Mechanism of Intrinsic Resistance to Vancomycin in Clostridium innocuum NCIB 10674

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Received 19 November 2003/Accepted 12 February 2004

We have studied the basis for intrinsic resistance to low levels of vancomycin in *Clostridium innocuum* NCIB 10674 (MIC = $8 \mu g/ml$). 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 ddlc. 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 ddlc, 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 ddlc. 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 μ g/ml (intermediate resistance) for 28 clinical isolates of C. innocuum and C. innocuum NCIB 10674 while teicoplanin remained active (MICs = 0.25 to 1 μ g/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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Primer	Sequence ^a	Position ^b	Primer use
A	-5' GTATGGGATGCCACTCAGCTCAAAC 3'	378-354	Inverse PCR
В	+5' TGCCGGAAGCAGCTTTGGAATTCAC 3'	567-591	Inverse PCR
С	+5' AAGGCATATCGAGCCATGAACTGCA 3'	867-892	Inverse PCR
D	-5' AGCCGTCAAAGGACTCCATCCTGTG 3'	613-591	Inverse PCR
LigBamHI	+5' CTGGATCCAGTGGTGAATGAGCTGG 3'	-5531	<i>ddl_c</i> innocuum cloning
LigSalI	-5' GCTTGTCGACGGAATCAGCTTCTG 3'	1178–1154	<i>ddl_c</i> innocuum cloning
RacBamHI	+5' CCGTTTCCGGATCCGATTGACAAG 3'	1018-1041	C. innocuum racemase
			cloning
RacSall	-5' ATT <u>GTCGAC</u> CTTCTCTTGAAAAATAG 3'	2265-2240	C. innocuum racemase
XYSacII	+5' TTGAGAGCTCTGGCAGAGGAG 3'	-289	vanXY cloning
XYBamHI	-5' GTTCGCATAATAAATAAA <u>GGATCC</u> GA 3'	589–564	$vanXY_c$ cloning

	ΤA	BLE	1.	Primers	used	in	this	study
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^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 C₁₈ 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 μ g/ml each in phosphate buffer (25 mM, pH: 6.5) containing MgCl₂ (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 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-MurNActetrapeptide 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-lysine₃. 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'-<u>AGTAAGGAGTN₈</u> 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

D 1		Alanine		m/z^c				
Реак	мигорершае туре	no. ^a	Proposed structure ^o	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 \times 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 \times 3) ^f	3,028.5	3,028.3			
26	Dimer	[4]	BisDS-A2-tetra-A2-tri(OH) $(-42 \times 2)^d$	1,905.9	1,905.9			
