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vanE Gene Cluster of Vancomycin-Resistant *Enterococcus faecalis* BM4405

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Acquired VanE-type resistance to low levels of vancomycin (MIC = 16 μg/ml) in *Enterococcus faecalis* BM4405 is due to the inducible synthesis of peptidoglycan precursors terminating in D-alanine–D-serine (Fines, M., B. Périchon, P. Reynolds, D. Sahm, and P. Courvalin, Antimicrob. Agents Chemother. 43:2161-2164, 1999). A chromosomal location was assigned to the *vanE* operon by pulsed-field gel electrophoresis and hybridization, and its sequence was determined. Three genes, encoding the VanE ligase, the $VanXY_E$ DD-peptidase, and the $VanT_E$ serine racemase, that displayed 43 to 53% identity with the corresponding genes in the *vanC* operon were found. In addition, two genes coding for a two-component regulatory system, $VanR_E$-$VanS_E$, exhibiting 60 and 44% identity with $VanR_C$-$VanS_C$, were present downstream from $vanT_E$. However, because of a stop codon at position 78, $VanS_E$ was probably not functional. The five genes, with the same orientation, were shown to be cotranscribed by Northern analysis and reverse transcription-PCR. The *vanE*, $vanXY_E$, and $vanT_E$ genes conferred inducible low-level resistance to vancomycin after cloning in *E. faecalis* JH2-2, probably following cross talk with a two-component regulatory system of the host.

Glycopeptide antibiotics bind with high specificity to the D-alanyl–D-alanine (D-Ala–D-Ala) termini of peptidoglycan late precursors and block the transglycosylation and transpeptidation steps in cell wall synthesis by gram-positive bacteria (32). In enterococci, glycopeptide resistance is due to production of modified peptidoglycan precursors which exhibit a lower binding affinity for vancomycin (32). Two substituted precursors, D-alanyl–D-lactate (D-Ala–D-Lac) and D-alanyl–D-serine (D-Ala–D-Ser), have been identified (4, 9, 33). Three types of glycopeptide resistance, VanA, VanB, and VanD, result from the production of D-Ala–D-Lac-terminating peptidoglycan precursors, whereas the VanC, VanE, and VanG types are characterized by the synthesis of precursors ending in D-Ala–D-Ser. Production of D-Ala–D-Ser-containing precursors in *Enterococcus gallinarum* and *Enterococcus casseliflavus-flavescens*, which display intrinsic resistance to vancomycin, is due to the presence of the chromosomal *vanC* operons. The cluster is composed of five genes: *vanC*, $vanXY_C$, *vanT*, $vanR_C$, and $vanS_C$ (1). The *vanC* gene encodes a ligase that synthesizes the dipeptide D-Ala–D-Ser; $vanXY_C$ specifies a bifunctional enzyme with DD-dipeptidase activity, responsible for hydrolysis of the dipeptide D-Ala–D-Ala, and DD-carboxypeptidase activity that removes D-Ala from UDP-MurNAc-pentapeptide-[D-Ala] (33); and the *vanT* gene directs the synthesis of a membrane-bound serine racemase that provides D-Ser for the resistance pathway (1). Expression of the resistance genes of the *van* operons is controlled by two-component regulatory systems (6). These systems comprise response regulators (VanR type) and histidine kinases which act as sensor proteins (VanS type) (6). Recently, VanE- and VanG-type vancomycin resistance was detected in strains of *Enterococcus faecalis*, a species which is not intrinsically resistant to vancomycin (15, 25). Clinical isolate BM4405, the first VanE-type strain, is resistant to a low level of vancomycin (MIC = 16 μg/ml) and susceptible to teicoplanin (MIC = 0.5 μg/ml). Attempts to transfer vancomycin resistance from BM4405 to a susceptible *E. faecalis* strain by filter mating were unsuccessful (15). We describe the genetic organization of the *vanE* operon in *E. faecalis* BM4405. The cluster includes three genes which are sufficient to confer resistance to vancomycin and two genes for a two-component system in which the sensor is most likely nonfunctional. We also demonstrated that the five genes are cotranscribed from a single promoter.

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MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The bacterial strains and plasmids are described in Table 1. *E. faecalis* clinical isolate BM4405 is resistant to low levels of vancomycin (MIC = 16 μg/ml) (15). VanC-type *E. gallinarum* BM4174 (12) and VanA-type *Enterococcus faecium* BM4147 (20) were used as controls in pulsed-field gel electrophoresis experiments. *E. faecalis* JH2-2, used in electrotransformation experiments, is susceptible to glycopeptides and resistant to fusidic acid and rifampin (19). *Escherichia coli* JM83 (42) and Top10 (Invitrogen, Groningen, The Netherlands) were used as the hosts in cloning experiments. Strains were cultured in brain heart infusion broth or agar (Difco Laboratories, Detroit, Mich.) at 37°C. Susceptibility to glycopeptides was determined by agar dilution with 10^5 CFU per spot on Mueller-Hinton agar (Bio-Rad, Marnes-La-Coquette, France) after 24 h of incubation.

Recombinant DNA techniques. Cleavage of DNA with restriction endonucleases (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England, and Gibco BRL-Life Technologies Inc.), purification of restriction fragments from agarose gel, and ligation with T4 DNA ligase (Amersham Pharmacia Biotech) were performed by standard methods (35).

Plasmid construction. The plasmids were constructed as follows (Fig. 1).

(i) Plasmid pAT664. Total DNA from BM4405 was partially digested with *Sau*3AI and ligated with pUC18 DNA cleaved by *Bam*HI. Clones harboring recombinant plasmids were screened by colony hybridization (35) with the 513-bp fragment internal to *vanE* purified from pAT663 (15) as a probe.

(ii) Plasmids pAT667 and pAT668. A fragment encompassing the *vanE*, $vanXY_E$, and $vanT_E$ genes, with or without 600 bp upstream from *vanE*, was amplified by using primer pairs E35-TE5 and E43-TE5, respectively, and *E. faecalis* BM4405 DNA as a template. Oligodeoxynucleotides E35 and E43 con-

