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Mechanism of Intrinsic Resistance to Vancomycin in *Clostridium innocuum* NCIB 10674

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We have studied the basis for intrinsic resistance to low levels of vancomycin in *Clostridium innocuum* NCIB 10674 (MIC = 8 μg/ml). Analysis by high-pressure liquid chromatography (HPLC) and mass spectrometry of peptidoglycan nucleotide precursors pools revealed the presence of two types of UDP-MurNac-pentapeptide precursors constitutively produced, an UDP-MurNAc-pentapeptide with a serine at the C terminus which represented 93% of the pool and an UDP-MurNAc-pentapeptide with an alanine at the C terminus which represented the rest of the pool. *C. innocuum* cell wall muropeptides containing pentapeptide[Ser], either dialanine substituted on the epsilon amino group of lysine or not, were identified and represented about 10% of the monomers while only 1% of pentapeptide[D-Ala] monomers were found. The sequence of a 2,465-bp chromosomal fragment from *C. innocuum* was determined and revealed the presence of $ddl_{c.\ innocuum}$ and *C. innocuum* racemase genes putatively encoding homologues of D-Ala:D-X ligases and amino acid racemases, respectively. Analysis of the pool of precursors of *Enterococcus faecalis* JH2-2, containing cloned $ddl_{c.\ innocuum}$ and *C. innocuum* racemase genes showed in addition to the UDP-MurNAc-pentapeptide[D-Ala], the presence of an UDP-MurNAc-pentapeptide[D-Ser] precursor. However, the expression of low-level resistance to vancomycin was observed only when both genes were cloned in *E. faecalis* JH2-2 together with the $vanXY_c$ gene from *Enterococcus gallinarum* BM4174 which encodes a D,D-peptidase which eliminates preferentially the high affinity vancomycin UDP-MurNAc-pentapeptide [D-Ala] precursors produced by the host. We conclude that resistance to vancomycin in *C. innocuum* NCIB 10674 was related to the presence of the two chromosomal $ddl_{c.\ innocuum}$ and *C. innocuum* racemase genes allowing the synthesis of a peptidoglycan precursor terminating in serine with low affinity for vancomycin.

Members of the genus *Clostridium* are a major part of the anaerobic microflora of humans and are a potential cause of human infections. *Clostridium innocuum* belongs to the normal intestinal flora of human infants and adults and is one of the species which have been reported to cause human infections such as intra-abdominal sepsis, bacteremia, and endocarditis (11, 25). *Clostridium* spp. are considered susceptible to glycopeptides, vancomycin, and teicoplanin. However, a recent report has shown that MICs of vancomycin were equal to 8 or 16 μg/ml (intermediate resistance) for 28 clinical isolates of *C. innocuum* and *C. innocuum* NCIB 10674 while teicoplanin remained active (MICs = 0.25 to 1 μg/ml), suggesting that low-level vancomycin resistance is intrinsic in this species (23). Resistance to glycopeptide antibiotics among gram-positive organisms may be either acquired or naturally expressed (5). Acquired resistance to glycopeptides is generally observed in enterococci and has recently spread to *Staphylococcus aureus* (10). The VanA, VanB, and VanD types of resistance result from the synthesis of a new pentadepsipeptide peptidoglycan precursors ending in D-lactate [D-Lac] and the elimination of the high-affinity vancomycin pentapeptide[D-Ala] precursor ending in D-alanine and synthesized by the host (27). Low-level resistance to vancomycin is acquired in enterococci with the VanE or VanG phenotypes and is intrinsic in the VanC types *Enterococcus gallinarum* and *Enterococcus casseliflavus-Enterococcus flavescens* (5). The basis for this resistance is the synthesis of D-Ala–D-serine (D-Ser) which is substituted for D-Ala–D-Ala in the pentapeptide precursor (6, 7) and in the muropeptides (18). Again, the pentapeptide[D-Ala] precursor with high affinity for vancomycin is completely eliminated by D,D-peptidases and/or D,D-carboxypeptidases, a condition necessary for full expression of resistance (28). In *Enterococcus gallinarum*, synthesis of D-Ser is carried out by a pyridoxal phosphate-dependent and membrane-bound serine racemase (VanT) (3).

In this report, we analyzed the pool of precursors and the peptidoglycan structure of *C. innocuum* NCIB 10674. We identified a $ddl_{c.\ innocuum}$ gene and a *C. innocuum* racemase gene with homology to genes encoding D-Ala–D-X ligases and amino acid racemases, respectively, and responsible for the synthesis of a precursor and different muropeptides ending in D-Ala–D-Ser.

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TABLE 1. Primers used in this study

Primer	Sequence[a]	Position[b]	Primer use
A	−5′ GTATGGGATGCCACTCAGCTCAAAC 3′	378–354	Inverse PCR
B	+5′ TGCCGGAAGCAGCTTTGGAATTCAC 3′	567–591	Inverse PCR
C	+5′ AAGGCATATCGAGCCATGAACTGCA 3′	867–892	Inverse PCR
D	−5′ AGCCGTCAAAGGACTCCATCCTGTG 3′	613–591	Inverse PCR
LigBamHI	+5′ CTGGATCCAGTGGTGAATGAGCTGG 3′	−55–−31	*ddl*$_{c.\ innocuum}$ cloning
LigSalI	−5′ GCTTGTCGACGGAATCAGCTTCTG 3′	1178–1154	*ddl*$_{c.\ innocuum}$ cloning
RacBamHI	+5′ CCGTTTCCGGATCCGATTGACAAG 3′	1018–1041	*C. innocuum* racemase cloning
RacSalI	−5′ ATTGTCGACCTTCTCTTGAAAAATAG 3′	2265–2240	*C. innocuum* racemase cloning
XYSacII	+5′ TTGAGAGCTCTGGCAGAGGAG 3′	−28–−9	*vanXY*$_c$ cloning
XYBamHI	−5′ GTTCGCATAATAAATAAAGGATCCGA 3′	589–564	*vanXY*$_c$ cloning