	Dimer	[3]	BisDS— $(-42 \times 2)^e$	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 \times 2)(-42 \times 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 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'-T<u>GGAAGAATGN₆GTG</u>) 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 UDPlinked 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-

$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	tcagtgactgtctgaataagaaaaattgtgcttcaccaatcaccagattgcgttgtttgaccgtgttctgccggatttgcgtccaaaaggat tgccccggtacaaggcatctgcagaccagctatgaattttggaaatgaagctgaataagtggtgaatgagctggtaaagggaccacc <u>agtaa</u>										92 184 19													
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GAC GGA AGG ATT CAG ACT TTG TTT GAG CTG AGT GGC ATT CCA TAG GTC GGC TGC GGC ATG AGG ATG AGG ATG AGG AGG AGG AGG AGG	D	G	т	I	Q	Т	\mathbf{L}	F	E	L	S	G	I	P	Y	V	G	С	G	Н	M	R	F	134
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GU ALC 19, Y Y S 2 A G AL A G AL A G AL A G AL A C AL A G AL A A A G AL A A G AL A A G AL A A G AL A A A A	A	1	C	M	D	K DDC	E CDD	M NTC	A	H CAT	1 2 T C	V CTC	M ATC	E GAG	САТ	A CCC	GGT	፲ ፲	TCG	тGT	GCC	CCG	ድ ጥጥጥ	668
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CCG GCA CCA ATC AGT CCG AAG CTG TTT TAG GAA GCA AGA GCA ATA ATG GCA AAA AAG GCA TTT CGA GCA ATT CGA CCA TT TG GAA CCA ATT TGA ATA TGA AGC ATG ATG TT GCG ATA GTA TGT TT GTG ACA CCG GAG GAT ATG CTT GTG ACA CCG GAG GAT CA CT ATA TGG ATG GAG GTG TT ACG GTT T T GTG ACA CCG GAG GAT CA CT CA TA TG AAT GAA GTC 1151 N T I P G F T D T S R Y P T M M K E A G I P F 341 ATA ACC ATT CCT GGC TTT ACC GAC ACC TCC CGT TAT CG ACT ATG ATG AAG GAG GCC GGT ATT CCG TT 1220 P E L I D K L V A L A M E E * M E M E N V L K 320 CCG GAA CTG ATT GAC AGC GTG TT GCA CTG GCA A <u>TG GAG AAGA GAA GAA GAG GAG AGA GAA GTG CTG AAG</u> 1288 S N T P S W L E I D L T R I S H N I K E V Q K 365 CCG ATG CG CG TCC TGG CTG GAA ATT GGT CTG ACA GGG ATC CTA ACA ATA AGA GAA AGG GAG ACG ATG GAA ATG CC CTC TCA AGC AGC AAG ATT TATG GTG ACA GGG ATC CTA AGC GAT AGA GG CA CTG GGA AGA GAG TG CGA AGA GAT CTG CTG AAG GGA CG GC TTT GGG GTG GGG GTT GGG GTA GGG GAT GGG CA GG GAT GGG AAG GGG CTT GG GGA GG GTT GGG GAA AGC GTG GAA TGC CC CTC ACA AGC AGC AGC AGG GG GAT GGC TTT TTT GGA GGA AGC GGC TAT GGG GCA GGC GTT GGG GAA AGC GTG GAA TGC CCC CGA ATG AGC GAG CAG GGG GAT GAC TTT TTT GGA GAA AGC GAC GCC CT GT CAT TTT CAT 1154 C I R E