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
E. coli JM83	F⁻ *ara* Δ(*lac-proAB*) *rpsL* (Str^r) [ϕ80d*lac*Δ(*lacZ*)M15]	43
E. coli Top10	F⁻ [*lac*I^{q}Tn*10*(Tet^r)] *mcr*A Δ(*mrr-hsdRMS mcrBC*) ϕ80*lacZ*ΔM15Δ*lac*X74 *recA* *araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nup*G	Invitrogen 15
E. faecalis BM4405	Vm^r (VanE type)	19
E. faecalis JH2-2	Fus^r Rif^r	
E. faecalis V583	Vm^r (VanB type)	13
E. gallinarum BM4174	Vm^r (VanC type)	12
E. faecium BM4147	Vm^r Te^r (VanA type)	20
Plasmids		
pCR2.1	Ap^r Km^r; *lacZ*α *oriR* from ColE1	Invitrogen
pUC18	Ap^r, *lacZ*α vector	41
pAT29	*oriR*pAMβ1 *oriR*pUC *oriT*RK2 Sp^r *lacZ*α	39
pAT663	0.5-kb PCR fragment (*vanE*) of BM4405 cloned in pCR2.1	15
pAT664	*Sau*3AI fragment (*vanE'XY*$_E$*T*$_E$*'*) of BM4405 cloned in pUC18	This work
pAT667	4.4-kb fragment (600 bp, *vanEXY*$_E$*T*$_E$) of BM4405 cloned in pAT29	This work
pAT668	3.8-kb fragment (*vanEXY*$_E$*T*$_E$) of BM4405 cloned in pAT29	This work

[a] Fus^r, fusidic acid resistance; Rif^r, rifampin resistance; Sp^r, spectinomycin resistance; Str^r, streptomycin resistance; Te^r, teicoplanin resistance; Tet^r, tetracycline resistance; Vm^r, vancomycin resistance.

tained a *Sac*I site, and TE5 contained *Sph*I site. The PCR products were digested with *Sac*I and *Sph*I and cloned in pAT29.

Plasmid pAT667 (600 bp upstream from *vanE*, *vanEXY*$_E$*T*$_E$) and pAT668 (*vanEXY*$_E$*T*$_E$) were introduced into *E. faecalis* JH2-2 by electrotransformation, and transformants were selected with spectinomycin (60 μg/ml).

Probes and hybridization. DNA was transferred onto Hybond N$^+$ membranes (Amersham Pharmacia Biotech) and fixed under UV illumination. Plasmid pAT663 DNA labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) by nick translation was used as a probe for colony hybridization, and Southern experiments were carried out under stringent conditions (35).

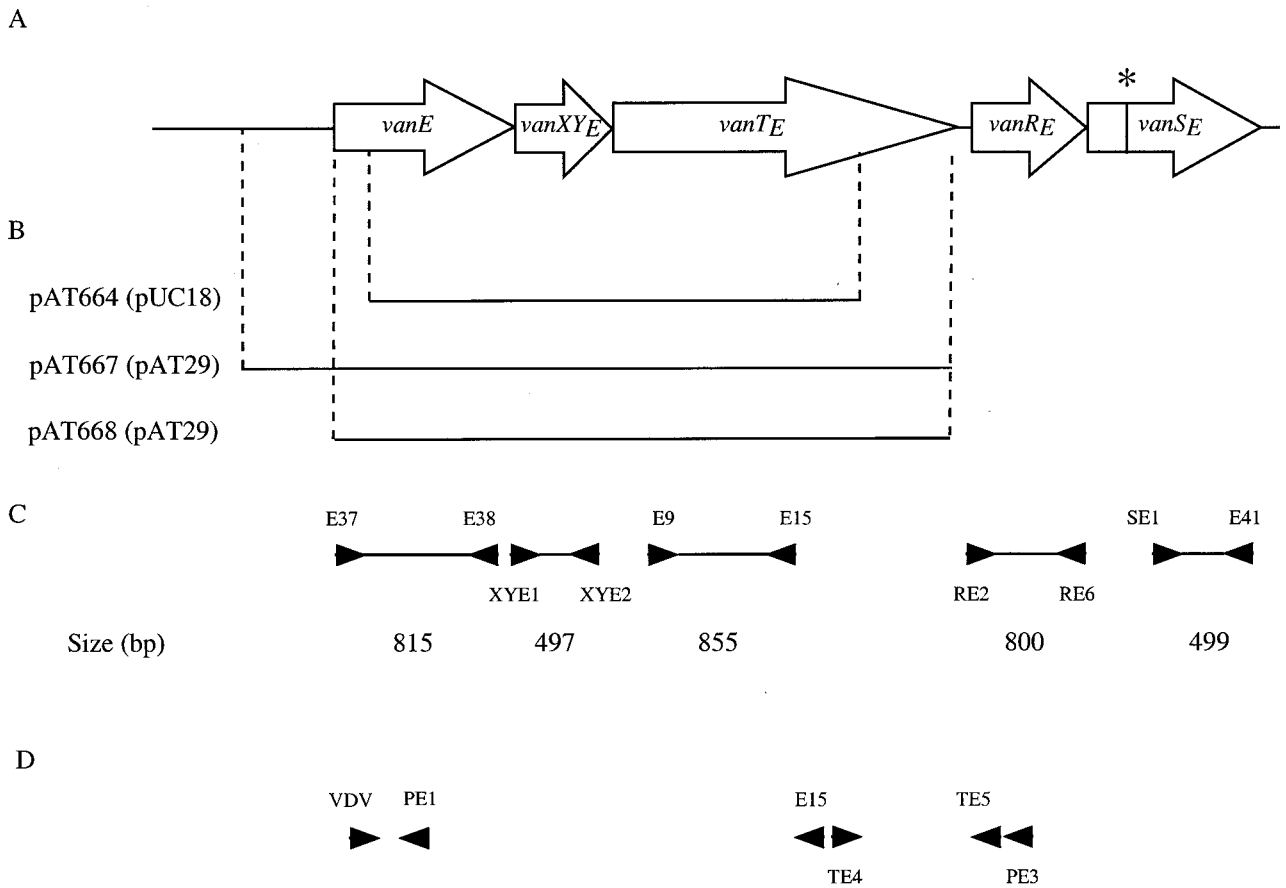

FIG. 1. Schematic representation of the *vanE* gene cluster and of recombinant plasmids. (A) Open arrows represent coding sequences and the direction of transcription. The asterisk indicates the stop codon in *vanS*$_E$. (B) The inserts in the recombinant plasmids are represented by solid lines, and the vectors are indicated in parentheses. (C) PCR fragments used as probes in Northern hybridization. (D) Oligonucleotides used in RT-PCR and in primer extension. Arrowheads indicate positions and orientations of primers.