[a] Restriction sites introduced in the primer sequence are underlined; +, direct primer; −, reverse primer.
[b] Position relative to the ATG start codon in the *ddl*$_{c.\ innocuum}$ or in the *vanXY*$_c$ genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *C. innocuum* NCIB 10674 (MICs of vancomycin and teicoplanin equal to 8 and 0.5 μg/ml, respectively) was grown in Wilkins-Chalgren broth or agar (Difco Laboratories, Detroit, Mich.) at 37°C under anaerobic conditions. For testing the inducible or constitutive mode of expression of vancomycin resistance, strain NCIB 10674 was grown anaerobically overnight in broth without or with subinhibitory concentrations of vancomycin (2 or 4 μg/ml). Induced and noninduced cells were diluted 1/50 in 10 ml of fresh broth in the presence of vancomycin at 0, 2, or 4 μg/ml. Bacterial growth was then measured for 12 h by spectrophotometry at 650 nm (Sequioa-Turner photometer [model 340]) and growth curves were plotted. *Enterococcus faecalis* JH2-2 was grown in brain heart infusion broth or agar (Difco Laboratories) at 37°C. Amplified genes were cloned in the shuttle multicopy vector pJIM2246 (which confers chloramphenicol resistance) (26). The MICs of vancomycin for enterococci containing various constructs were determined on Mueller-Hinton agar by the E-test method as recommended by the manufacturer (AB Biodisk, Uppsala, Sweden) and read after 48 h of incubation at 37°C. *E. coli* DH10B was used in transformation experiments. The *vanXYc* gene was amplified from total DNA of *E. gallinarum* BM4174 (28).

DNA manipulations. *C. innocuum* NCIB 10674 total DNA was extracted as previously described (23). Amplification of fragments internal to genes encoding related ligases with degenerate V1 and V2 primers was performed as previously described (15). Digestion with restriction endonucleases (New England Biolabs Inc., Beverly, Mass.), isolation of plasmid DNA, ligation, and transformation were carried out by standard methods (29). Sequencing was carried out with an ABI 377 automatic sequencer (Applied Biosystems). The entire sequence of the *ddl*$_{c.\ innocuum}$ and *C. innocuum* racemase genes was obtained by inverse PCR (24). Briefly, a digoxigenin-labeled probe (Roche Applied Science, Mannheim, Germany) from the amplified product was obtained with oligonucleotides V1 and V2. This probe hybridized in Southern experiments to a 5-kb SacII fragment, a 6.7-kb DraI fragment, and a 3-kb EcoRI fragment from *C. innocuum* NCIB 10674 chromosomal DNA. *Clostridium* DNA was digested with these enzymes and self-ligated at 15°C for 18 h. DNA was also digested with both DraI and SacII and treated with T4 DNA polymerase to generate blunt ends before ligation. The inverse PCR was performed with primers A, B, C, and D (Table 1). The *ddl*$_{c.\ innocuum}$, *C. innocuum* racemase, and *vanXY*$_C$ genes were cloned in plasmid pJIM2246 using primers shown in Table 1. Nucleotide and amino acid sequences were analyzed by using the BLAST and FASTA softwares available over the Internet at the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignment and phylogenetic tree were performed with the ClustalX and PHYLIP programs.

Preparation and analysis of the peptidoglycan nucleotide precursor pools. Enterococcal cells grown to an optical density at 650 nm of 0.7 were treated with vancomycin at 50 times the MIC for 90 min. Peptidoglycan precursors were extracted with formic acid as previously described (6) and analyzed by reverse-phase high-pressure liquid chromatography (RP-HPLC) with a μBondapack C_{18} column (3.9 by 300 nm; Waters) at a flow rate of 0.5 ml min^{-1} with 50 mM ammonium acetate, pH 5.0. Products were detected by absorbance at 262 nm. The UDP-MurNAc structures were deduced from their molecular mass determined by liquid chromatography-mass spectrometry and mass spectrometry-mass spectroscopy (MS/MS) as previously described (9).

Peptidoglycan structure analysis. Muropeptides were prepared from cell walls as described previously (8) except that hydrofluoric acid was used during the peptidoglycan purification (12, 18) and cellosyl (generous gift from Hoechst) was added to mutanolysin (Sigma, Saint-Quentin Fallavier, France) and lysozyme (Sigma) at 250 μg/ml each in phosphate buffer (25 mM, pH: 6.5) containing $MgCl_2$ (10 mM) during the hydrolysis step. The resulting muropeptides were reduced with sodium borohydride and separated by RP-HPLC coupled to mass spectrometry as previously described (8, 20). The structure of the muropeptides were deduced either from their molecular masses or after coelution with other structures previously identified in *E. faecalis* (13, 19). Some muropeptides were further purified by RP-HPLC and analyzed by MS/MS using the nanoelectrospray source kit for the Finnigam TSQ 7000 Protona A/S (San Jose, Calif.) as previously described (20).