N G I Q S P I L V L G Y T P P V H F F Y 454 CTC GCT GAA AAC GCA CAC GAC GG GG TT CTG GTG CGG GG GTA AGC GCC TGT CAT TTT CAT TAT 1564 L N E A S L I Q T L V S K E Y A E E K L N A Y A 477 TTG AAC GAC GAC ACC CAA GCC CAT GCC AAG GGG GAT ACC GG CT GTG CAT AGC CGA ATT CG AAA GAG CAC AAT GT GTT GAA CAC CAA AGC CTG GTA CTG AAA GGG AAT CCC GG CCT TTT CAT TTT CAT TAT 1564 A C Q N V V V K A H A K V N T G M S R I G I S 500 AAA GAG CAC AAT GTT GTT CAA AGC GCA CTT GCC GAG GTT GGA GAA AGC CGC ATT CG ATT CG AGG GAA AGC CTT CTC CACA AGC CAT TCG AGA GAA AGC CCA TTT CG CGA CAT AGC CAA GGC CTT GTA GAA AAA CGG CAC CTT CAC CAA GG CAT TTCG AGA GAA AGC CT GGA CAT CGG ATT CGG GAT ATC CAAG GAA AGC CTT CGG GAT ATC CAAG GGA TT CG GGA ATT CG GAA GGC CTT GTA CGA AGG GAT CTG CTG AAA GGG ATT CTG AGA GAA AAT CTG CGG AAA GGC CTG GTA CGC CTT CAC CAAA CGC ATT CG AAA GAG CA	Ρ	А	R	I	S	Ρ	К	L	F	E	Е	А	R	E	I	А	К	K	А	Y	R	А	М	295
N C C K G M T R V D M F V T P E D T I I L N E V 318 AC TGC AGG GGA AGC GGT GG GAT TG GAT ATT GAA GTC TTT GGA GCA CG GGA GC GAT ACC ATC ATT GAA GAG GTC 1151 N T I P G F T D T S R Y P T M M K E A G I P F 341 AT ACC ATT CCT GC TTT ACC GAC ACC CC CG TTA TC GAT ATTG ATA TTG ATA TTG ATA TC GAT TTC CG TT1 120 P E L I D K L V A L A M E E * M M E C N V L K 326 CG GAA CTG ATT GAC AAG CTT GTT GCA CTG GC ATG GGA AGT GA agaa GTG GAA AAT GTG CTG AAG 1288 S N T P S W L E I D L T R I S H N I K E V Q K 385 AGC AAT ACG CCG TC TG CG GAA ATT GAT CG AC AGG ATC CAC CAT AAG GAA GAA ATA GAG ATC AGA AGA TA AGG GAA GAA TGA CTG AAG ATG AGA GTG CTG AAG 1357 L I P S T S K I M A I V K A N G Y G GA ATT GG CAAT CGA AGA GAA GAA ATA AGA GAA GAA GAA GAA G	CCG	GCA	CGA	ATC	AGT	CCG	AAG	CTG	TTT	GAG	GAA	GCA	AGA	GAA	ATC	GCA	AAA	AAG	GCA	TAT	CGA	GCC	ATG	1082
ARC TYCE ARG GGA ATG ACC CGF GTT GTT GTT GTG TT GTG ACA CGG GGA GAG GAG CAT ATG ATG AG GTC ATA TGG AG GTC TT LOC GAG ACC TTG CTG TT GTG CT TAT GTG ACT AGG GAG GCC GGT ATT CGG TTT LOC GAC ACC TTG CC CTT TAT CGG ACT ATG ATG AGG GAG GCC GGT ATT CGG TTT LOC GAC TTG GTG GCA ATG GCA TTG CGA CTG GAC ATG GCA ATG GAG AGA TGA AGG GC GGTA ATA GTG CTG AGG AGG ATG GC TT T TA CG GAC TTG GCA CTG GCA ATG GCA ATG GCA ATG AGA GAA TGA AGG GC GAA AAT GTG CTG AAG AGG ATG GC GAA ATT GAT CTG ACC AGG ATC CTG CCA CATA AAC GAA ATT AAG GAA ATA AAG GAA CTA CAA ACA ACA ACA ACA AGC AATA AAG GAA CTA CAA ACA ACA ACA ACA ACA ACA ACA A	N	С	K	G	M	Т	R	V	D	M	F	V	T	P	E	D	T	I	I	L	N	E	V	318
