ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1994, p. 1788–1793
0066-4804/94/$04.00+0

Vol. 38, No. 8

Analysis of Genes Encoding D-Alanine–D-Alanine Ligase-Related Enzymes in *Enterococcus casseliflavus* and *Enterococcus flavescens*

FERRAN NAVARRO† AND PATRICE COURVALIN*

Unité des Agents Antibactériens, Centre National de la Recherche Scientifique, EP J0058, Institut Pasteur, 75724 Paris Cedex 15, France

Received 22 March 1994/Returned for modification 19 May 1994/Accepted 7 June 1994

Using degenerate oligonucleotides complementary to sequences encoding conserved amino acid motifs in D-alanine–D-alanine (Ddl) ligases, we have amplified ca. 600-bp fragments from *Enterococcus casseliflavus* ATCC 25788 and *Enterococcus flavescens* CCM439. Sequence analysis of the amplification products indicated that each strain possessed two genes, $ddl_{E.\ cass.}$ and *vanC-2*, and $ddl_{E.\ flav.}$ and *vanC-3*, respectively, encoding Ddl-related enzymes. The fragments internal to the *vanC* genes were 98.3% identical. The *vanC-2* gene was cloned into *Escherichia coli* and sequenced. Extensive similarity (66% nucleotide identity) was detected between this gene and *vanC-1* from *Enterococcus gallinarum* (S. Dutka-Malen, C. Molinas, M. Arthur, and P. Courvalin, Gene 112:53–58, 1992), suggesting that the *vanC* genes are required for intrinsic low-level resistance to vancomycin. The partial deduced amino acid sequences of $ddl_{E.\ cass.}$ and $ddl_{E.\ flav.}$ were identical and closely related to that of the Ddl ligase of *Enterococcus faecalis* (79% identity). In Southern hybridization experiments, only DNA from *E. casseliflavus* and *E. flavescens* hybridized to probes internal to the *vanC-2* and $ddl_{E.\ cass.}$ genes.

The glycopeptides vancomycin and teicoplanin constitute an important class of antibiotics for the treatment of severe infections due to gram-positive bacteria. Glycopeptides bind to the peptidyl-D-alanyl–D-alanine (D-Ala–D-Ala) termini of peptidoglycan precursors at the cell surface, thereby inhibiting cell wall synthesis (2). The D-Ala residues that interact with glycopeptides are incorporated into peptidoglycan precursors as a dipeptide synthesized by D-alanine–D-alanine ligases (Ddl). Acquired resistance to glycopeptides in enterococci is due to production of D-Ala–D-Ala structurally related ligase VanA (6) or VanB (15), which synthesizes the depsipeptide D-alanyl–D-lactate (D-Ala–D-Lac) (3, 18). The depsipeptide replaces the dipeptide D-Ala–D-Ala, leading to synthesis of peptidoglycan precursors that bind vancomycin with greatly reduced affinity (6, 23). D-Ala–D-Ala and D-Ala–D-Lac ligases are structurally related (12, 15).

Enterococci with acquired glycopeptide resistance harbor two related but distinct gene clusters, *vanA* and *vanB*, defined by hybridization with DNA probes derived from the structural genes for the VanA and VanB ligases, respectively (11, 28). Strains possessing the *vanA* operon are inducibly resistant to high levels of vancomycin and teicoplanin (VanA phenotype). Strains with the *vanB* gene cluster are inducibly resistant to various levels of vancomycin but remain susceptible to teicoplanin (VanB phenotype). Three species of enterococci are intrinsically resistant to low levels of vancomycin only: *Enterococcus gallinarum*, *Enterococcus casseliflavus*, and *Enterococcus flavescens*. The *vanC-1* gene of *E. gallinarum* BM4174 encodes a D-Ala–D-serine ligase required for low-level vancomycin resistance (13, 29). The *vanC-1* gene was detected in all strains of *E. gallinarum* tested but not in *E. casseliflavus* and *E. flavescens* (21). In this study, we analyze the nucleotide sequence of genes encoding D-Ala–D-Ala ligase-related proteins from these two intrinsically resistant species.