Nucleotide accession number. The DNA sequences of the *ddl*$_{c.\ innocuum}$ and *C. innocuum* racemase genes have been deposited with GenBank accession number AY479979.

RESULTS

Pool of UDP-linked cytoplasmic precursors in *C. innocuum*. Precursors were studied in *C. innocuum* NCIB 10674 grown in the presence (2 μg/ml) or absence of vancomycin. Chromatograms of precursor pools of *C. innocuum* NCIB 10674 that were induced or noninduced were similar and showed that two types of UDP-MurNAc-pentapeptide precursors were present. One had a molecular mass of 1,165.8 Da, corresponding to a UDP-MurNAc-pentapeptide with a serine at the C terminus (pentapeptide[D-Ser]) and represented 93% of the pool. The other had a molecular mass of 1,149.4 Da, corresponding to a UDP-MurNAc-pentapeptide with an alanine at the C terminus (pentapetide [D-Ala]) and represented only 7% of the pool. The presence of the serine or an alanine at position 5 as well as the presence of a lysine residue at position 3 was demonstrated by MS/MS (data not shown). Neither UDP-MurNAc-tetrapeptide nor UDP-MurNAc-tripeptide precursors were found. Lack of tetrapeptide precursors suggested the absence of D,D-carboxypeptidase activity in *C. innocuum*. The quantitative similarity of the precursor pool for induced and noninduced cells suggested that expression of resistance to vancomycin was constitutive in *C. innocuum* NCIB 10674. This observation was consistent with the finding that growth curves for cells induced or not induced with vancomycin and challenged with subinhibitory concentrations of vancomycin were similar (data not shown).

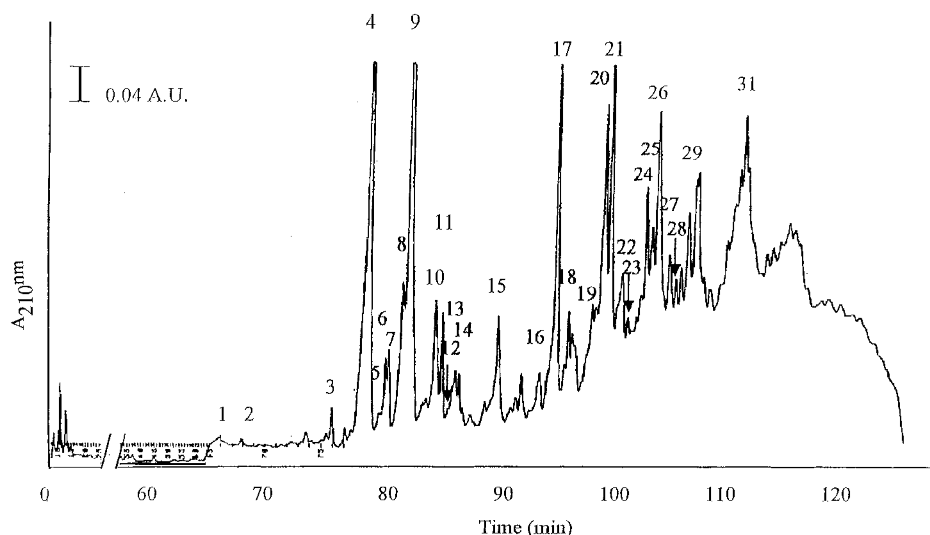

FIG. 1. Separation of *C. innocuum* cell wall muropeptides by RP-HPLC.

Muropeptide composition of *C. innocuum* NCIB 10674. The structure of 29 muropeptides of *C. innocuum* was identified (Fig. 1) and their deduced structures are shown in Table 2. Among the monomers two major muropeptides (peak 4 and 9) representing about 60% of the monomers were identified by their molecular mass and MS/MS as disaccharide tripeptides with two alanines branched on the ε-amino group of the L-lysine$_3$. Peak 9 differed from peak 4 by a mass difference of −42, corresponding to the loss of the *N*-acetyl residue from the *N*-acetylglucosaminyl moiety of the disaccharide. This suggested that two alanines could be part of the interpeptide bridge in different oligomers. The presence of such dialanine interpeptide bridges was confirmed by MS/MS in two tetra-tridimers present in peak 20 and 26.

As expected from the presence or cytoplasmic pentapeptide[D-Ser] precursors, different monomers containing pentapeptide[D-Ser]) either dialanine substituted or not were also identified in peaks 8, 9, 12, and 14, and represented about 10% of the monomers. Only small quantities (1%) of monomers pentapeptide[D-Ala] were found in agreement with the low amount of pentapeptide[D-Ala] precursor present in the pool. Structures containing pentapeptide[D-Ser] were also identified among the dimers (peaks 17, 18, 22, 23, 25, and 28). Since the detailed structure of different dimers and trimers could not be exactly determined due to the unknown precise number of alanine present in the interpeptide-bridge or at the C terminus (tetra or tri) only some structures are proposed (Table 2).