TABLE 2. Oligonucleotides used in this study

Primer	Sequence	Position[a]
E9	5′ ACT GTG TTT CGG GTA GC	1722–1738
E12	5′ TAT GGG AGT TGT GAA	2711–2725
E15	5′ CAG AAG CTG AGC TAG T	2576–2561
E35	5′ GCG AGC TCA CAG ATC AGG AAA TCG GA[b]	NA[c]
E37	5′ GGA TCA CCG AAG AAG GT	119–135
E38	5′ CCA GGC ATT GTA TTG ATC T	932–914
E41	5′ GCA ATT GCT AAC CCT AGA CC	5464–5445
E43	5′ GCG AGC TCA ACA AAT ACT GGA GGT A[b]	NA
XYE1	5′ GTT CAG GCT CCG TTT GCG C	1103–1121
XYE2	5′ GCA GTT CCT CTT ATT GAC TC	1599–1580
TE4	5′ GCA GCG GTT CAG GTG TTT	2159–2174
TE5	5′ GCG CAT GCA GCC ATT AAA CAT CCT[d]	3785–3770
RE1	5′ CCG AGA CAG CCA AAT	4410–4424
RE2	5′ TCG ACT GTC GAC AAA T	4214–4199
RE6	5′ AGA TCG ATT TAG CCA TAC	3913–3930
SE1	5′ AGC TAA CAG ATT AGA ACA T	5027–5045
SE2	5′ GGA GTT CTT AAG TCA TGT	4921–4904
VDV	5′ GAT CGT TGG TTT TTA GAT	178–195
RDeg2	5′ CCN ACH CCS CRB ACV GTT[e]	NA
PE1	5′ CCA ATG ACC TTC TTC GGT GAT CC	120–96
PE3	5′ AAG CTT TCT TTT CCT GAC ATA GCC TC	3877–3851
SDEg1	5′ ATS GSM ARH CCM ARW CC[f]	NA

[a] Nucleotide numbering begins at the first base of the *vanE* gene.
[b] The *Sac*I site is underlined.
[c] NA, not applicable.
[d] The *Sph*I site is underlined.
[e] B = C, G, or T; H = A, C, or T; N = A, C, G, or T; R = A or G; S = G or T; V = A, C, or G.
[f] H = A, C or T; M = A or C; R = A or G; S = G or T; W = A or T.

PCR and nucleotide sequencing. The PCR mixture consisted of reaction buffer (final concentrations of 1.5 mM $MgCl_2$ and 10 mM Tris-HCl at pH 8.3); 500 μM (each) dATP, dCTP, dTTP, and dGTP; 40 pmol of each primer; 2 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech); and 100 ng of enterococcal DNA in a total volume of 50 μl. DNA amplification was carried out in a GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). DNA sequencing was performed by the dideoxynucleotide chain termination method (36) with α-^{35}S-dATP (Amersham) and the T7 Sequenase version 2.0 DNA sequencing kit (Amersham). Plasmid DNA used as a template was extracted with the commercial Wizard Plus Minipreps DNA purification system (Promega, Madison, Wis.).

Computer analysis of sequence data. Sequence data were analyzed with the Sequence Analysis Software Package (version 7; Genetics Computer Group, Madison, Wis.). Phylogenetic analysis was carried out with the PHYLIP program package (14).

Analysis of peptidoglycan precursors. Extraction and analysis of peptidoglycan precursors were performed as described previously (26). Enterococci were grown in brain heart infusion broth overnight at 37°C in the presence (4 μg/ml) or absence of vancomycin with gentle agitation to an optical density at 600 nm of 1 (mid-exponential phase). Ramoplanin was added to a concentration of 3 μg/ml, and incubation was continued for 30 min. Bacteria were harvested, and the cytoplasmic precursors were extracted with 8% trichloroacetic acid (15 min at 4°C), desalted, and analyzed by high-performance liquid chromatography. Results were expressed as the percentages of total late peptidoglycan precursors represented by UDP-MurNAc-tetrapeptide, UDP-MurNAc-pentapeptide, and UDP-MurNAc-pentapeptide–D-Ser that were determined from the integrated peak areas.

Pulsed-field gel electrophoresis. Genomic DNA embedded in agarose plugs (24) was digested for 3 h at 37°C with 0.01 U of I-*Ceu*I, an intron-encoded endonuclease specific for rRNA genes. Fragments were separated on a 1.2% agarose gel with a contour-clamped homogeneous electric field DR III system (Bio-Rad Laboratories, Hercules, Calif.) under the following conditions: total migration, 24 h; initial pulse, 60s; final pulse, 120s; voltage, 6 V/cm; included angle, 120°; and temperature, 16°C. Fragments were blotted onto Hybond N^+ membranes (Amersham Pharmacia Biotech) and hybridized (i) with an [α-^{32}P]dCTP-labeled 16S rRNA (*rrs*) probe obtained by amplification of an internal portion of the *rrs* gene (17) and (ii) with a *vanE*-specific probe (15).

RNA techniques. (i) Extraction of total RNA. *E. faecalis* BM4405 was grown to an optical density at 600 nm of 0.7, and bacteria were disrupted with a Mickle disintegrator by using 3.5-g (106-μm-diameter) glass beads (Sigma Chemical Co., St. Louis, Mo.) in the presence of 0.25 ml of 10% sodium dodecyl sulfate, 1 ml of 2% macaloid (National Lead Co., New York, N.Y.), and 3 ml of phenol (16). The mixture was shaken three times for 1 min each at 4°C and centrifuged for 15 min at 8,500 × *g*. The supernatant was extracted with phenol and chloroform. Total RNA was precipitated by addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 3 volumes of ice-cold 100% ethanol. RNA pellets were resuspended in diethyl pyrocarbonate-water.

(ii) Northern analysis. Equal amounts of total RNA (20 μg) were separated under denaturing conditions in 1.2% agarose-formaldehyde-MOPS (morpholinepropanesulfonic acid) gel, stained with ethidium bromide, and blotted onto Hybond N^+ membranes (Amersham Pharmacia Biotech) (35). DNA probes obtained by PCR with total DNA from BM4405 as a template and primers E37-E38 (*vanE*), XY_{E1}-XY_{E2} (*vanXY*$_E$), E9-E15 (*vanT*$_E$), R2-R6 (*vanR*$_E$), and S1-E41 (*vanS*$_E$) (Fig. 1C; Table 2) were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Pharmacia Biotech) by using the Megaprime DNA labeling system (Amersham Pharmacia Biotech). Hybridizations were carried out under stringent conditions, and washes were performed as described previously (22). The size of the transcripts was determined according to RNA molecular weight marker I (Boehringer, Mannheim, Germany).

