when members of at least three out of the eight different indicator genera were inhibited by the producers, and (ii) isolates producing bacteriocin with high intraspecific activity, considered as such when they showed antimicrobial activity against at least 10 out of the 20 *Enterococcus* isolates used as indicators.

Bacteriocin producer isolates with broad interspecific activity and high intraspecific activity were more frequently detected among *vanA*-containing *Enterococcus* isolates (44.4 and 88.8%, respectively) than among Van^s isolates (13 and 26%, respectively) obtained from human clinical samples. When other origins were considered, the proportion was lower for *vanA*-containing strains (0 and 40%) and similar for Van^s isolates (24.3 and 24.3%) (Table 6). Strains with simultaneously broad interspecific and high intraspecific activities were much more frequently found among *vanA*-containing isolates (33.3%) than among Van^s isolates (6.5%) from clinical samples. The BAC$^+$ *E. faecalis* isolates showed more frequent high intraspecific activity than the BAC$^+$ *E. faecium* isolates (29 of 75 [38.6%] and 7 of 26 [27%], respectively).

Mating experiments. In all 12 *vanA*-containing *Enterococcus* isolates tested (nine *E. faecalis* and three *E. faecium*), bacteriocin production was cotransferred together with vancomycin and erythromyein resistance to the recipient *E. faecalis* strain JH2-2, with a mating frequency ranging from 5×10^{-2} to 6.6×10^{-8}. In 8 of these 12 isolates, high-level kanamycin and streptomycin resistance was also cotransferred. In all cases, vancomycin-resistant transconjugants showed the same spectrum of inhibitory activity against indicator isolates as the donors, sug-

TABLE 6. Broad interspecific activity[a] and high intraspecific activity[b] of BAC$^+$ enterococcal isolates from different origins and vancomycin resistance phenotypes

Isolates origin (no. of BAC$^+$ isolates)	Phenotype (no.)	No. (%) of isolates with:		
		Broad interspecific activity	High intraspecific activity	Broad interspecific activity plus high intraspecific activity
Human clinical samples (55)	Van^r (9)	4 (44.4)	8 (88.8)	3 (33.3)
	Van^s (46)	6 (13)	12 (26)	3 (6.5)
Human fecal samples (29)	Van^r (1)	0	0	0
	Van^s (28)	8 (28.6)	5 (17.9)	2 (7.1)
Sewage (11)	Van^r (2)	0	1 (50)	0
	Van^s (9)	1 (11.1)	4 (44.4)	1 (11.1)
Chicken samples (7)	Van^r (7)	0	3 (42.8)	0
Total (102)	Van^r (19)	4 (21)	12 (63.1)	3 (15.8)
	Van^s (83)	14 (16.9)	21 (25.3)	6 (7.2)

[a] Broad interspecific activity, inhibition of at least three of the eight different genera tested as indicators.
[b] High intraspecific activity, inhibition of at least 10 of the 20 different *Enterococcus* isolates tested as indicators.

gesting cotransference of the same bacteriocin(s) genetic determinant(s).

Bacteriocin RC714 characterization and purification. The bacteriocin produced by the *vanA*-containing *E. faecium* RC714 strain (which was isolated from a human exudate sample) was chosen for further characterization. Bacteriocin RC714 showed inhibitory activity against all *E. faecalis* (eight Van^s and one *vanA*- and one *vanB*-containing isolate) and *E. faecium* (six Van^s and two *vanA*-containing isolates) strains tested as indicators, as well as against *L. monocytogenes, Listeria innocua, Listeria murrayi, Listeria grayi, Lactobacillus paracasei, Lactobacillus plantarum, Leuconostoc* sp., and *P. pentosaceus*. However, no inhibitory activity was detected against the *vanA*-containing *E. hirae, vanC-1*-containing *E. gallinarum, S. epidermidis, S. aureus, S. haemolyticus, E. coli*, and *B. subtilis. E. faecium* RC714 did not show β-hemolysis, and negative PCR results for the previously reported bacteriocins AS-48 and enterocin I were also obtained. The RC714 strain consistently cotransferred vancomycin and erythromycin resistance and bacteriocin production to the *E. faecalis* strain JH2-2. This bacteriocin was resistant to heat treatment (100°C for 20 min) and was stable in a wide range of pH values (3 to 11). Bacteriocin RC714 was susceptible to the proteolytic activity of trypsin, α-chemotrypsin, papain, alkaline protease, and proteinase K, but it was resistant to lysozyme.