Identification of the *ddl*$_{c.\ innocuum}$ and *C. innocuum* racemase genes homologous to *ddl* and *alr* genes. No amplification product was observed with DNA of the strain, using a PCR assay with primers specific for resistance genes *vanA*, *vanB*, *vanC1*, *vanC2*, *vanD*, *vanE*, and *vanG* (14). The degenerate primers V1 and V2 which allow amplification of fragments internal to genes that encode related ligases (15), were used in a PCR with total DNA of *C. innocuum* NCIB 10674 as a template. A ca. 600-bp fragment was amplified and cloned into *E. coli*. Nucleotide sequences of the fragment, determined on both strands, were identical in 10 clones. The deduced amino acid sequence was compared with those encoded by various *ddl* genes, the D-Ala:D-Ala ligases from *E. coli*, the VanA and VanB D-Ala:D-Lac ligases, and the VanC1 and VanE D-Ala:D-Ser ligases. The sequence displayed between 28% and 39% of identity with the corresponding portion of those proteins. The motifs conserved in the related ligases were present, suggesting that the amplified fragment was internal to a ligase gene possibly involved in vancomycin resistance. Fragments similar in size were also amplified with oligonucleotides V1 and V2 from two clinical isolates of *C. innocuum*. The deduced amino acid sequence was found identical to that for *C. innocuum* NCIB 10674 except for one amino acid substitution (V235A).

The sequence of the regions upstream and downstream from the V1-V2 PCR product was obtained by inverse PCR as follows. The upstream sequence was obtained from a DraI-SacII fragment and the downstream sequence from an EcoRI fragment. In the 2,465-bp sequenced fragment, two open reading frames (ORF) were identified (Fig. 2). The 1,068-bp upstream ORF (nucleotides [nt] 198 to 1265) was preceded by a putative ribosome binding site (RBS) (5′-AGTAAGGAGTN$_8$ ATG) that displayed complementarity (underlined) to the *Bacillus subtilis* RBS consensus sequence (3′-OH UCUUUCC UCC) (22). The percentages of identity of the putative product, called Ddl$_{c.\ innocuum}$, with various D-Ala:D-Lac, D-Ala:D-Ser, and D-Ala:D-Ala ligases were calculated from the sequence alignment. Percentages of identity ranged from 39 to 45% with the D-Ala:D-Ser ligases (VanE, Van C1, VanC2, and VanG), from 36 to 41% with D-Ala:D-lactate (D-Lac) ligases (VanA, Van B, VanD, and ligases from *Paenibacillus popillae*, *Streptomyces toyocaensis*, *Amycolatopsis orientalis*), and from 36 to 38% with the putative D-Ala:D-Ala ligases from clostridia (*Clostridium acetobutylicum*, *Clostridium perfringens*, *Clostridium tetani*, and *Desulfitobacterium hafniense*). The highest degree of identity (45%) was with the VanG (D-Ala:D-Ser) ligase. The motifs conserved in the related amino acid ligases were found in the deduced 355-amino acid sequence. Of four amino acids that are present in the D-Ala:D-Ser ligases (EKYQ), two (KY) at positions 262 to 263 were conserved (16). Alignment

TABLE 2. Molecular mass and structure of muropeptides from *C. innocuum* NCIB 10476

Peak	Muropeptide type	Alanine no.[a]	Proposed structure[b]	*m/z*[c]	
				Observed	Calculated
1	Monomer		DS-mono	570.2	570.2
2	Monomer		DS-di	698.3	698.4
3	Monomer		DS-tetra	897.4	897.6
4	Monomer		DS-A2-tri[d]	968.5	968.7
5	Monomer		DS-tetra(−42)	855.4	855.7
6	Monomer		DS-A-tetra	968.5	968.5
7	Monomer		DS-A-tetra(OH)[d]	969.5	969.7
8	Monomer		DS-penta[A](−42)	926.6	926.6
	Monomer		DS-A2-penta[S](OH)[d]	1,127.5	1,128.0
9	Monomer		DS-A2-tri(−42)	926.5	926.7
	Monomer		DS-A2-penta[S]	1,126.5	1,127.2
10	Monomer		DS-A-tetra(OH)(−42)	927.4	927.6
11	Monomer		DS-A2-tetra	1,039.8	1,039.8
12	Monomer		DS-A2-penta[S](OH)(−42)	1,085.5	1,085.4
13	Monomer		DS-A-tetra(−42)	926.5	926.8
14	Monomer		DS-A2-penta[S](−42)[d]	1,084.5	1,084.8
	Monomer		DS-A2-penta[A](OH)[d]	1,111.5	1,111.5
15	Monomer		DS-A2-tetra(−42)	997.7	998.4
16	Dimer	[3]	BisDS[e]	1,917.9	1,918.2
17	Dimer	[4]	BisDS[e]	1,990.0	1,990.1
	Dimer	[3]	DS-A2-tetra-A-penta[S](OH)(−42)[f]	2,035.0	2,034.7
18	Dimer	[4]	BisDS-A2-tetra-A2-penta[S]	2,148.0	2,148.3
19	Dimer	[3]	BisDS—(OH)(−42)	1,876.9	1,877.0
20	Dimer	[4]	BisDS-A2-tetra-A2-tri(−42)[d]	1,947.9	1,947.8
21	Dimer	[4]	BisDS—(−42)[e]	1,947.9	1,947.0
22	Dimer	[4]	BisDS-A2-tetra-A2-penta[S](OH)(−42)	2,105.0	2,105.0
23	Dimer	[4]	BisDS-A2-tetra-A2-penta[S](OH × 2)(−42)	2,106.0	2,105.7
24	Trimer	[6]	TerDS—(OH)[e]	3,010.5	3,010.9
25	Trimer	[4]	TerDS-A2-tetra-A-tetra-A-penta[S](OH × 3)[f]	3,028.5	3,028.3
26	Dimer	[4]	BisDS-A2-tetra-A2-tri(OH)(−42 × 2)[d]	1,905.9	1,905.9
	Dimer	[3]	BisDS—(−42 × 2)[e]	1,833.9	1,833.2
27	Dimer	[4]	BisDS—(OH)(−42 × 2)	1,905.9	1,906.2
28	Dimer	[4]	BisDS-A2-tetra-A2-penta[S](OH × 2)(−42 × 2)	2,064.9	2,065.3
29	Trimer	[6]	TerDS-tetra-tetra-tri (OH) (−42)[e]	2,969.4	2,968.9

[a] Data in brackets are total number of alanines present in the cross-bridge, in the free N- terminal and C-terminal ends of oligomers.