MATERIALS AND METHODS

Bacterial strains. Twenty-four *E. casseliflavus* strains and two *E. flavescens* strains were collected from various sources (Table 1). As controls, we included well-characterized glycopeptide-resistant enterococcal strains belonging to phenotypes VanA, VanB, and VanC. These were *Enterococcus faecium* BM4147 (VanA [12]), *Enterococcus faecalis* V583 (VanB [15]), and five strains of *E. gallinarum*: NCDO 2313, A13 (from the laboratory collection of L. A. Devriese), BM4115 (from our laboratory collection), 6764 (from the bioMérieux collection [Marcy l'Etoile, France]), and BM4174 (VanC [13]). The following susceptible strains belonging to 16 different species were used: *Enterococcus avium* NCDO 2369, *Enterococcus cecorum* NCDO 2674, *Enterococcus columbae* STR 345, *Enterococcus dispar* NCIMB 13000, *Enterococcus durans* NCDO 596, *E. faecalis* NCDO 581, *E. faecium* NCDO 942, *Enterococcus hirae* NCDO 1258, *Enterococcus malodoratus* NCDO 846, *E. mundtii* NCDO 2375 and API 9001120, *Enterococcus pseudoavium* NCDO 2138, *Enterococcus raffinosus* NCTC 12192, *Enterococcus saccharolyticus* NCDO 2594, *Enterococcus solitarius* NCTC 12193, *Enterococcus sulfureus* NCDO 2379, and *Enterococcus seriolicida* ATCC 49156.

Identification of enterococci. Enterococci were identified as described previously (10) with the 30°C motility test and API 50 CH strips (bioMérieux).

Culture conditions and antibiotic susceptibility tests. All cultures were performed at 37°C in brain heart infusion broth and agar (Difco Laboratories, Detroit, Mich.). The method of Steers et al. (33), with 10^4 CFU per spot, was used to determine the MICs of antibiotics on Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France).

DNA manipulation. Total (20) and plasmid (5) DNA was prepared as described previously. Restriction fragments were

* Corresponding author. Mailing address: Unité des Agents Antibactériens, Institut Pasteur, 28, rue du Docteur Roux, 75724 Paris Cedex 15, France. Phone: (33) (1) 45 68 83 20. Fax: (33) (1) 45 68 83 19.

† Present address: Servei de Microbiologia, Hospital de la Santa Creui Sant Pau, Barcelona, Spain.

TABLE 1. Properties of the *E. casseliflavus* and *E. flavescens* strains

Strain	Yellow pigment	Motility	MIC (μg/ml)[a]		Acid production from ribose	Fragment hybridizing with[b]:		Source or reference[c]
			Vm	Te		*vanC-2*	$ddl_{E.\ cass.}$	
E. casseliflavus								
ATCC 25788	+	+	8	0.5	+	2.5	4.1	37
8410085	+	+	4	0.5	+	2.5	4.1	API
8407106	+	+	8	0.5	+	2.5	4.1	API
A22	+	+	1	0.25	+	4.2	6.8	8
9001123	+	+	4	0.5	+	9.5	2.6	API
9001122	+	+	4	0.5	+	8.6	2.6	API
9001119	+	+	4	0.25	+	4.2	6.8	API
8911032	+	+	8	1	+	7.9	3.3	API
8910035	+	+	8	0.5	+	2.5	4.1	API
ATCC 25789	+	−	4	0.5	+	2.5	4.1	37
LT52	+	−	8	0.5	+	7.4	3.3	37
LT62	+	+	8	0.5	+	7.4	3.9	37
LT31	−	+	4	0.5	+	7.4	7	37
C7	+	+	8	0.5	+	7.4	6.5	37
G13	−	+	1	0.25	+	7.4	7	37
NCDO 2154	+	+	4	1	+	2.5	4.1	NCDO
NCDO 2310	+	+	4	0.5	+	2.5	4.1	NCDO
NCDO 2376	+	+	4	1	+	2.5	4.1	NCDO
NCDO 2378	+	+	8	0.5	+	8.3	7.5	NCDO
NCDO 2380	+	+	8	0.5	+	2.3	4.2	NCDO
NCDO 2725	+	+	2	0.5	+	7.9	7.7	NCDO
ATCC 14436	+	+	8	1	+	8.1	3.2	26
ATCC 12817	+	+	8	0.5	+	7.9	3.2	26
ATCC 13638	+	+	8	0.5	+	7.9	4	7
E. flavescens								
CCM 439	+	−	8	1	−	4.1	8.2	26
CCM 441	+	−	8	0.5	−	4.1	8	26

[a] Vm, vancomycin; Te, teicoplanin.
[b] Size in kilobases of the *Kpn*I fragment hybridizing with the *vanC-2* and *ddl* probes.
[c] API, API-bioMérieux collection; NCDO, National Collection of Dairy Organisms, Reading, United Kingdom.

analyzed by 0.8% agarose gel electrophoresis. DNA fragments were extracted from gels with the QIAEX Gel Extraction Kit (Qiagen Inc., Chatsworth, Calif.). For Southern hybridization, DNA was transferred by vacuum onto Nytran membranes (Schleicher & Schuell, Dassel, Germany) with a Trans Vac TE80 apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). Prehybridization and hybridization were performed under stringent conditions at 68°C in 0.1% sodium dodecyl sulfate (SDS)–0.7% nonfat dry milk–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (21). Membranes were washed at 68°C in 0.1% SDS–2× SSC. Probes were labeled with [α-^{32}P]dCTP (Radiochemical Centre, Amersham, England) with a nick-translation kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.).