The purification scheme for bacteriocin RC714 is shown in Table 7. After the second reverse-phase chromatography step, a final yield of 1.1% of the initial activity and a 29-fold increase in the specific activity of bacteriocin RC714 was obtained. The overall purification procedure resulted in a single peak upon C_2/C_{18} reverse-phase liquid chromatography (Fig. 1). SDS analysis showed an electrophoretically pure protein with an apparent molecular size of ca. 3,000 Da and with inhibitory activity against *E. faecium* AR9 (Fig. 2). The N-terminal sequencing of purified bacteriocin RC714 allowed determination of a total of 42 amino acid residues. This sequence showed an identity of 88% and a similarity of 92% with bacteriocin 31 previously described by Tomita et al. (53) in an *E. faecalis* strain (Fig. 3). A difference of 5 out of 42 amino acids with respect to bacteriocin 31 was observed.

DISCUSSION

Bacteriocin production has been shown to confer an ecological advantage on the producer strain (48). In this study, a higher proportion of BAC$^+$ isolates was detected among *E. faecalis* (80.6%) than among *E. faecium* (21.6%) isolates. Similarly, Tomita et al. (53) found that 54% of their *E. faecalis* isolates were BAC$^+$. Both in feces and in invasive isolates, *E. faecalis* was more commonly found and was at a higher proportion than *E. faecium* (43). In accordance with our results, this fact may be explained, at least in part, by the ecological advantage of the BAC$^+$ *E. faecalis* strains.

Our data indicate that the proportion of BAC$^+$ isolates

TABLE 7. Purification of bacteriocin RC714 from *E. faecium vanA* RC714

Fraction	Vol (ml)	A_{280}[a]	Total activity (10^5 BU[b])	Sp act[c]	Fold increase in sp act	Yield (%)
Culture supernatant	2,000	388.5	6.4	1.6×10^3	1	100
Ammonium sulfate precipitation (fraction I)	91.5	67.15	9.4	1.4×10^4	8.7	147
Binding to SP-Sepharose fast flow (fraction II)	30.5	37.5	7.7	12.0×10^4	12.5	120
Binding to phenyl-Sepharose CL-4B (fraction III)	23	7.5	0.77	1.0×10^4	6.25	12
FPLC[d] (C_2/C_{18} reverse-phase chromatography)						
First run	3.5	1.21	0.5	4.1×10^4	25.6	7.8
Second run	0.6	0.15	0.07	4.6×10^4	29.0	1.1

[a] Total A_{280} is the A_{280} multiplied by the volume in milliliters.
[b] BU, bacteriocin units.
[c] Specific activity is the BU divided by the A_{280}.
[d] FPLC, fast-performance liquid chromatography.

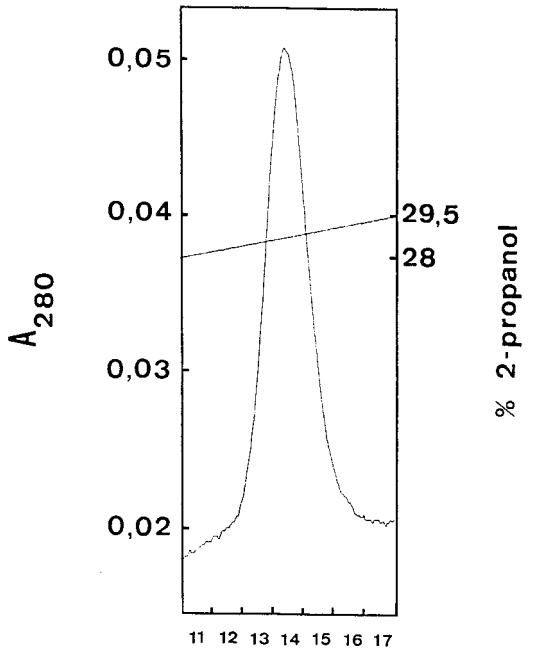

FIG. 1. C_2/C_{18} reverse-phase chromatography of bacteriocin RC714 (second run). Numbers below the graph indicate the fraction (0.15 ml each) exhibiting bacteriocin RC714 activity.