[b] Proposed structure deduced from the molecular mass or MS/MS. DS, disaccharide (GlcNAC-MurNAC); BisDS, dimeric form; TerDS, trimeric form; mono, monopeptide (L-Ala); di, dipeptide (L-Ala–D-iGln); tri, tripeptide (L-Ala–D-iGln–L-Lys); tetra, tetrapeptide (L-Ala–D-iGln–L-Lys–D-Ala); penta [A], pentapeptide (L-Ala–D-iGln–L-Lys–D-Ala–D-Ala); penta [S], pentapeptide (L-Ala–D-iGln–L-Lys–D-Ala–D-Ser); A, one alanine branched on L-Lys; A2, two alanines branched on L-Lys; OH indicates the presence of Glu instead of iGln; (−42), monomer without acetyl group on GlcNAC, (−42 × 2), dimer without acetyl group on both GlcNAC of the dimer. For other trimers, tetramers, and pentamers (peaks >29), no structure is proposed.

[c] $(M + H)^+$ ion of the reduced muropeptide.

[d] Structure determined after MS/MS.

[e] No structure is proposed, due to the unknown distribution of the alanine(s) (see footnote *a*) in the interpeptide bridge or at the C terminus: for a dimer it can be either a BisDS-tetra-tetra or a BisDS-tetra-tri, and for a trimer it can be either a TerDS-tetra-tetra-tetra or a TerDS-tetra-tetra-tri.

[f] Assignment of the number of alanine in the cross bridge is arbitrary.

of ligases was used to construct a phylogenetic tree, confirming that $Ddl_{c.\ innocuum}$ was related to D-Ala:D-X ligases (Fig. 3) (17).

Immediately downstream $ddl_{c.\ innocuum}$, another ORF (nt 1271 to 2422) was identified that was preceded by an RBS (5′-TGGAAGAATGN$_6$GTG) that displayed complementarity to the 3′ extremity (underlined) to the 3-OH terminus of *B. subtilis* 16S rRNA (22) and began by an unusual GTG initiation codon. This ORF could possibly code for a 383-amino-acid protein that displayed homology with alanine racemases of different microorganisms encoded by *alr* genes and was therefore a hypothetical *C. innocuum* racemase gene. No potential transmembrane domains was detected using a hydrophobicity plot of the predicted amino acid sequence, suggesting that it was a soluble protein. Percentages of identity with serine racemases VanT of *E. gallinarum* BM4174, $VanT_E$ of *E. faecalis*, $VanT_{C2}$ from *E. casseliflavus* and the putative serine racemase $VanT_G$ ranged from 30 to 34%. Percentages of identity with putative alanine racemases of clostridia (*C. acetobutylicum*, *C. perfringens*, *C. tetani*, *Clostridium thermocellum*, and *D. hafniense*) ranged from 28 to 33%. Analysis revealed the presence of motifs conserved in racemases, in particular the putative pyridoxal attachment site which is highly conserved in alanine racemases (Fig. 2).

Expression of glycopeptide resistance and pool of UDP-linked cytoplasmic precursors in *E. faecalis* harboring plasmid encoded genes from *C. innocuum*. We tested if the putative $ddl_{c.\ innocuum}$ ligase and *C. innocuum* racemase genes could confer vancomycin resistance in an heterologous host. They were first amplified from *C. innocuum*, then cloned either alone or combined on the shuttle plasmid pJIM2246 where they were expressed under the control of the promoter of the chloramphen-