DNA amplification, cloning, and sequencing. DNA amplification by PCR was performed with oligonucleotides V1 and V2 (13). The PCR products were purified by gel electrophoresis and cloned into the *Sma*I site of pUC18 (24). Nucleotide sequencing was by the dideoxynucleotide chain terminator technique (32) with modified T7 DNA polymerase (Sequenase; U.S. Biochemicals, Cleveland, Ohio), α-^{35}S-dATP (Radiochemical Centre), and synthetic oligodeoxyribonucleotides complementary to the sequence (Unité de Chimie Organique, Institut Pasteur, Paris, France).

Computer analysis of sequence data. Nucleotide and amino acid sequences were compared by using GenBank, EMBL, and Swiss-Prot databases with the Genetics Computer Group software (9) and with the program FASTA (25).

Enzymes and chemicals. Restriction endonucleases and T4 DNA ligase were purchased from Amersham Corp., Arlington Heights, Ill. RNase A (bovine pancreas) and proteinase K were from Calbiochem-Behring (La Jolla, Calif.). SeaKem GTG agarose was from FMC Bioproducts (Rockland, Maine). Teicoplanin was provided by Gruppo Lepetit (Milan, Italy), and vancomycin was from Eli Lilly & Co. (Indianapolis, Ind.).

Nucleotide sequence accession numbers. The nucleotide sequence of *vanC-2* and the partial sequences of *vanC-3*, $ddl_{E.\ cass.}$, and $ddl_{E.\ flav.}$ genes have been deposited in the GenBank data library (Los Alamos, N.Mex.) under accession numbers L29638, L29639, L29641, and L29640, respectively.

RESULTS AND DISCUSSION

Identification of *Enterococcus* spp. Differentiation among *E. faecium*, *E. gallinarum*, *E. casseliflavus*, and *E. flavescens* is based on a few physiological tests, the most discriminative being motility at 30°C and production of a yellow pigment (10). However, certain strains of *E. casseliflavus* were nonpigmented, and the motility test was not totally reliable (Table 1), as already reported (37). Absence of these traits can lead to misidentification of enterococcal clinical isolates.

Intrinsic resistance of *E. casseliflavus* and *E. flavescens* to vancomycin. The MIC of vancomycin for the reference strain *E. casseliflavus* ATCC 25788 was 8 μg/ml. The MICs for the 23 other *E. casseliflavus* strains ranged from 1 to 8 μg/ml (Table 1). The MICs for two strains were only 1 μg/ml, and that for one strain was 2 μg/ml. The two strains of *E. flavescens* were also resistant to low levels of the antibiotic, with vancomycin