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L I P S T S K I M A I V K A A G G G H G G H G J V L 400 CTG ATT CCC TCT ACA AGC AG ATT ATG GCA ATG GCA CTC GTC AAC GCG TAT GGC CAC GGC GAC GGC GAA 431 TGC TCC CCA ATG ATG GAA CAC CAG GGG ATT GAC TTT TTT GGA GTA AGC GAT GTG CAC GGC GAT GTG GAA 1426 CTG CTC CCA ATG ATG GAA CAC CAG GGG ATT GAC TTT TTT GGA GTA AGC AGT GTT GAC GAA GGC GTT CAA 1495 L R E N G I Q S P I L V L G Y T P P V H F H Y 454 CTG GGT GAA AAC GGC ATC CAA AGT CCG ATT CTG GTG GGG TAT ACG CCG CT GTT CAT TTT CAT TAT 1564 L N E A S L I Q T L V S K E Y A E K L N A Y A 477 TG GAC GAC GCT TCC CTG ATC CAA AGT CCG ATT TCA AGG GTG AAT GCG GAA AGC CTG AAC GCA TAT GCT 1633 K E Q N V V V K A A H A K V N T G M S R I G I S CAC AGG ATC ATG GAT ATC ATA ACG CAG GTG AAT ACC GT ATG CAC AAA GCC ATT TCG 1702 H R I M H I I S R I S K A L V R K L K H L N V S CAC AGG ATC ATG CAT ATC ATA TCG AGG ATA TCA AAA GCG CTT GTA CGT CTG AAG CAT CTG AAT GCT 1702 H R I M H I I S R I S V S D C L D E E N C A F T N H Q 546 GT ATT TCC CAC TTC AGT GTC AGT GAC TTT CG GGA GAT TAG CAC CAT CTG AAT GCT 1771 G I F S H F S V S D C L D E E N C A F T N H Q 546 GT ATT TC CC CAT TAG GT GTT GTA GAC TTT GGA GTA AAA GCG TTG AG GAT ATG TGC CAA CAC CCA TAT CCC 1711 G I F S H F S V S D C L D D E E N C A F T N H Q 546 GT ATT TC TCC CAC TTC AGT GTC AGT GAC TTT CG GGA GAT ATA TGT GCC CTG CAA CAC CCC GTA CAC CCC AAT CCC 1711 G I F S H F S V L A D L R P E G L T P V Q R I C 569 ATT GCG TTG TTG GAA CGT GTT CTG GCG GAT TTG CGT CAG AGA CAT CTG CAAT CAC CCC GTA CAA CCC AAT CCC 1760 170 A L F F R R V L A D L R P E G L T P V Q R I C 569 ATT GCG ATG GAA TT <u>TT AAA</u> TTT TC GAA CCC CGC ATT GGA TTA GAT TAT GTA CCC CCC GAA CAC CCC GAA TTA GCT CTG 178 G V P A M M H W Q F F A R H R S F S P H G M E G 1998 GA CA C TA TGG AAT T <u>TT AAA</u> TTT TA CC GAA CCC CGC GAT TTA GCT GCG GAT TTT CAA GCC GGA AT 1978 G A C V P A M M M H W Q F A R R H R S F S P H G M E G AAA GGA CTA TGG AGG ATA TTT CAG CTG GCA GT GT GCC GGT GT TAC CGC GCA TTT CCAA GCG GAA CA 1978 G A C V P A M M M H W Q F A R R V P I	AGC	AAT	ACG	CCG	TCC	TGG	CTG	GAA	TTA	GAT	CTG	ACC	AGG	ATC	TCA	CAT	AAC	ATA	AAG	GAA	GTA	CAG	AAG	1357
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The theorem is the set of the term of the set of the s	CrG	ATT C	p	M	ACA M	AGC F	AAG	ATT O	ATG	GCA T	AIC D	EIC E	AAA F	909 G	V	S	S	v	D	E	G	v	R	431