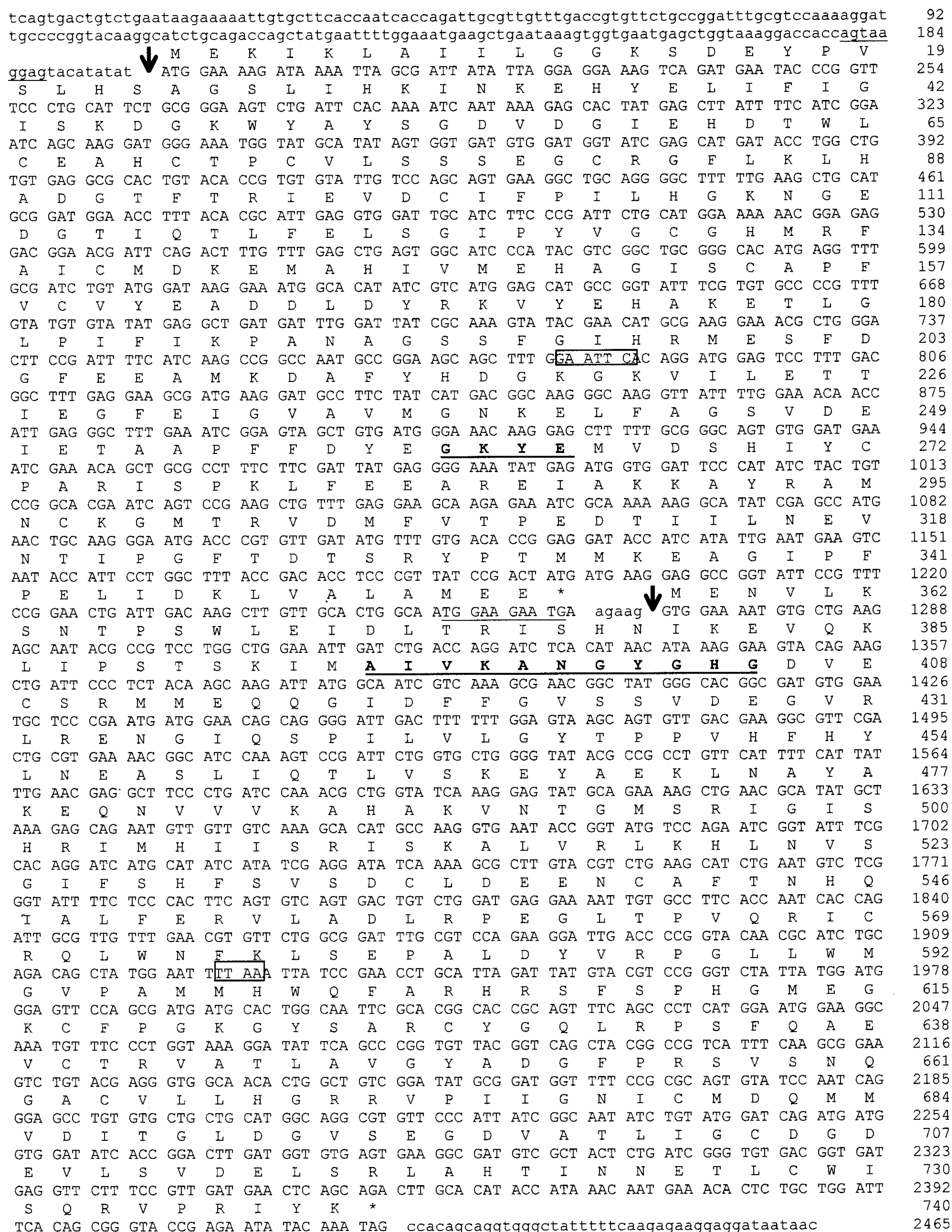

FIG. 2. Sequence of the $ddl_{c.\ innocuum}$ and *C. innocuum* racemase genes. The putative ribosome binding sites are underlined. The deduced amino acid sequence of $Ddl_{c.\ innocuum}$ and *C. innocuum* racemase are shown above the nucleotide sequence. Start of the proteins is indicated by an arrow. The EcoRI (nt 781 to 786) and the DraI (nt 1923 to 1926) sites used for inverse PCR are boxed. The conserved motif in D-Ala:D-Ser ligases and the putative pyridoxal attachment site in alanine racemases are in boldface type and are underlined.