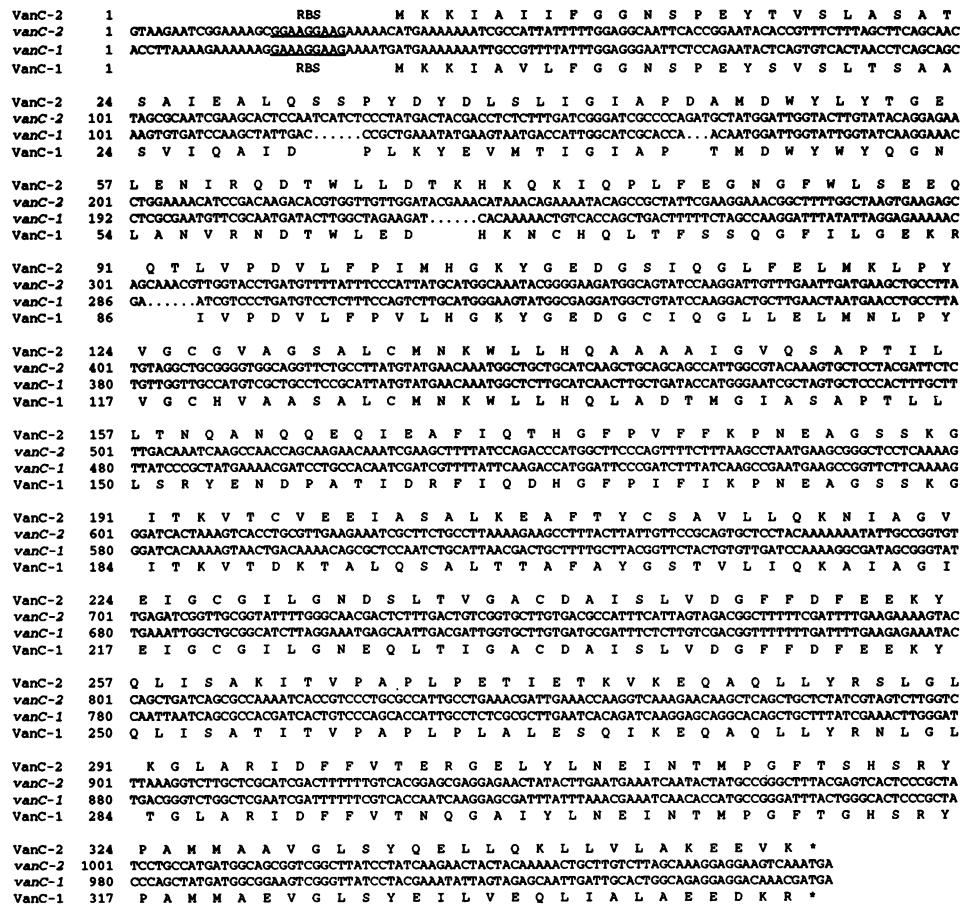

FIG. 1. Comparison of DNA and deduced amino acid sequences of *vanC-2* from *E. casseliflavus* ATCC 25788 and *vanC-1* from *E. gallinarum* BM4174 (13). The putative ribosome binding sites (RBS) are underlined. Dots indicate gaps.

MICs of 8 μg/ml. By contrast, the MICs of vancomycin for strains belonging to the other enterococcal species studied were between 0.25 and 2 μg/ml. These results confirm that vancomycin MICs for *E. casseliflavus* and *E. flavescens* are usually higher than those for other enterococcal species (34, 36). For the former species, the low MIC breakpoint of vancomycin (4 μg/ml) (1, 22) divides the bacterial population into two parts, and half of the isolates are categorized as susceptible (Table 1).

Nucleotide sequence of the *vanC-2* gene from *E. casseliflavus* ATCC 25788. Degenerate oligonucleotides V1 and V2 (13) are complementary to sequences encoding amino acid motifs that are conserved in DdlA and DdlB ligases of *Escherichia coli* and in resistance proteins VanA, VanB, and VanC. The oligonucleotides allow amplification by PCR of fragments internal to genes encoding Ddl-related enzymes (13). Amplification of ca. 600-bp fragments from *E. casseliflavus* ATCC 25788 was obtained with V1 and V2 (data not shown). The PCR products were cloned into pUC18 in *E. coli*, and the sequence of the insert of a recombinant plasmid was determined. Sequence comparison indicated that the insert corresponded to a fragment internal to a Ddl-related gene that was designated *vanC-2*. A genomic library from *E. casseliflavus* ATCC 25788 was constructed by cloning total DNA digested partially with *Sau*3AI into bacteriophage λEMBL3 (17). Recombinant phages containing the *vanC-2* gene were identified by plaque hybridization with the cloned PCR fragment as a probe (4). The DNA of a recombinant bacteriophage was purified and digested with *Sph*I, and a 2-kb fragment containing the *vanC-2* gene was subcloned into pUC18. The sequence of *vanC-2* was determined, and the nucleotide and the deduced amino acid sequences were compared with those of the *vanC-1* gene from *E. gallinarum* BM4174 (Fig. 1). Extensive similarity was detected between *vanC-1* and *vanC-2* (66% nucleotide identity) and the corresponding gene products (69% amino acid identity), suggesting that VanC-2 is involved in low-level vancomycin resistance like VanC-1.

Nucleotide sequence of fragments internal to genes encoding D-Ala–D-Ala ligase-related enzymes from *E. casseliflavus* and *E. flavescens*. Sequencing of other cloned PCR products indicated that V1 and V2 amplified fragments of two genes from *E. casseliflavus* ATCC 25788, one corresponding to *vanC-2* and the other to a gene designated $ddl_{E.\ cass.}$ Similarly, two amplification products were obtained with DNA from *E. flavescens* CCM 439. The sequence of these fragments was determined, and the corresponding genes were designated *vanC-3* and $ddl_{E.\ flav.}$ The fragments internal to the *vanC-2* and *vanC-3* genes differed at 10 positions, leading to four amino acid differences (Fig. 2A). The fragments of $ddl_{E.\ cass.}$ and $ddl_{E.\ flav.}$ displayed 25 differences, but the deduced amino acid sequences were identical (Fig. 2B). This observation suggests that the *ddl* genes are under a strong selective pressure which eliminates missense mutations.