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CTG GTG GAA AAC GGC ATC CAA AGT CCG ATT CTG GTG CTG GGG TAT ACG CCG CT GTT CAT TTT CAT TAT 1564 L N E A S L I Q T L V S K E Y A E K L N A Y A 477 TG AAC GAG GCT CC GTG CTG CAA CG CTG GTA TCA AAG GAG TAT GCA GAA AAG CTG AAC GCA TAT GCT 1633 K E Q N V V V K A H A K V N T G M S R I G I S 500 AAA GAG CAG AAT GTT GTT GTC AAA GCA CAT GCC AAG GTG AAT ACC GGT ATG TCC AGA ATC GGT ATT TCG 1102 H R I M H I I S R I S K A L V R L K H L N V S 523 CAC AGG ATC ATG CAT ATC ATA TCG AGG GAT TCA AAA GCG CTT GTA CGT CTG AAG CAT CGT AAT GTC TCG 1771 G I F S H F S V S D C L D E E N C A F T N H Q 546 GTT ATT TTC TCC CAC TTC AGT GTC TG GAG GAC TGT CGG GAG GAA AAT TGT GCC TTC ACC AAT CAC CAG 174 A L F E R V L A D L R P E G L T P V Q R I C 569 ATT GCG TTG TTT GAA CGT GTT CTG GGG GAT TTG CGT CGA GAA GGA TTG ACC CGG GTC TTA TTG GCC TTA CCT ACC AAT GCC 1909 R Q L W N F K L S E P A L D Y V R P G L L W M 592 AGA CAG GTA GTG ATG CAT GTG GGA AAT TCC GAA GGA CAT TGT GCC CG GTA TAT TGG ATG GAT GG GTA TAT GG ATG GAT ATG CAT GTG GGA CAT TCC GAA CGC GCA CTT AGT TAT GTA GAT TAT GTA GAT GAT GAG GAT ATT GGA AGG CTA TTA GGA AG A L F E R V L S S E P A L D Y V R P G L W M 592 AGA CAG CTA TGG AAT T <u>TT AAA</u> TTA TCC GAA CCT GCA TAT GAT TAT GTA GAT TAT GTA CGT CCG GGT CTA TTA TGG ATG G V P A M M H W Q F A R H R S F S S P H H G M E G 615 GGA GTT CCA GGG ATG ATG CAC TGG CAA TTC GCA CGG CAC CCC AGT TTC AGC CCT CAT GGA ATG GAA GGC 2047 K C F P G G K G Y S A R C Y G Q L R P S F Q A E 638 AAA TGT TC CCT GGT AAA GGA TAT TCA GGC GTA TTA GGG TAT GCG CGC TCA TTT CAA GCG GAA 2116 GTC TT ACG AGG GTG GCA ACA CTG GCT GTC GGT GTT CCC ATT ATC GGC CAG TAT CCA ATT CAG CGG GAA 2116 GTC TT R V A T L A V G Y A D G F P R S V S N Q 661 GTC TGT ACG AGG GTG GCA ACA CTG GCT GTC GTT GCC ATT ATC GGC AAT ATC TGT ATG GAT GAT GAT ATG CGT ATT CCA ATT CAG GGA GCT CTG TGG CTG CTG CAT GGC AGG CTG TT CCC ATT ATC GGC CAT ATC TGT ATG GAT GAT ATC CAT CAG CGG GT ATT CCA ATT CAG GGA GTT CTT TCC GTT GAT GAT GGT GTG GAG GAT GTT CCC ATT ATC GGC AAT ATC TGT ATG GAT GAT CAG CGG G	L	R	Е	N	G	I	Q	S	Р	I	L	v	L	G	Y	т	Ρ	Р	v	Н	F	Н	Y	454