among human clinical isolates was significantly higher (63.2%) than that among human fecal isolates (37.2%). Among Van^r isolates, bacteriocin production was found in 81.8% of the isolates from human clinical samples and in 6.7% of those from human fecal samples. In addition to the low frequency of bacteriocin production by *vanA*-containing *Enterococcus* isolates from human fecal origin, Van^s BAC^+ enterococcal isolates of fecal origin showed a high inhibitory activity against *vanA*-containing *E. faecium* isolates (Table 5). In fact, more than half (60.3%) of all our BAC^+ human fecal *Enterococcus* isolates, all of them Van^s, inhibited both clones of *vanA*-containing *E. faecium* used as indicators. Only 6.19% of these isolates inhibited the *vanA*-containing *E. faecalis* H1 strain used as indicator. Moreover, not only most *vanA* enterococcal isolates obtained from human clinical samples were BAC^+, but their bacteriocin activities showed broad interspecific activity and high intraspecific activity in a higher proportion than BAC^+ isolates from the other origins. Most BAC^+ and *vanA*-containing *E. faecium* and *E. faecalis* isolates corresponded to different PFGE patterns, thus indicating that the results were not severely biased by the predominance of a particular widespread clone. These observations suggest an ecological advantage of bacteriocinogenic strains for colonization and for invasion, as previously postulated (30, 39).

L. monocytogenes was the *Listeria* species most inhibited by BAC^+ enterococcal isolates of different origins. The activities of BAC^+ enterococcal isolates against lactic acid bacteria indicate that *P. pentosaceus* is the most susceptible strain to this antimicrobial inhibition. The moderate activities of BAC^+ isolates against lactic acid bacteria and the absence of activity against *Bacillus* or staphylococci suggest high bacteriocin specificity, preferentially mediating amensalistic interactions among different enterococcal populations in the intestinal habitat.

The bacteriocins detected in *E. faecalis* frequently correspond to the bacteriocin/hemolysin encoded by the plasmid pAD1 (32), which usually also confers a sex pheromone response (13, 60). The bacteriocin/hemolysin has been associated with virulence in animal models (10, 29, 33). Nine of our 32 BAC^+ tested isolates (eight *E. faecalis* and one *E. faecium*) (28%) were β-hemolytic, and all of them were Van^s and of clinical origin. None of our BAC^+ Van^r enterococcal isolates showed this β-hemolytic activity. Tomita et al. (53) detected a high proportion of BAC^+ isolates among their *E. faecalis* isolates (54%), and 68% of these bacteriocin producers showed β-hemolytic activity. The occurrence of β-hemolysin in clinical isolates of *E. faecalis* varied from 17 to 60% in different studies (14, 21, 30). Similarly, Coque et al. (15) detected β-hemolysin activity among *E. faecalis* strains of different origins (16 to 37%) but not in non-*E. faecalis* isolates.

This study has demonstrated the cotransference of vancomycin and erythromycin resistance with bacteriocin production (but not β-hemolysin) in 12 *vanA*-containing enterococcal isolates, corresponding to nine different PFGE patterns of *E. faecalis* (nine isolates, six clones) and *E. faecium* (three isolates,

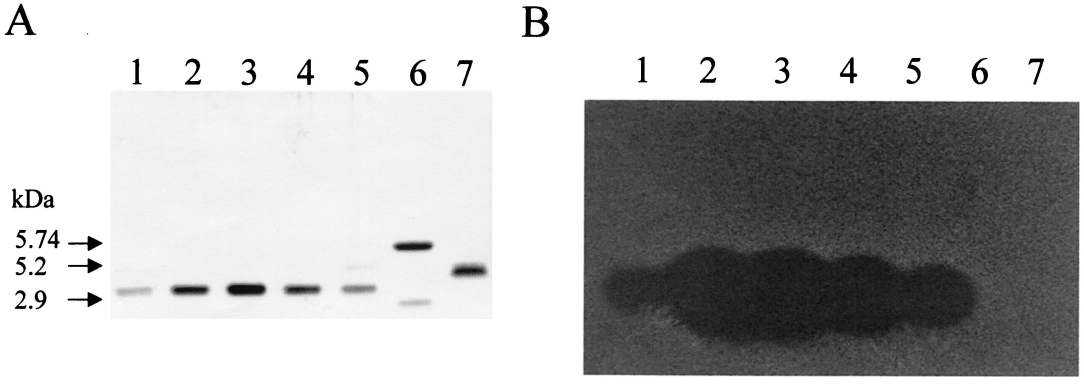

FIG. 2. SDS-PAGE of bacteriocin RC714 and detection of antimicrobial activity. (A) Silver-stained gel. (B) Gel fixed in 20% 2-propanol–10% acetic acid and washed in deionized water as described by Bhunia et al. (6). The gel was then placed on an MRS agar plate and overlaid with MRS soft agar containing *E. faecium* AR9. Lanes 1 to 5, fraction numbers 13 to 17 of C_2/C_{18} second run-purified bacteriocin RC714 (see Fig. 1); lane 6, purified pIS β peptide (35); lane 7, purified enterocin I (22). Size standards are indicated on the left.