Analysis of the sequence of the PCR products showed that

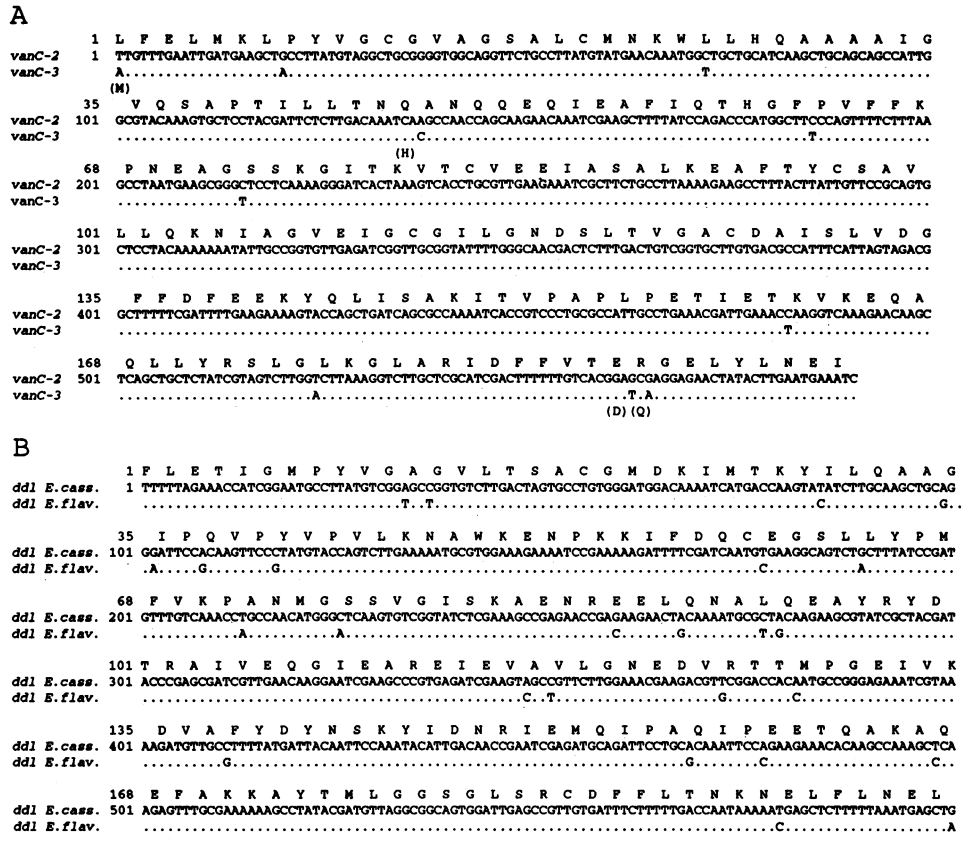

FIG. 2. Comparison of partial DNA and deduced amino acid sequences of *vanC-2* of *E. casseliflavus* and *vanC-3* of *E. flavescens* (A) and *ddl* of *E. casseliflavus* (*ddl E. cass.*) and *ddl* of *E. flavescens* (*ddl E. flav.*) (B). Dots indicate positions where identical nucleotides were present. The deduced amino acid sequences of *vanC-2* and *ddl E. cass.* are indicated above the nucleotide sequence. Amino acid substitutions in VanC-3 are indicated in parentheses below the sequence of *vanC-3*.

E. casseliflavus and *E. flavescens* each possess two genes encoding D-Ala–D-Ala ligase-related enzymes. The interspecies nucleotide sequence divergence between the *vanC* (1.7%) and *ddl* (4.2%) genes was surprisingly low compared with that expected for housekeeping genes from different species or relative to estimates of sequence diversity between the genomes of *E. casseliflavus* and *E. flavescens* based on DNA-DNA hybridization experiments (26).