L N E A S L I Q T L V S K E Y A E K L N A Y A 477 TTG AAC GAG GCT TCC CTG ATC CAA ACG CTG GTA TCA AAG GAG TAT GCA GAA AAG CTG AAC GCA TAT GCT 1633 K E Q N V V V K K A H A K V N T G M S R I G L S 500 AAA GAG CAG AAT GTT GTT GTC AAA GCA CAT GCC AAG GTG AAT ACC GGT ATG TCC AGA ATC GGT ATT TCG 1702 H R I M H I I S R I S K A L V R L K H L N V S CAC AGG ATC ATG CAT ATC ATA TCG AGG ATA TCA AAA GCG CTT GTA CGT CTG AAG CAT CTG AAT GTC TCG 1771 G I F S H F S V S D C L D E E N C A F T N H Q 546 GGT ATT TTC TCC CAC TTC AGT GTC GTG GAG GAC TGT CTG GAA GGA TTG GCC TTC ACC AAT CAC CAG 1840 T A L F E R V L A D L R P E G L T P V Q R I C AGG CAG CTA TGG AAT GTT GTG GCG GAT TTG CGA CAT GCA GAA GGA TTG ACC CCG GTA CAA CGC ATC TGC 1909 R Q L W N F K L S E P A L D V V R P G L L W M 592 AGA CAG CTA TGG AAT GTT CTG GC GAA TTC GCA CAC GCA CGC AGT TTA GAA CGC CTT ACC AAT GGA ATG 1978 G V P A M M H W Q F A R H R S F S P H G M E G GGA GTT CCA GGG ATG GCA CAT TCG GCC CGC GT TTA CAG CCT CAT GGA ATG GAA GGA 2047 K C F P G G K G Y S A R C Y G A R C C GG CAT TTC AGC CGC AGT TTC AGC CTT AGG AATG GAA GGC 2047 K C F P G G K G Y S A R C Y G A R R C Y G Q L R P S F Q A E 638 AAA TGT TTC CCT GGT AAA GGA TAT TCA GCC GCG GT GTT ACG GCA GAA GGC CCG CAG TTT CAG CCG CAG TAT CAA CGC GAA 204 C T R V A T L A V G Y A D G Y A D G F P R S V S N Q 661 GTC TGT ACG AGG GTG GCA GAA CGC GGT GTT GCC GGA GTT TTC CGC CGC AGT TTT CAG CCG CGA GTA TCC AAT CAG 2185 G A C V L L H G R R V P I I G N I C M D Q M M 684 GGA GCC TGT GTG GTG CTG GAT GGC AGT GTG GCA GAT GTG GCG GAT ATC CTG TTG ACG GAT GAA GGC GAA 2116 V C T R V A T L A V G Y A D G Y A D G F P R R S V S N Q GTC TGT ACG AGG GTG GCA ACA CTG GCC AGT GTC GCA GTA GGT TTT CGG GCG ATT TCC AAT CAG 2185 G A C V L L L H G R R V P I I G G N I C M D Q M M 684 GGA GCC TGT GTG GTG GTG GAT GGC AGT GTG GAT ATT CCC ATT ATC GGG TGT GAC GAT GAT GAT CAA GGA GCC TGT GTG GTG GTG GAT GAC GCT GGC AGT GTT CCC ATT ACC ATT ACC ATT AAC AAT GAA ACA CTC TGC GGG ATT 730 GAG GTT CTT TCC GTT GAT GAA CCC AGG CAG GTT GTA GC	CTG	CGT	GAA	AAC	GGC	ATC	CAA	AGT	CCG	ATT	CTG	GTG	CTG	GGG	TAT	ACG	CCG	CCT	GTT	CAT	$\mathbf{T}\mathbf{T}\mathbf{T}$	CAT	TAT	1564
TTG AAC GAG GCT TCC CTG ATC CAA ACG CTG GTA TCA AAG GAG TAT GCA GAA AAG CTG AAC GCA TAT GCT TAT GCT TAT GCT AA ACG CTG GTA TCA AAG GAG TAT GCA GAA AAG CTG AAC GCA TAT GCT TAT GCT AAA GCA CAT GCA CAG GAG TAT GCA GAA AAG CTG AAC GCA TAT GCT TC 500 AAA GAG CAG AAT GTT GTT GTC AAA GCA CAT GCC AAG GTG AAT ACC GGT ATG TCC AGA ATC GGT ATT TCG 1702 H R I M H I I I S R I S K A L V R L K H L N V S 523 CAC AGG ATC ATG CAT ATC ATA TCG AGG ATA TCA AAA GCG CTT GTA CGT CTG AAG CAT CTG AAT GTC TCG G I F S H F S V S D C L D E E N C A F T N H Q 546 GGT ATT TTC TCC CAC TTC AGT GTC AGT GAC TGT CTG GAT GAG