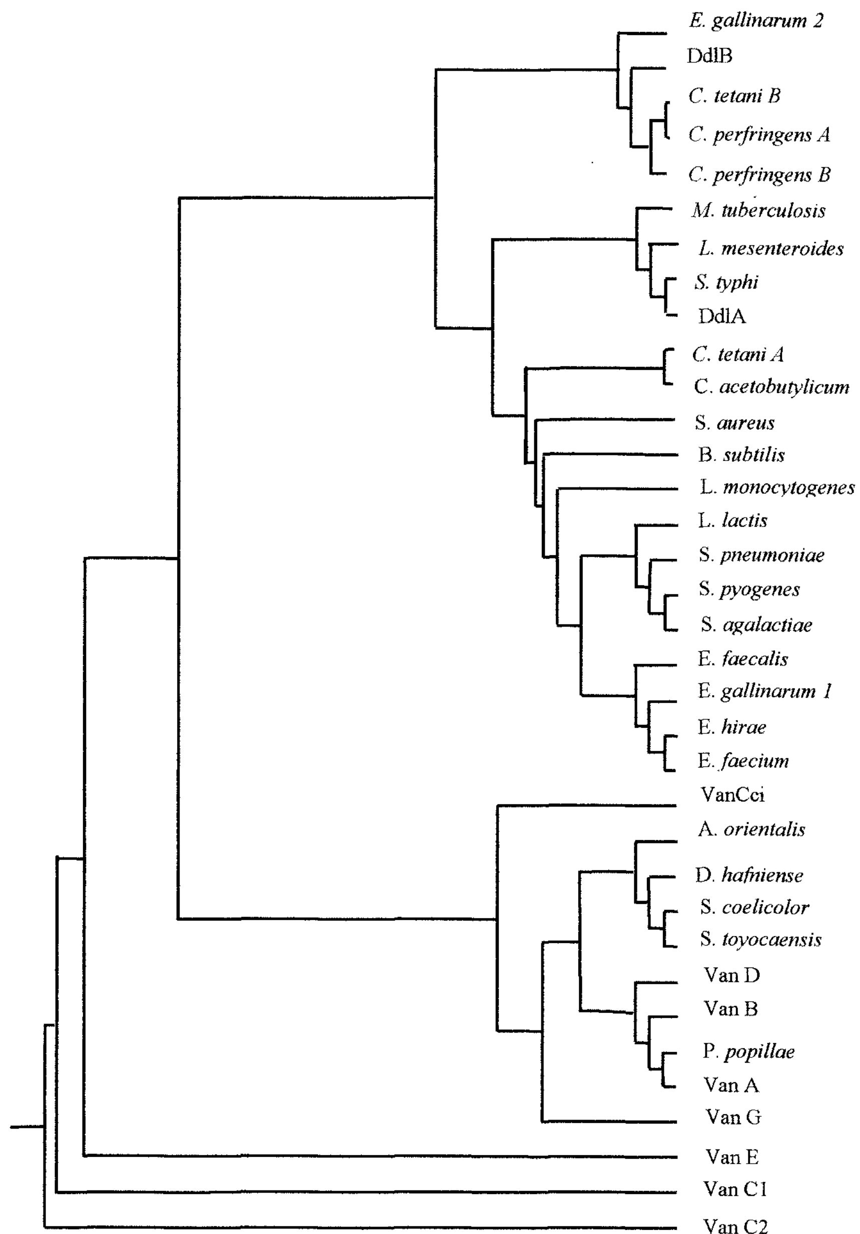

FIG. 3. Phylogenetic tree derived from the alignment of D-Ala:D-Lac, D-Ala:D-Ser, and selected D-Ala:D-Ala ligases. The tree was constructed by the neighbor-joining method, taking into account the results of maximum-parsimony and bootstrapping analysis. Sequences of the ligases are from *Amycolatopsis orientalis* (AAD19835), *Bacillus subtilis* 168 (CAB12263), *C. acetobutylicum* [Ddl] (AAK80837), *C. innocuum* [Ddl $_{c.\ innocuum}$] (), *C. perfringens* [DdlA] (BAB81021), *C. perfringens* [DdlB] (BAB80525), *C. tetani* [DdlA] (AAO34934), *C. tetani* [DdlB] (AAO35288), *D. hafniense* [Ddl] (ZP_00099215), *E. coli* K12 [DdlA] (NP_414915), *E. coli* K12 [DdlB] (NP_414634), *E. casseliflavus* [VanC2] (AAA60990), *E. faecalis* V583 [VanB] (2007289A), *E. gallinarum* BM4174 [VanC1] (AAA24786), *E. faecalis* [VanD] (AAM09849), *E. faecalis* BM4405 [VanE] (AAL27442), *E. faecalis* WCH9 [VanG] (AAF71281), *E. faecalis* [Ddl] (AAC43218), *E. faecium* BM4147 [VanA] (AAA65956), *E. faecium* [Ddl] (ZP_00036460), *E. gallinarum* BM4174 [Ddl1] (AAN62561), *E. gallinarum* BM4174 [Ddl2] (AAK97387), *E. hirae* [Ddl] (Q47827), *Lactococcus lactis* (AAK04439), *Leuconostoc mesenteroides* [Ddl] (Q48745), *Listeria monocytogenes* (CAC98933), *M. tuberculosis* [Ddl] (CAB05431), *P. popillae* (AAF36803), *Salmonella enterica* serovar Typhi [DdlA] (AA070072), *S. aureus* [Ddl] (BAB43170), *S. agalactiae* [Ddl] (AAM99654), *S. pneumoniae* [Ddl] (CAB64467), *Streptomyces coelicolor* [Ddl] (NP_627790), and *Streptomyces toyocaensis* [Ddl] (AAC23582).

icol acetyltransferase gene and finally introduced into *E. faecalis* JH2-2 (Table 3). *E. faecalis* JH2-2 harboring pJIM2246 containing the cloned $ddl_{c.\ innocuum}$ ligase or *C. innocuum* racemase genes showed only the presence of pentapeptide[D-Ala] precursor and no change in the vancomycin MICs. Combination of *C. innocuum* racemase gene and $ddl_{c.\ innocuum}$ resulted in the production of pentapeptide[D-Ala] and pentapeptide[D-Ser] whereas resistance to vancomycin was still not expressed. This result was, however, not surprising since synthesis of modified precursors by the cloned genes could result in vancomycin resistance only if the high-affinity vancomycin pentapeptide[D-Ala]precursor produced by the host was eliminated (5, 27, 28).

TABLE 3. Peptidoglycan precursors in extracts of *E. faecalis* JH2-2 harboring various plasmids and for which MICs of vancomycin differ

Plasmid	MIC of vancomycin (μg/ml)	Type of peptidoglycan precursors (%)[a]		
		Tetra	Penta[Ser]	Penta[Ala]
pJIM2246	2	—[b]	—	100
pJIM2246Ω $ddl_{c.\ innocuum}$	2	—	—	100
pJIM2246Ω *C. innocuum* racemase gene	2	—	—	100
pJIM2246Ω $ddl_{c.\ innocuum}$, *C. innocuum* racemase gene	2	—	9	91
pJIM2246Ω $vanXY_c$	2	90.8	—	9.2
pJIM2246Ω $ddl_{c.innocuum}$, $vanXY_c$	2	ND[c]	ND	ND
pJIM2246Ω *C. innocuum* racemase gene, $vanXY_c$	2	92.5	—	7.5
pJIM2246Ω $ddl_{c.\ innocuum}$, *C. innocuum* racemase gene, $vanXY_c$	6	28.5	11.7	59.8

[a] Tetra, UDP-MurNac–L-Ala–γ-D-Glu–L-Lys–D-Ala; Penta[Ser], UDP-MurNac–L-Ala–γ-D-Glu–L- Lys–D-Ala–D-Ser; Penta[Ala], UDP-MurNac–L-Ala–γ-D-Glu–L-Lys–D-Ala–D-Ala.
[b] —, not detected.
[c] ND, not done.