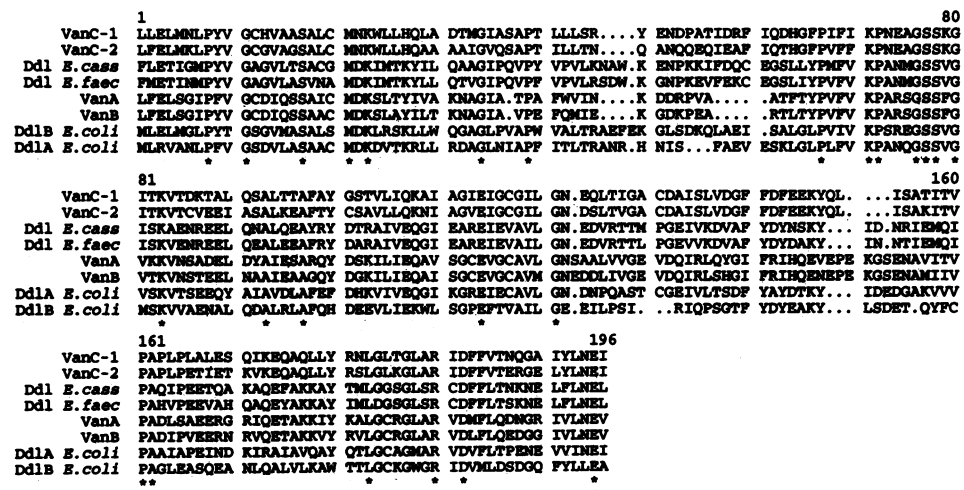

FIG. 3. Alignment of the deduced partial amino acid sequences of VanC-1, VanC-2, Ddl of *E. casseliflavus* (Ddl *E. cass*), Ddl of *E. faecalis* (Ddl *E. faec*) (14), VanA (12), VanB (14), and DdlA (38) and DdlB (30) of *E. coli*. Stars indicate positions where identical amino acids were present in all sequences.

TABLE 2. Sequence identity between the deduced partial amino acid sequences of ligases[a]

Sequence compared	% Sequence identity with:								
	VanC-1	VanC-2	VanC-3	$Ddl_{E.\ cass.}$[b]	$Ddl_{E.\ faec.}$	VanA	VanB	$DdlA_{E.\ coli}$	$DdlB_{E.\ coli}$
VanC-1		73	73	32	34	38	38	34	35
VanC-2			98	33	34	37	39	36	33
VanC-3				33	34	37	38	37	34
$Ddl_{E.\ cass.}$					79	33	35	42	35
$Ddl_{E.\ faec.}$						31	35	42	36
VanA							77	34	30
VanB								35	32
$DdlA_{E.\ coli}$									39
$DdlB_{E.\ coli}$									

[a] Identity between pairs of sequences was derived from the alignment in Fig. 3.
[b] The deduced partial amino acid sequences of $Ddl_{E.\ cass.}$ and $Ddl_{E.\ flav.}$ were identical.

The distinction between *E. casseliflavus* and *E. flavescens* is tenuous since the two species are differentiated only on the basis of acid production from ribose (26). The high degree of identity between the ligase genes (Fig. 2B) is not consistent with the assignation of clinical isolates of *E. casseliflavus* and *E. flavescens* to two different species.

Amino acid sequence comparison. The deduced amino acid sequences of the amplification products of *vanC-2* and $ddl_{E.\ cass.}$ were aligned with the corresponding portions of other ligases (Fig. 3), and the percentages of identity were derived for pairs of sequences (Table 2). Identity was significantly higher between VanC-1 and VanC-2 (73%) than between these proteins and any of the other ligases (33 to 39%). Likewise, Ddl from *E. casseliflavus* was closely related to the D-Ala–D-Ala ligase of *E. faecalis* (79%) and displayed a lower level of identity with other ligases (32 to 42%). These results are expected if the Ddl proteins from *E. faecalis* and from *E. casseliflavus* correspond to D-Ala–D-Ala ligases from related species whereas VanC-1 and VanC-2 correspond to ligases involved in vancomycin resistance that have no counterpart in glycopeptide-susceptible enterococci.

Distribution of the *vanC-2* and *ddl* genes from *E. casseliflavus*. Genomic DNA from enterococcal strains was digested with *Kpn*I or *Kpn*I plus *Hin*dIII and analyzed by Southern hybridization with the *vanC-2* and $ddl_{E.\ cass.}$ gene fragments as probes (data not shown). The probes hybridized with DNA of the 24 *E. casseliflavus* strains and of the 2 *E. flavescens* strains (Table 1). A minimum of five hybridization profiles was detected in *E. casseliflavus*. The two *E. flavescens* strains were indistinguishable and had profiles distinct from those of the *E. casseliflavus* isolates. The probes did not hybridize with DNA from strains of *E. gallinarum* and *E. faecalis*, from *E. faecium* with VanA- or VanB-type resistance, and from susceptible enterococci belonging to 16 species.

E. casseliflavus is pathogenic for humans with frequencies ranging from 0.3 to 1% of clinically relevant enterococcal isolates (27, 31). The frequency of isolation of yellow-pigmented enterococci, mainly *E. casseliflavus*, from wounds (approximately 4%) is similar to that of *E. faecium* (27, 30). Low-level vancomycin resistance in enterococci is frequently not detected by disk diffusion or by automated antibiotic susceptibility techniques (34–36). The possibility of treatment failure because of intrinsic low-level resistance in infections has been documented previously (16, 19, 21). These two observations stress the need for accurate identification of bacterial species intrinsically resistant to this drug.