(iii) RT-PCR experiments. Total RNA samples were digested with RNase-free DNase I (5 U/μg of RNA) (Amersham Pharmacia Biotech) in a final volume of 1 ml for 10 min at 37°C. Samples were treated with proteinase K (0.2 mg/ml) (Boehringer), extracted with phenol-chloroform, and precipitated with ethanol. Reverse transcription (RT) was carried out with 2 μg of purified RNA in a 20-μl final volume containing 1× enzyme buffer (Superscript II; Gibco), 50 mM magnesium chloride, 0.1 mg of bovine serum albumin (New England Biolabs Inc., Beverly, Mass.) per ml, 1 mM (each) of four deoxyribonucleoside triphosphates (Amersham Pharmacia Biotech), 50 pmol of the primer TE5 or PE3 (Fig. 1D; Table 2), 20 U of RNase inhibitor (RNAguard; Amersham Pharmacia Biotech), and 200 U of Moloney murine leukemia virus modified reverse transcriptase (Superscript II; Gibco). Samples were incubated for 30 min at 37°C, and the enzyme was inactivated at 95°C for 5 min. The DNA products were amplified by PCR in an 80-μl reaction volume containing the previous 20-μl samples, 50 pmol each of the VDV and E15 primers or TE4 and PE3 (Fig. 1D; Table 2), 1× enzyme buffer (Amersham Pharmacia Biotech), and 2 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech). PCR (30 cycles) was performed in a GeneAmp PCR system 2400 (Perkin-Elmer Cetus, Norwalk, Conn.). PCR prod-

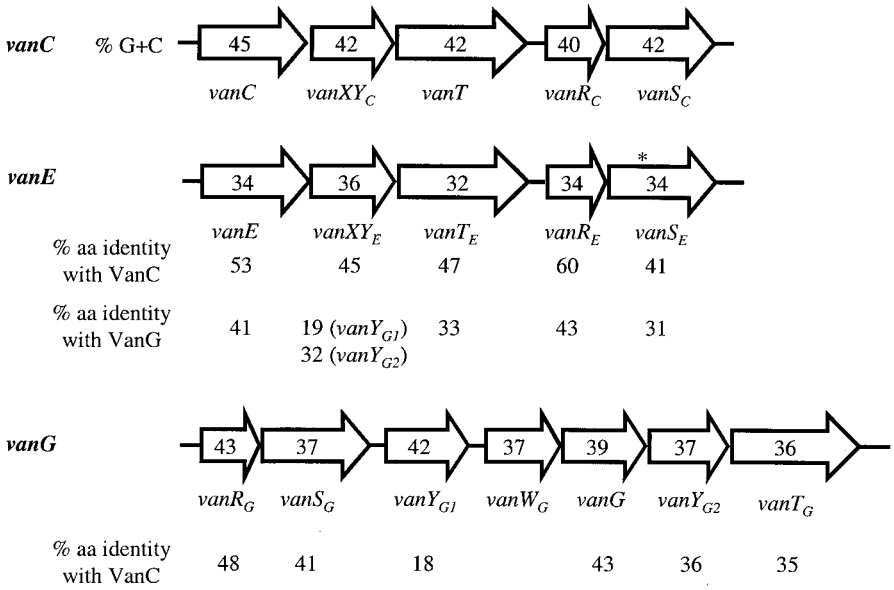

FIG. 2. Comparison of the D-Ala–D-Ser gene clusters. Arrows represent coding sequences and indicate the direction of transcription. The asterisk indicates the stop codon in *vanS*$_E$. The guanosine-plus-cytosine content (% G+C) is indicated in the arrows. The percentages of amino acid (aa) identity between the deduced proteins are indicated under the arrows.

ucts were transferred from agarose gel to a Hybond N$^+$ membrane (Amersham Pharmacia Biotech) and hybridized with specific probes (Fig. 1C).

(iv) Primer extension analysis. The synthetic oligodeoxynucleotide PE1 (Fig. 1D; Table 2) was 5′ end labeled with [γ-^{32}P]ATP (4,500 Ci/mmol; Amersham Pharmacia Biotech) and T4 polynucleotide kinase (Amersham Pharmacia Biotech). After phenol-chloroform extraction, the labeled primer was precipitated with ethanol and redissolved in sterile water to a final concentration of 1 pmol/μl. Labeled primer (1 pmol) was annealed to 50 μg of total RNA at 65°C for 3 min, and extension was performed in a 20-μl final volume with 40 U of Moloney murine leukemia virus modified reverse transcriptase (Superscript II; Gibco) for 45 min at 50°C. After addition of 5 μl of stop solution (Amersham Pharmacia Biotech) and heat denaturation, the sample was immediately loaded onto 6% polyacrylamide–urea sequencing gels for electrophoresis. Sequencing reactions using the same primer and appropriate plasmid DNA templates were run in parallel to allow determination of the endpoints of extension products.

RESULTS AND DISCUSSION

Cloning of the *vanE* gene cluster. Fragments obtained after partial digestion of *E. faecalis* BM4405 total DNA with *Sau*3AI were cloned in pUC18 DNA cleaved with *Bam*HI into *E. coli*, and transformants were screened by hybridization with a *vanE* internal probe (Fig. 1A). Plasmid pAT664 (*vanE′XY*$_E$*T*$_E$*′*) carried an insert of 8 kb that was sequenced. Three open reading frames (ORFs), designated *vanE*, *vanXY*$_E$, and *vanT*$_E$, were found, but the two distal ones were truncated (Fig. 1). The 5′ portion of *vanE* and 1.5 kb upstream were obtained by successive inverted PCRs. We assumed that the *vanE* cluster had the same gene organization as the *vanC* operon, i.e., that *vanT*$_E$ should be followed by the *vanR*$_E$ and *vanS*$_E$ genes. We thus amplified BM4405 DNA by using oligodeoxynucleotide E12, specific for *vanT*$_E$, and degenerate oligodeoxynucleotide RDeg2, complementary to the sequence encoding a conserved motif in the C-terminal part of VanR-type proteins (Table 2). The PCR product obtained, with the expected size of 1.7 kb, was sequenced, providing the 3′ end of *vanT*$_E$ and entire *vanR*$_E$. To sequence further downstream from *vanR*$_E$, specific primer RE1, deduced from the sequence obtained, and degenerate oligodeoxynuleotide SDeg1, complementary to the sequence encoding a conserved motif in the C-terminal part of VanS-type proteins, were used to amplify total DNA from BM4405 (Table 2). Determination of the sequence of the PCR product indicated the presence of the *vanS*$_E$ gene. A 1.5-kb fragment downstream from *vanS*$_E$ was obtained by inverted PCR and sequenced, but no ORF was found. The gene organization of the *vanE* cluster is shown in Fig. 1. Recently, the sequence of the *vanE* gene cluster of *E. faecalis* N00-410 (40) was released, and it exhibits 96 to 98% identity with that of BM4405 (unpublished data; accession number AF 430807).

Analysis of the proteins encoded by the *vanE* gene cluster. The deduced amino acid sequence of *vanE* exhibited 53 and 41% identity with the VanC (2) and VanG (25) D-Ala:D-Ser ligases, respectively (Fig. 2). The EKYQ motif conserved in the VanC-type resistance ligases (13) was found in VanE (EKYN) at positions 198 to 201. The phylogenetic tree based on the alignment of the D-Ala:D-Lac and D-Ala:D-Ser ligases confirmed that VanE was related to VanC (Fig. 3).