GAA AAT TGT GCC TTC ACC AAT CAC CAG 1840 T A L F E R V L A D L R P E G L T P V Q R I C 569 ATT GCG TTG TTT GAA CGT GTT CTG GGC GAT TTG CGT GCA GAA GAG TTG ACC CCG GTA CAA CGC ATC TGC 1909 R Q L W N F K L S E P A L D Y V R P G L L W M 592 AGG CAG CTA TGG AAT T <u>TT AAA</u> TTA TCC GAA CCT GCA TTA GAT TAT GTA CGT CCG GGT CTA TTA TGG ATG 1978 G V P A M M H W Q F A R H R S F S P H G M E G 615 GGA GTT CCA GCG ATG GAC TGC CGG CAA TTC GCA CGG CAC CGC AGT CTA GAT GGA AGG C2047 K C F F G G K G Y S A R C Y G Q L R P S F Q A E 638 AAA TGT TTC CCT GGT AAA GGA TAT TCA GCC CGG TAT AGC TAT CGG CCG TCA TTT CAA GCG GAA GGA GAG CTA CCC GGT AAA GGA TAT TCA GCC CGG TAT AGC GGT CAG CTA CGG CCG TCA TTT CAA GCG GAA 2116 V C T R V A T L A V G Y A D G F P R S V S N Q 661 GTC TGT ACG AGG GTG GCA ACA CTG GCT GTC GGA TAT GCG GAT GCG CAG TTC CGG CGA TAT CAG CCG GAA 2116 V C T T R V A T L A V G Y A D G F P R S V S N Q 661 GTC TGT ACG AGG GTG GCA ACA CTG GCT GTC GGA TAT GCG GAT GTC GGC AGT TAT CGG CAG TCA ATC CAG 2185 G A C V V L L L H G R R V P I I G N I C M D Q M M 684 GGA GCC TGT GTG GCG GCA GCA GCT GGT GTG CGA TAT GCG ATT ATC GGC CGG TGT TCC AAT CAG 2185 G A C V V L L L H G G R R V P I I I G N I C M D Q M M 684 GGA GCC TGT GTG GCG GCA GCT GGT GGC GGT GTT CCC ATT ATC GGC AAT ATC TGT ATG GAT CAG ATG ATG 2254 V D I I T G G L D G V S E G D V A T L L I G C C D G D 707 GTG GAT ATC ACC GGA CTT GAT GGT GGA GAG GCT GTT CCC ATT ATC GGT ATC GAT GAA CAC CTC TGC GGT GAT 33	L	N	Е	А	S	\mathbf{L}	I	Q	т	L	V	S	K	Е	Y	A	E	K	L	N	A	Y	A	477
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H R I GAG GAG AAT GIT GIT GIT GIT GAA GAG AAT GAG AAT GAG AAT GAG AAT ACC ST AAT ACC AAT GAG AAT GIT GIT GIT GIT AT GAG AAT GAG CAT AT GAG AAT TG GAG AAT TTC TCC ACC AAT CAC AAT GAG ACT CAG AAT CAC AAA GCG CAT TTC CAC CAAT CAC CAG CAG CAC CAG TAT TTC TCC CAC TTC AGT GAC GAT GAG CAT CTG GAG GAA AAT TGT GCC TTC ACC AAT CAC CAG 1840 T A L F E R V L A D L R P E G L T P V Q R I C 569 ATT GCG TTG TTT GAA CGT GTT CTG GCG GAT TTG CGT CCA GAA GGA TTG ACC CCG GTA CAA CGC ATC TGC 1909 R Q L W N F K L S E P A L D Y V R P G L L W M 592 AGA CAG CTA TGG AAT TTT TAT TTA TAT GTA CGT CCG GGT CTA TTA TGG ATG 1978 G V P A M M H W Q F A R H R S F S P H G M E G 615 GGA GTT CCA GCG ATG ATG CAC TGG CAA TTC GCA CGG CAC CGC AGT TTC AGC CCT CAT GGA ATG GAA GGC 2047 K C F P G K G Y S A R C Y G Q L R P S F Q A E 638 AAA TGT TTC CCT GGT AAA GGA TAT TCA