ACKNOWLEDGMENTS

We thank F. Depardieu and S. Evers for introducing F.N. to the techniques of molecular biology, S. Dutka-Malen for continuous interest throughout this project, M. Arthur for help with the writing of the manuscript, and L. A. Devriese for the gift of strains and help with bacterial identification.

REFERENCES

1. **Acar, J., E. Bergogne-Bérézin, J. M. Brogard, Y. Chabbert, R. Cluzel, P. Courvalin, H. Dabernat, H. Drugeon, L. Dubreuil, J. Duval, J. P. Flandrois, J. Fleurette, F. Goldstein, M. Meyran, C. Morel, A. Philippon, J. Sirot, C. J. Soussy, A. Thabaut, and M. Véron.** 1993. Communiqué 1993 du Comité de l'Antibiogramme de la Société Française de Microbiologie. Path. Biol. **41**:741–748.
2. **Arthur, M., and P. Courvalin.** 1993. Genetics and mechanisms of glycopeptide resistance in enterococci. Antimicrob. Agents Chemother. **37**:1563–1571.
3. **Arthur, M., C. Molinas, T. D. H. Bugg, G. D. Wright, C. T. Walsh, and P. Courvalin.** 1992. Evidence for in vivo incorporation of D-lactate into peptidoglycan precursors of vancomycin-resistant enterococci. Antimicrob. Agents Chemother. **36**:867–869.
4. **Benton, W. D., and R. W. Davis.** 1977. Screening λgt recombinant clones by hybridization to single plaques in situ. Science **196**:180–182.
5. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
6. **Bugg, T. D. H., G. D. Wright, S. Dutka-Malen, M. Arthur, P. Courvalin, and C. T. Walsh.** 1991. Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. Biochemistry **30**:10408–10415.
7. **Collins, M. D., J. A. E. Farrow, and D. Jones.** 1986. *Enterococcus mundtii* sp. nov. Int. J. Syst. Bacteriol. **36**:8–12.
8. **Costa, Y., M. Galimand, R. Leclercq, J. Duval, and P. Courvalin.** 1993. Characterization of the chromosomal *aac(6')-Ii* gene specific for *Enterococcus faecium*. Antimicrob. Agents Chemother. **37**:1896–1903.
9. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
10. **Devriese, L. A., B. Pot, and M. D. Collins.** 1993. Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species groups. J. Appl. Bacteriol. **75**:399–408.
11. **Dutka-Malen, S., R. Leclercq, V. Coutant, J. Duval, and P. Courvalin.** 1990. Phenotypic and genotypic heterogeneity of glycopeptide resistance determinants in gram-positive bacteria. Antimicrob. Agents Chemother. **34**:1875–1879.
12. **Dutka-Malen, S., C. Molinas, M. Arthur, and P. Courvalin.** 1990. The VanA glycopeptide resistance protein is related to D-alanyl-D-alanine ligase cell wall biosynthesis enzymes. Mol. Gen. Genet. **224**:364–372.