The second putative protein, VanXY$_E$, was 45% identical

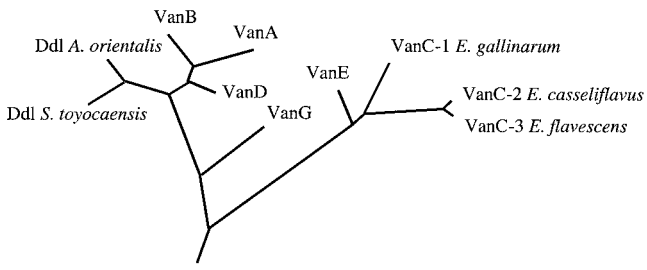

FIG. 3. Phylogenetic tree derived from the alignment of D-Ala:Lac and D-Ala:D-Ser ligases. The tree was constructed by the neighbor-joining method, taking into account the results of maximum-parsimony and bootstrapping analysis.

with the $VanXY_C$ bifunctional enzyme (1) (Fig. 2). $VanXY_E$ displayed higher identity with VanY and $VanY_B$ DD-carboxypeptidases (23 and 16%, respectively) than with VanX and $VanX_B$ DD-dipeptidases (13 and 16%, respectively). The identity was lower with $VanY_{G1}$ than with $VanY_{G2}$ (19 and 32%, respectively). The consensus sequences found in VanX DD-dipeptidases (23), VanY DD-carboxypeptidases (5), and $VanXY_C$ DD-peptidases (34) were also present in $VanXY_E$. An SxHxxGx AxD motif, in which the histidine and aspartate are zinc ligands, was found in $VanXY_E$ (S_{95}EHEIGLAVD$_{104}$). Furthermore, another histidine ligand to zinc, conserved in the zinc binding domain of DD-peptidases (33), was found in $VanXY_E$ (H157). A conserved glutamate/aspartate residue functioning as a catalytic base (1) was present in $VanXY_E$ (E154). The hydrophobicity profile of $VanXY_E$ suggested a cytoplasmic localization for the protein (data not shown). Previous study of vancomycin-induced *E. faecalis* BM4405 indicated weak DD-dipeptidase and DD-carboxypeptidase activities in cytoplasmic extracts (15). Both activities are also found in the cytoplasm of VanC-type *E. gallinarum* BM4174 (1).

The third ORF, *vanT*$_E$, encoded a putative protein with 47 and 33% identity with $VanT_C$ (2) and $VanT_G$ (25) serine racemases, respectively (Fig. 2). The N-terminal half of $VanT_E$ contained 11 clusters of hydrophobic amino acids, suggesting that, like $VanT_C$, it may be a membrane-associated protein. The serine racemase activity present in the membrane fractions of BM4405 is ca. 10-fold higher than that of *E. gallinarum* BM4174 (15). The C-terminal domain of $VanT_E$ had substantial sequence identity (28%) with that of alanine racemase Alr1 from *E. coli*. The putative pyridoxal 5′-phosphate attachment motif, which is highly conserved in alanine racemases and in VanT (33), was found in $VanT_E$ (V_{373}VKANAYGCG$_{382}$). Furthermore, the residues implicated in the hydrogen-bonding interactions with the phosphate group of pyridoxal 5′-phosphate in VanT (Y379, S540, and N688) (2) were present in $VanT_E$. Finally, residues which putatively play a structural role and maintain the geometry of the active site of alanine racemases and VanT (2) were identified in $VanT_E$: A377, A379, Y380, R410, G619, D622, R626, and E688.

The two genes downstream from the three resistance determinants are likely to encode a two-component regulatory system (Fig. 1A). The putative $VanR_E$ protein exhibited 61 and 43% identity with $VanR_C$ (1) and $VanR_G$ (25), respectively (Fig. 2). The conserved aspartate and lysine residues typical of response regulators in two-component systems from gram-positive bacteria (28) were present in $VanR_E$ (D10, D53, and K102). $VanR_E$ displayed 44% identity with the CheY-like response regulator of *Clostridium acetobutylicum* (29).

The deduced amino acid sequence of *vanS*$_E$ showed 41 and 31% identity with $VanS_C$ (1) and $VanS_G$ (25), respectively (Fig. 2). The N-terminal part of $VanS_E$ contained transmembrane segments characteristic of the sensor proteins of two-component systems (6). The carboxyl-terminal part of $VanS_E$ had four of the five conserved amino acid motifs (H, N, F, and G2) characteristic of transmitter modules of histidine protein kinases (30, 38). However, a stop codon at position 78 of *vanS*$_E$ will result in the production of a truncated protein, suggesting that $VanS_E$ is nonfunctional. The level of phosphorylation of VanR-type proteins is controlled by the kinase and phosphatase activities of VanS-type sensors (3, 7, 42). However, kinases encoded by the host chromosome are able to activate the VanR response regulator (3, 8, 37), and it has been demonstrated that both PhoR and acetylphosphate are capable of activating VanR (18). In the absence of a functional $VanS_E$, inducibility of vancomycin resistance expression in BM4405 (15) could be due to cross talk either with another two-component system or of $VanR_E$ with an heterologous histidine kinase.

TABLE 3. Glycopeptide MICs and nature of peptidoglycan precursors in *E. faecalis* strains

E. faecalis	MIC (μg/ml) of vancomycin	Precursors[a] (%)		
		Tetra	Penta-D-Ser	Penta-D-Ala
BM4405	16	2	8	90
BM4405 (Vm4)[b]	16	10	90	0
JH2-2	2	ND[c]	ND	ND
JH2-2/pAT667	2	8	3	89
JH2-2/pAT667 (Vm4)	6	13	53	34
JH2-2/pAT668	2	ND	ND	ND

[a] Tetra, UDP-MurNAc–L-Ala–γ-D-Glu–L-Lys–D-Ala; penta-D-Ser, UDP-MurNAc–L-Ala–γ-D-Glu–L-Lys–D-Ala–D-Ser; penta-D-Ala, UDP-MurNAc–L-Ala–γ-D-Glu–L-Lys–D-Ala–D-Ala.
[b] BM4405 induced with 4 μg of vancomycin per ml.
[c] ND, not determined.

Location of the *vanE* gene cluster. Fragments of *E. faecalis* BM4405 total DNA digested with I-*Ceu*I, an intron-encoded endonuclease specific for rRNA genes (21), were separated by pulsed-field gel electrophoresis and transferred onto a nylon membrane which was hybridized successively to 16S rRNA (*rrs*)- and *vanE*-specific probes. The probes cohybridized with a ca. 350-kb fragment from BM4405, indicating a chromosomal location for the *vanE* cluster (data not shown).