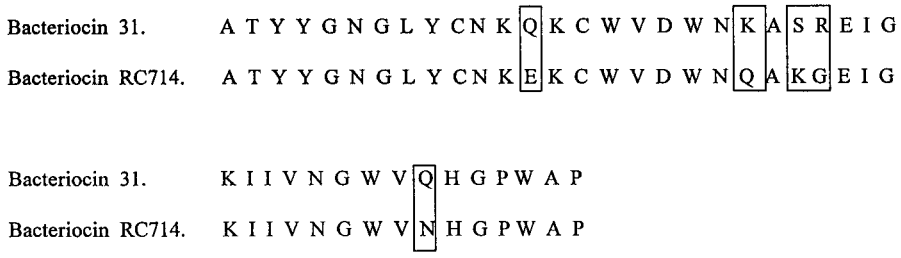

FIG. 3. Comparison of bacteriocin RC714 amino acid sequence with that corresponding to bacteriocin 31 (53). The different amino acids are indicated.

three clones). In all cases, the selection for transconjugants was first performed for vancomycin resistance, and bacteriocin production and other antibiotic resistance determinants were then evaluated in Van^r transconjugants. Notably cotransference does not mean association between bacteriocin production and antibiotic resistance, and the eventual presence of more than one bacteriocin in the transconjugants cannot be ruled out. Nevertheless, the frequent cotransference may have ecological consequences. In 1990, Handwerger et al. (28) described the cotransference of vancomycin resistance and a pheromone response as well as β-hemolytic activity in *E. faecium*. Cotransference of high-level aminoglycoside, erythromycin, and chloramphenicol resistance and bacteriocin/hemolysin has also been previously reported (30, 39, 40).

Sequence analysis of the bacteriocin RC714 revealed that our bacteriocin belongs to the class II bacteriocins (small, heat-stable, non-lantionine-containing peptides) (20). A bacteriocin similar to RC714 was previously described in an *E. faecalis* strain from Japan by Tomita et al. (53) and was named bacteriocin 31. Bacteriocin RC714 has a difference of 5 amino acids from the deduced sequence of 42 amino acids of bacteriocin 31 (Fig. 3) and originated from a clinical *vanA*-containing *E. faecium* strain. Also, our bacteriocin showed a wide range of activity against different indicator isolates of different genera (5) and species (10) of gram-positive bacteria. On the basis of the N-terminal amino acid sequences, the bacteriocin RC714 could represent a new bacteriocin (enterocin) different from bacteriocin 31.

The use of glycopeptides in humans and animals has been previously associated with the dissemination of Van^r enterococci (45). The present work suggests that other factors should be taken into account. The very frequent association of bacteriocin production and vancomycin resistance in different enterococcal clones isolated from human clinical samples suggests that the production of amensalistic substances may enhance extra-intestinal colonization. In contrast, bacteriocin production was infrequently found among our vancomycin-resistant enterococcal strains from human fecal samples. That may suggest that these nonbacteriocinogenic vancomycin-resistant fecal isolates may remain at low density in the intestinal tract. Whether these observations help explain the discrepancies in the rates of vancomycin resistance among enterococcal isolates from invasive infections versus fecal isolates in Europe and the United States remains to be evaluated. Such evaluation will require a broader sampling of vancomycin-resistant strains of different origins and/or the use of animal models. This work indicates that a complete understanding of the epidemiology of vancomycin resistance in *Enterococcus* will probably require the simultaneous consideration of different factors involved in the dissemination and selection of particular strains in different environmental compartments.

ACKNOWLEDGMENTS

We are grateful to F. Marco, B. Robledo, J. Castillo, E. Cercenado, M. Lantero, and A. Fleites, as well as to the Spanish Culture Type Collection, for providing us with some of the strains used in this study. We also thank M. Martínez-Bueno for providing us with the plasmid pAM401-61, A. Portillo and C. Martinez for technical assistance, and L. de Rafael, M. Morosini, and T. Coque for critical review of the manuscript.

R. D. C. was supported by a grant from the Diputación General de Aragón of Spain (project P49/97) and from the Sociedad Española de Quimioterapia. This work has been supported in part by a grant from the Fondo de Investigaciones Sanitarias (00/0545) of Spain.

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