13. **Dutka-Malen, S., C. Molinas, M. Arthur, and P. Courvalin.** 1992. Sequence of the *vanC* gene of *Enterococcus gallinarum* BM4174 encoding a D-alanine:D-alanine ligase-related protein necessary for vancomycin resistance. Gene **112**:53–58.
14. **Evers, S., P. E. Reynolds, and P. Courvalin.** 1994. Sequence of the *vanB* and *ddl* genes encoding D-alanine:D-lactate and D-alanine:D-alanine ligases in vancomycin-resistant *Enterococcus faecalis* V583. Gene **140**:97–102.
15. **Evers, S., D. F. Sahm, and P. Courvalin.** 1993. The *vanB* gene of vancomycin-resistant *Enterococcus faecalis* V583 is structurally related to genes encoding D-Ala-D-Ala ligases and glycopeptide-resistance proteins VanA and VanC. Gene **124**:143–144.
16. **Fantin, B., R. Leclercq, M. Arthur, J. Duval, and C. Carbon.** 1991. Influence of low-level resistance to vancomycin on efficacy of teicoplanin and vancomycin for treatment of experimental endocarditis due to *Enterococcus faecium*. Antimicrob. Agents Chemother. **35**:1570–1575.
17. **Frischauf, A. M., H. Lehrach, A. Poustka, and N. Murray.** 1983. Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. **170**:827–842.
18. **Handwerger, S. M. J., M. J. Pucci, K. J. Vol, J. Liu, and M. S. Lee.** 1992. The cytoplasmic peptidoglycan precursor of vancomycin-resistant *Enterococcus faecalis* terminates in lactate. J. Bacteriol. **174**:5982–5984.
19. **Kaplan, A. H., P. H. Gilligan, and R. R. Facklam.** 1988. Recovery of resistant enterococci during vancomycin prophylaxis. J. Clin. Microbiol. **26**:1216–1218.
20. **Le Bouguénec, C., G. de Cespedes, and T. Horaud.** 1990. Presence of chromosomal elements resembling the composite structure Tn*3701* in streptococci. J. Bacteriol. **172**:727–734.
21. **Leclercq, R., S. Dutka-Malen, J. Duval, and P. Courvalin.** 1992. Vancomycin resistance gene *vanC* is specific to *Enterococcus gallinarum*. Antimicrob. Agents Chemother. **36**:2005–2008.
22. **National Committee for Clinical Laboratory Standards.** 1993. Methods for dilution antimicrobial disk susceptibility tests for bacteria that grow aerobically. M7-A3. National Committee for Clinical Laboratory Standards, Villanova, Pa.
23. **Nieto, M., and H. R. Perkins.** 1971. Modifications of the acyl-D-alanyl-D-alanine terminus affecting complex-formation with vancomycin. Biochem. J. **123**:789–803.
24. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26**:101–106.
25. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence analysis. Proc. Natl. Acad. Sci. USA **85**:2444–2448.
26. **Pompei, R., F. Berlutti, M. C. Thaller, A. Ingianni, G. Cortis, and B. Dainelli.** 1992. *Enterococcus flavescens* sp. nov., a new species of enterococci of clinical origin. Int. J. Syst. Bacteriol. **42**:365–369.
27. **Pompei, R., G. Lampis, F. Berlutti, and M. C. Thaller.** 1991. Characterization of yellow-pigmented enterococci from severe human infections. J. Clin. Microbiol. **29**:2884–2886.
28. **Quintiliani, R., Jr., S. Evers, and P. Courvalin.** 1993. The *vanB* gene confers various levels of self-transferable resistance to vancomycin in enterococci. J. Infect. Dis. **167**:1220–1223.
29. **Reynolds, P. E., H. M. Snaith, A. J. Maguire, S. Dutka-Malen, and P. Courvalin.** 1994. Analysis of peptidoglycan precursors in vancomycin resistant *Enterococcus gallinarum* BM4174. Biochem. J. **301**:5–8.
30. **Robinson, A. C., D. J. Kenan, J. Sweeney, and W. D. Donachie.** 1986. Further evidence for overlapping transcriptional units in an *Escherichia coli* cell envelope-cell division gene cluster: DNA sequence and transcriptional organization of the *ddl ftsQ* region. J. Bacteriol. **167**:809–817.
31. **Ruoff, K. L., L. de la Maza, M. J. Murtagh, J. D. Spargo, and M. J. Ferraro.** 1990. Species identities of enterococci isolated from clinical specimens. J. Clin. Microbiol. **28**:435–437.
32. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
33. **Steers, E., E. L. Foltz, B. S. Gravies, and J. Riden.** 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. (Washington, D.C.) **9**:307–311.
34. **Swenson, J. M., M. J. Ferraro, D. F. Sahm, P. Charache, The National Committee for Clinical Laboratory Standards Working Group on Enterococci, and F. C. Tenover.** 1992. New vancomycin disk diffusion breakpoint for enterococci. J. Clin. Microbiol. **30**:2525–2528.
35. **Swenson, J. M., B. C. Hill, and C. Thornsberry.** 1989. Problems with the disk diffusion test for detection of vancomycin resistance in enterococci. J. Clin. Microbiol. **27**:2140–2142.
36. **Tenover, F. C., J. Tokars, J. Swenson, S. Paul, K. Spitalny, and W. Jarvis.** 1993. Ability of clinical laboratories to detect antimicrobial agent-resistant enterococci. J. Clin. Microbiol. **31**:1695–1699.
37. **Vincent, S., R. G. Knight, M. Green, D. F. Sahm, and D. M. Shlaes.** 1991. Vancomycin susceptibility and identification of motile enterococci. J. Clin. Microbiol. **29**:2335–2337.
38. **Zawadzke, L. E., T. D. H. Bugg, and C. T. Walsh.** 1991. Existence of two D-alanine:D-alanine ligases in *Escherichia coli*: cloning and sequencing of the *ddlA* gene, and purification and characterization of the DdlA and DdlB enzymes. Biochemistry **30**:1673–1682.