Genes necessary for vancomycin resistance in *E. faecalis* BM4405. To test if the *vanE*, *vanXY*$_E$, and *vanT*$_E$ genes were sufficient to confer vancomycin resistance to the host, a 3.8-kb fragment encompassing the three structural genes but devoid of any 5′ upstream sequence was cloned in pAT29, leading to plasmid pAT668 (*vanEXY*$_E$*T*$_E$). The plasmid was introduced into *E. faecalis* JH2-2 by electrotransformation, and, irrespective of the absence or presence of a low concentration of vancomycin in the culture medium (1 or 2 μg/ml), the transformants remained susceptible to vancomycin (MIC = 2 μg/ml) (Table 3). A 4.4-kb fragment containing the structural genes together with 600 bp upstream from *vanE* was then cloned in pAT29, generating plasmid pAT667 (600 bp, *vanEXY*$_E$*T*$_E$) (Fig. 1B). In the absence of induction, strain JH2-2 harboring pAT667 was susceptible to vancomycin. However, a reproducible threefold increase in the vancomycin MIC was observed after growth in the presence of vancomycin (Table 3). Taken together, these results indicate that the *vanE*, *vanXY*$_E$, and *vanT*$_E$ genes are sufficient to confer vancomycin resistance and that the region upstream from the *vanE* cluster may act as a promoter for activation of transcription of the resistance genes, as in the *vanC* operon (1). When JH2-2/pAT667 was grown in the absence of vancomycin, a very high proportion (89%) of precursors ending in D-Ala–D-Ala was found in the cells. In contrast, precursors ending in D-Ala–D-Ser represented 53% of total peptidoglycan precursors in cells grown in the presence of vancomycin (Table 3), confirming that the resistance genes were inducibly expressed. In vancomycin-in-

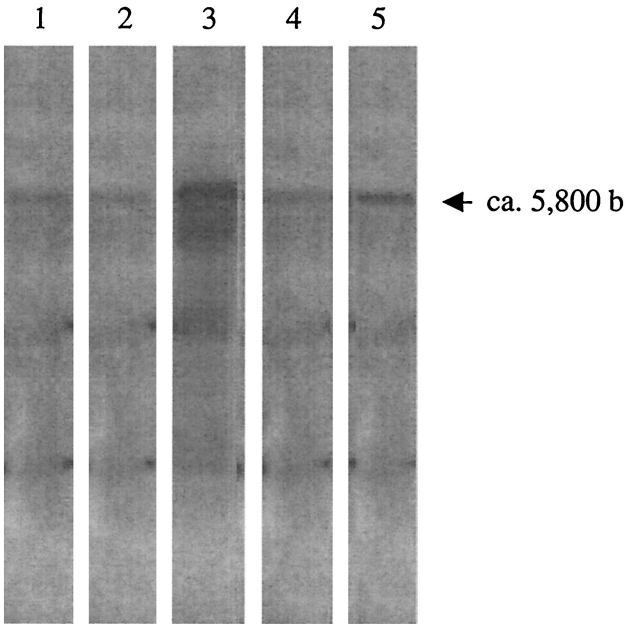

FIG. 4. Analysis of *vanE* gene cluster transcription by Northern hybridization. Total RNA from BM4405 was hybridized with the *vanE* (lane 1), *vanXY*$_E$ (lane 2), *vanT*$_E$ (lane 3), *vanR*$_E$ (lane 4), and *vanS*$_E$ (lane 5) probes. The sizes of the transcripts were determined according to RNA molecular weight marker I (Boehringer) (not shown). b, bases.

duced JH2-2/pAT667 cells, nearly half of the peptidoglycan precursors were of the susceptible type. This might indicate that, possibly due to inefficient cross talk, expression of the *vanE* operon was lower in induced JH2-2/pAT667 than in BM4405. This finding could account for the fact that the transformant was inhibited by a vancomycin concentration lower than that for strain BM4405 (Table 3).

Transcription analysis of the *vanE* gene cluster. The *vanA*, *vanB*, and *vanD* operons are cotranscribed from their respective P_H (6), P_{YB} (37), and P_{YD} (10) promoters. The start codons of the *vanXY*$_E$ and *vanT*$_E$ genes overlap the termination codons of *vanE* and *vanXY*$_E$, respectively, suggesting that the *vanE*, *vanXY*$_E$, and *vanT*$_E$ genes are cotranscribed. The *vanS*$_E$ start codon also overlaps the *vanR*$_E$ stop codon, suggesting the existence of a second transcription unit. Total RNA from BM4405 was extracted and analyzed by Northern hybridization with probes internal to every gene in the *vanE* operon (Fig. 1C). A single transcript of ca. 5800 nucleotides was observed, which hybridized with all of the probes, including those internal to *vanR*$_E$ and *vanS*$_E$ (Fig. 4). The size of the transcript and the absence of a smaller mRNA encompassing the last two *van* genes are consistent with the production of a single mRNA corresponding to the five genes and originating from a promoter upstream from *vanE*. Cotranscription of the entire *vanE* gene cluster was tested by RT of total RNA from BM4405 with primer TE5, internal to *vanT*$_E$ (Fig. 1D; Table 2). The cDNA was amplified by PCR with primers VDV and E15, internal to *vanE* and *vanT*$_E$, respectively (Fig. 1D; Table 2). A PCR product of the expected size of 2.4 kb that cohybridized with probes specific for *vanE*, *vanXY*$_E$, and *vanT*$_E$ (Fig. 1D and Fig. 5) was obtained. To confirm that a single transcript corresponded to the five genes, RT of total RNA from BM4405 with primer PE3, internal to *vanR*$_E$ (Fig. 1D; Table 2), was performed. The cDNA was then amplified using primers internal to *vanT*$_E$ (TE4) and *vanR*$_E$ (PE3) (Fig. 1D; Table 2). A PCR product of ca. 1.7 kb, which cohybridized with the *vanT*$_E$ and *vanR*$_E$ probes, was obtained (data not shown), indicating that the genes for the two-component system were cotranscribed with the resistance genes. Based on these observations, primer extension was performed to locate the transcriptional start site for *vanE* by using primer PE1, complementary to the 5′ end of that gene (Fig. 1D; Table 2) (Fig. 6). The proposed initiation codon for *vanE* was preceded by a putative ribosome binding site (5′ ATACTGGAGGN$_8$ATG) (Fig. 6) that displayed high complementarity to the 3′ extremity of *Bacillus subtilis* 16S rRNA (3′-OH-UCUUUCCUCC) (27). The P_E promoter region contained two overlapping putative −10 regions, TTT CAA and TTCAAT, similar to the −10 σ^{70} recognition consensus. Both regions were at a correct distance, 10 and 11 bp, respectively, from the transcription start. At bp 22 or 23 up-