Partial elimination of this latter was achieved, by cloning the $vanXY_c$ gene from *E. gallinarum* BM4174 downstream from the $ddl_{c.\ innocuum}$ and/or *C. innocuum* racemase genes (Table 3). $VanXY_c$ has a D,D-peptidase activity which degrades UDP-MurNAc-pentapeptide[D-Ala] to UDP-MurNAc-tetrapeptide and can hydrolyze D-Ala:D-Ala, although at a lesser efficiency (28). By contrast, this enzyme has a with very low dipeptidase activity against D-Ala:D-Ser and no activity against UDP-MurNAc-pentapeptide[D-Ser] (28). Introduction of this construct in *E. faecalis* JH2-2 resulted in the production of pentapeptide[D-Ser], pentapeptide[D-Ala], and tetrapeptide precursors, together with a reproducible threefold increase in the MIC of vancomycin. In the presence of the cloned $ddl_{c.\ innocuum}$ and $vanXY_c$ genes and in the absence of *C. innocuum* racemase gene similar increased MIC of vancomycin was observed for *E. faecalis* when D-serine (10 mM) was added to Mueller-Hinton agar. In contrast, the addition of L-serine (10 mM) did not affect susceptibility to vancomycin.

DISCUSSION

In this work, we have shown that intrinsic low-level resistance to vancomycin in *C. innocuum* is related to the synthesis of a high proportion of low-affinity precursors ending in D-Ala–D-Ser (4, 18). This is the first report of such a mechanism of resistance in an anaerobic bacteria.

The two genes encoding a putative $Ddl_{c.\ innocuum}$ ligase and *C. innocuum* racemase were found to be adjacent on the chromosome. The ligases from other *Clostridium* spp. form a group distinct from $Ddl_{c.\ innocuum}$ which was closely related to the VanG D-Ala:D-Ser ligase and to D-Ala:D-Lac ligases, although placed on a separate branch (Fig. 3). *C. innocuum* racemase was predicted to be a soluble protein, similar to "classical" racemases and therefore differs from the other serine racemases reported previously in enterococci—$VanT_C$ (1), $VanT_E$ (1), and $VanT_G$ (21—which contain 10 transmembrane domains and are probably membrane-bound. The reason for this difference is unknown.

As our results showed that resistance was related to the synthesis of precursors terminating in D-Ser, the presence of a small quantity of precursors ending in D-Ala and of pentapeptide[Ala] monomers in *C. innocuum* was surprising. It is possible that the $Ddl_{c.\ innocuum}$ ligase has also some activity of a D-Ala:D-Ala ligase. Alternatively, another D-Ala:D-Ala ligase could be encoded by the chromosome of *C. innocuum*. However a single gene encoding a D-Ala:D-Ser ligase was amplified by oligodeoxynucleotides V1 and V2 which does not exclude the presence of a second, more structurally remote, *ddl* gene. There is circumstantial evidence that a single ligase is present as no gene encoding a VanXY-type enzyme is present in the operon from *C. innocuum* between the ligase and racemase genes while when the two genes are cloned in *E. faecalis* (which has a D-Ala–D-Ala ligase), VanXYc has also to be added for the organism to become low-level resistant to vancomycin. In general, bacterial chromosomes encode a single enzyme, although there are exceptions such as *E. gallinarum* with one D-Ala:D-Ser and two D-Ala:D-Ala ligases (2), and enteric bacteria (*E. coli* and *Salmonella enterica* serovar Typhimurium) with two D-Ala:D-Ala ligases. The in silico analysis of the sequenced genome of clostridia showed that *C. acetobutylicum* and *D. hafniense* contained only one putative D-Ala:D-Ala ligase (GenBank access numbers AAK80837 and ZP_00099215, respectively), *C. perfringens* two (BAB81021 and BAB80525), and *C. tetani* two, as well (AAO34934 and AAO35288).

Cloning of the $ddl_{c.\ innocuum}$ and *C. innocuum* racemase genes in *E. faecalis* showed that cooperation of the two genes was necessary for the synthesis of the low vancomycin affinity pentapeptide[D-Ser] precursor in this host and that they confer resistance to vancomycin provided that, in the presence of the cloned $vanXY_c$ gene, the high affinity vancomycin pentapeptide[D-Ala] precursor synthesized by the heterologous host was partially eliminated. Homology of *C. innocuum* racemase with amino acid racemases and expression of vancomycin resistance without addition of D-Ser suggested that the protein catalyses synthesis of D-Ser in vivo from L-Serine available either from the culture medium or synthesized de novo. In confirmation of this hypothesis, the cloned $ddl_{c.\ innocuum}$ gene alone in presence of $vanXY_c$ was sufficient for expression of resistance if bypass of the absent *C. innocuum* racemase was obtained after addition of D-serine to the culture medium.

Thus, it can be concluded that in *C. innocuum*, cooperation of $ddl_{c.\ innocuum}$ and *C. innocuum* racemase lead to the expression of glycopeptide resistance since they allow the predominant production of cytoplasmic pentapeptide [D-Ser] precursor which is then processed by the cell wall machinery to be integrated in the peptidoglycan.

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