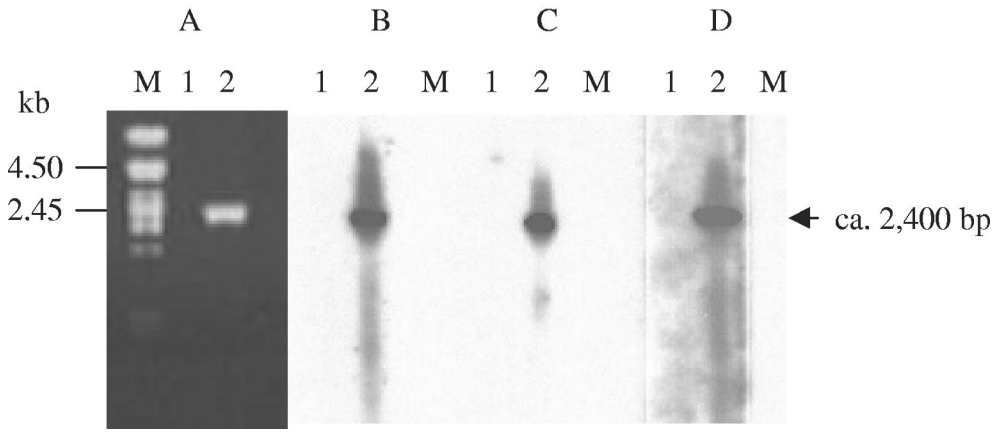

FIG. 5. Analysis of the transcription of the *vanE*, *vanXY*$_E$, and *vanT*$_E$ genes. Electrophoresis of the product obtained by RT-PCR with primers VDV and E15 (Fig. 1D and Table 2) (A) and corresponding Southern hybridizations with *vanE* (B), *vanXY*$_E$ (C), and *vanT*$_E$ (D) probes (Fig. 1C) are shown. Incubations were carried out in the absence (lanes 1) or presence (lanes 2) of reverse transcriptase. Lanes M, DNA from bacteriophage lambda digested by *Pst*I as a marker.

FIG. 6. Identification of the transcriptional start site for the *vanE*, *vanXY*$_E$, *vanT*$_E$, *vanR*$_E$, and *vanS*$_E$ genes in BM4405 by primer extension analysis. (Left panel) Lane 1, primer elongation product obtained with oligodeoxynucleotide PE1 and 50 μg of total RNA from BM4405 (arrowhead); lanes T, G, C, and A, results of sequencing reactions performed with the same primer. Right panel, sequence from nucleotide positions −353 to +141 (numbering from the A of the ATG start codon of *vanE*, negative in the 3′-to-5′ direction and positive in the 5′-to-3′ direction). The +1 transcriptional start site for the *vanE*, *vanXY*$_E$, *vanT*$_E$, *vanR*$_E$, and *vanS*$_E$ mRNA in BM4405 and the −35 and −10 promoter sequences located upstream are in boldface. The ATG start codon of *vanE* is indicated by an arrow, and the ribosome binding site (RBS) is in boldface and underlined.

stream from the proposed −10 sequences lies a TTGAGG putative −35 sequence. However, due to spacing, it remains open whether this sequence plays a role in the recognition of the promoter region by the σ^{70} RNA polymerase complex. Furthermore, expression of the *vanE* operon is likely to depend on the VanR$_E$ transcriptional activator, which is known to render the −35 sequence dispensable for expression (11).

In conclusion, the *vanE* operon comprises fives genes, with three of them being sufficient to confer vancomycin resistance whereas the last two encode a two-component system postulated to regulate expression of the operon. However, since VanS$_E$ appears not to be functional, inducibility of resistance by vancomycin is likely to be due to cross talk reactions with another two-component regulatory system of the host. Comparative analysis of the *vanE* operon indicated that VanE-type resistance in *E. faecalis* BM4405 was due to the presence of a chromosomal operon related to *vanC*. It has been demonstrated that transfer of vancomycin resistance among enterococci can be associated with the movement of large genetic elements from chromosome to chromosome (31). Our results suggest acquisition by *E. faecalis* of a cluster of genes from an intrinsically resistant species such as *E. gallinarum* or *E. casseliflavus-flavescens*. To find a clue as to the mechanism of acquisition of the resistance operon, we are determining the sequence of the flanking regions.

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REFERENCES

1. **Arias, C., P. Courvalin, and P. Reynolds.** 1999. The *vanC* gene cluster of vancomycin-resistant *Enterococcus gallinarum* BM4174. Antimicrob. Agents Chemother. **44:**1660–1666.
2. **Arias, C., M. Martín-Martinez, T. Blundell, M. Arthur, P. Courvalin, and P. Reynolds.** 1999. Characterization and modelling of VanT: a novel membrane-bound serine racemase from vancomycin resistant *Enterococcus gallinarum* BM4174. Mol. Microbiol. **31:**1653–1654.
3. **Arthur, M., F. Depardieu, G. Gerbaud, M. Galimand, R. Leclercq, and P. Courvalin.** 1997. The VanS sensor negatively controls VanR-mediated transcriptional activation of glycopeptide resistance genes of Tn*1546* and related elements in the absence of induction. J. Bacteriol. **179:**97–106.
4. **Arthur, M., C. Molinas, T. Bugg, G. Wright, C. 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.
5. **Arthur, M., C. Molinas, and P. Courvalin.** 1992. Sequence of the *vanY* gene required for production of a vancomycin-inducible D,D-carboxypeptidase in *Enterococcus faecium* BM4147. Gene **120:**11–114.
6. **Arthur, M., C. Molinas, and P. Courvalin.** 1992. The VanS-VanR two-component regulatory system controls synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J. Bacteriol. **173:**2582–2591.
7. **Baptista, M., F. Depardieu, P. Reynolds, P. Courvalin, and M. Arthur.** 1997. Mutations leading to increased levels of resistance to glycopeptide antibiotics in VanB-type enterococci. Mol. Microbiol. **25:**93–105.
8. **Baptista, M., P. Rodriguez, F. Depardieu, P. Courvalin, and M. Arthur.** 1999. Single-cell analysis of glycopeptide resistance gene phenotype in teicoplanine-resistant mutants of VanB-type *Enterococcus faecalis*. Mol. Microbiol. **32:**17–28.
9. **Billot-Klein, D., L. Gutmann, S. Sable, E. Guittet, and J. van Heijenoort.** 1994. Modification of peptidoglycan precursors is a common feature of the low-level vancomycin-resistance VANB-type *Enterococcus* sp. strain D366 and of the naturally glycopeptide-resistant species *Lactobacillus casei*, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, and *Enterococcus gallinarum*. J. Bacteriol. **176:**2398–2405.
10. **Casadewall, B., P. Reynolds, and P. Courvalin.** 2001. Regulation of expression of the *vanD* glycopeptide resistance gene cluster from *Enterococcus faecium* BM4339. J. Bacteriol. **183:**3436–3446.
11. **DeHaseth, P., M. Zupancic, and M. Record, Jr.** 1998. RNA polymerase-promoter interactions: the comings and goings of RNA polymerase. J. Bacteriol. **180:**3019–3025.
12. **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.
13. **Evers, S., B. Casadewall, M. Charles, S. Dutka-Malen, M. Galimand, and P.**

Courvalin. 1996. Evolution of structure and substrate specificity in D-alanine: D-alanine ligases and related enzymes. J. Mol. Evol. **42:**706–712.
14. **Felsenstein, J.** 1993. PHYLIP version 3.5c. University of Washington, Seattle.
15. **Fines, M., B. Périchon, P. Reynolds, D. Sahm, and P. Courvalin.** 1999. VanE, a new type of acquired glycopeptide resistance in *Enterococcus faecalis* BM4405. Antimicrob. Agents Chemother. **43:**2161–2164.
16. **Glatron, M. F., and G. Rapoport.** 1972. Biosynthesis of the parasporal inclusion of *Bacillus thuringiensis*: half-life of its corresponding messenger RNA. Biochimie **54:**1291–1301.
17. **Greisen, K., M. Loeffelholz, A. Purohit, and D. Leong.** 1994. PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. J. Clin. Microbiol. **32:**335–351.
18. **Haldimann, A., S. Fisher, L. Daniels, C. Walsh, and B. Wanner.** 1997. Transcriptional regulation of the *Enterococcus faecium* BM4147 vancomycin resistance gene cluster by the VanS-VanR two-component regulatory system in *Escherichia coli* K-12. J. Bacteriol. **179:**5903–5913.
19. **Jacob, A., and S. Hobbs.** 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes*. J. Bacteriol. **117:**360–372.
20. **Leclercq, R., E. Derlot, J. Duval, and P. Courvalin.** 1988. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. N. Engl. J. Med. **319:**157–161.
21. **Liu, S., A. Hessel, and K. Sanderson.** 1993. Genomic mapping with *I-Ceu*I, an intron-encoded endonuclease specific for genes for ribosomal RNA, in *Salmonella* spp., *Escherichia coli*, and other bacteria. Proc. Natl. Acad. Sci. USA **90:**6874–6878.
22. **Magnet, S., P. Courvalin, and T. Lambert.** 1999. Activation of the cryptic *aac*(*6′*)-*Iy* aminoglycoside resistance gene of *Salmonella* by a chromosomal deletion generating a transcriptional fusion. J. Bacteriol. **181:**6650–6655.
23. **McCafferty, D., I. Lessard, and C. Walsh.** 1997. Mutational analysis of potential zinc-binding residues in the active site of the enterococcal D-Ala-D-Ala dipeptidase VanX. Biochemistry **36:**10498–10505.
24. **McCarthy, A., G. Victor, K. Ramotor, and B. Toye.** 1994. Risk factors for acquiring ampicillin-resistant enterococci and clinical outcomes at a Canadian tertiary-care hospital. J. Clin. Microbiol. **32:**2671–2676.
25. **McKessar, S., A. Berry, J. Bell, J. Turnidge, and J. Paton.** 2000. Genetic characterization of *vanG*, a novel vancomycin resistance locus of *Enterococcus faecalis*. Antimicrob. Agents Chemother. **44:**3224–3228.
26. **Messer, J., and P. Reynolds.** 1992. Modified peptidoglycan precursors produced by glycopeptide-resistant enterococci. FEMS Microbiol. Lett. **94:**195–200.
27. **Moran, C., N. Lang, S. LeGrice, G. Lee, M. Stephens, A. Sonenshein, J. Pero, and R. Losick.** 1982. Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. Mol. Gen. Genet. **186:**339–346.
28. **Msadek, T., F. Kunst, and G. Rapopport.** 1993. Two-component regulatory systems, p. 729–745. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C.
29. **Nölling, J., G. Breton, M. V. Omelchenko, K. S. Makarova, Q. Zeng, R. Gibson, H. M. Lee, J. Dubois, D. Qiu, J. Hitti, Y. I. Wolf, R. L. Tatusov, F. Sabathe, L. Doucette-Stamm, P. Soucaille, M. J. Daly, G. N. Bennett, E. V. Koonin, and D. R. Smith.** 2001. Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. J. Bacteriol. **183:**4823–4838.
30. **Parkinson, J. S., and E. C. Kofoid.** 1992. Communication modules in bacterial signaling proteins. Annu. Rev. Genet. **26:**71–112.
31. **Quintiliani, J. R., and P. Courvalin.** 1994. Conjugal transfer of the vancomycin resistance determinant *vanB* between enterococci involves the movement of large genetic elements from chromosome to chromosome. FEMS Microbiol. Lett. **119:**359–364.
32. **Reynolds, P.** 1985. Inhibitors of bacterial cell wall synthesis. Symp. Soc. Gen. Microbiol. **38:**13–40.
33. **Reynolds, P., C. Arias, and P. Courvalin.** 1999. Gene $vanXY_C$ encodes D,D-dipeptidase (VanX) and D,D-carboxypeptidase (VanY) activities in vancomycin-resistant *Enterococcus gallinarum* BM4174. Mol. Microbiol. **34:**341–349.
34. **Reynolds, P., H. Snaith, A. Maguire, S. Dutka-Malen, and P. Courvalin.** 1994. Analysis of peptidoglycan precursors in vancomycin-resistant *Enterococcus gallinarum* BM4174. Biochem. J. **301:**5–8.
35. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Sanger, F., S. Nicklen, and A. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
37. **Silva, J. A. Haldimann, M. Prahalad, C. Walsh, and W. Banner.** 1998. *In vivo* characterization of the type A and B vancomycin-resistant enterococci (VRE) VanRS two-component systems in *Escherichia coli*: a nonpathogenic model for studying the VRE signal transduction pathways. Proc. Natl. Acad. Sci. USA **95:**11951–11956.
38. **Stock, J. B., A. J. Ninfa, and A. M. Stock.** 1989. Protein phosphorylation and regulation of adaptative responses in bacteria. Microbiol. Rev. **53:**450–490.
39. **Trieu-Cuot, P., C. Carlier, C. Poyart-Salmeron, and P. Courvalin.** 1990. A pair of mobilizable shuttle vectors conferring resistance to spectinomycin for molecular cloning in *Escherichia coli* and Gram-positive bacteria. Nucleic Acids Res. **18:**4296.
40. **Van Caeseele, P., S. Giercke, J. Wylie, D. Boyd, M. Mulvey, S. Amin, and M. Ofner-Agostini.** 2001. Identification of the first vancomycin-resistant *Enterococcus faecalis* harbouring *vanE* in Canada. Can. Commun. Dis. Rep. **27:**101–104.
41. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
42. **Wright, G. D., T. R. Holman, and C. T. Walsh.** 1993. Purification and characterization of VanR and the cytosolic domain of VanS: a two-component regulatory system required for vancomycin resistance in *Enterococcus faecium* BM4147. Biochemistry **32:**5057–5063.
43. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.