GCC CGG GTT TAT GGA CGG CCG TCA TTT CAA GCG GAA 2116 V C T R V A T L A V G Y A D G F P R S V S N Q 661 GCT TGT ACG AGG GTG GCA ACA CTG GCT GTC TCC GGA TAT CGG CAT GGA TGT TTC CAA GCG GAA 2116 V C T R V A T L A V G Y A D G F P R S V S N Q 661 GGA GTC TGT ACG GGA GAC ACT GGC TGT GCT GTA TAT GGA ATG CAA CAA CAA CAA CAA CAA CAA CAA CAA CA	К 777	E CDC	Q	N N	V CUUT	V CTTT	V CTC	<u>к</u> ллл	A	H CAT	A	AAC	V CTTC	N DDT	ACC	GCT	M አጥር	а тсс	ACA	ATC	GGT	፲ ስጥጥ	TCG	1702
CAC AGG ATC ATG CAT ATC ATA TCG AGG ATA TCA AAA GCG CTT GTA CGT CTG AAG CAT CTG AAT GTC TCG 1771 G I F S H F S V S D C L D E E N C A F T N H Q 546 GGT ATT TTC TCC CAC TTC AGT GTC AGT GAC TGT CTG GAT GAG GAA AAT TGT GCC TTC ACC AAT CAC CAG 1840 T A L F E R V L A D L R P E G L T P V Q R I C 569 ATT GCG TTG GTT GAA CGT GTT CTG GCG GAT TTG CGT CCA GAA GGA TGA CCC CG GTA CAA CGC ATC TGC 1909 R Q L W N F K L S E P A L D Y V R P G L L W M 592 AGA CAG CTA TGG AAT TTT AAA TTA TCC GAA CCT GCA TTA GAT TAT GTA CGT CCG GGT CTA TTA TGG ATG 1978 G V P A M M H W Q F A R H R S F S P H G M E G 615 GGA GTT CCC GGT AAA GGA TAT CC GCA CGC CGG CAC CGC AGT TTC AGC CCT CAT TGA GGA GGA GGC 2047 K C F P G K G Y S A R C Y G Q L R P S F Q A E 638 AAA TGT TTC CCT GGT AAA GGA TAT TCA GCC CGG TGT TAC GGT CAG CTA CGC CAG GGA AGGC 2047 K C T R V A T L A V G Y A D G F P R S V S N Q 661 GTC TGT ACG AGG GTG GCA ACA CTG GCT GTC GGA TAT GCG GAT GGT TTT CCG CGC AGT TTC CAA GCG GAA 2116 V C T R V A T L A V G Y A D G F P R S V S N Q 661 GTC TGT ACG AGG GTG GCA ACA CTG GCT GTC GGA TAT GCG GAT GGT TTT CCG CGC AGT GTA TCC AAT CAG 2185 G A C V L L L H G R R V P I I G N I C M D Q M M 684 V D I T G L D G V S E G D V A T L I G C D G D 707 GTG GAT ATC ACC GGA CTT GAT GGT GTG AGT GAA GGC GAT GTG TATC GGT CAT CTG ATC GGT GAA GAA ATG ATG ATG 2254 V D I S V D E L S R L A H T I N N E T L C W I 730 GAG GTT CTT TCC GTG GAT GAT GAT CTA GC GAA CTT GCA CAT ACC ATA AAC AAT GAA ACA CTC TGC TGG GAT TAC C G G G G G G G G G G G G G G G G G G G	H	R	T	M	Н	T	T	S	R	T	S	K	A	L	V	R	L	K	H	L	N	v	S	523
G I F S H F S V S D C L D E E N C A F T N H Q 546 GGT ATT TC CC CA TT AGT GGC ATT GGT ATT GGT ATT GGT ATT GGT ATT GGT GTG GTG GGG GAT TG CGT GGT CC AA D L R P E G L T P V Q R I C 569 ATT GCG TT GA CGT TG GGT CC GA GA TA CC GA CG GA CG GA C L D Y V R P G L L W M 592 AGA CA TGG AA M M M Q F A R H R S F	CAC	AGG	ATC	ATG	CAT	ATC	ATA	TCG	AGG	ATA	TCA	AAA	GCG	CTT	GTA	CGT	CTG	AAG	CAT	CTG	AAT	GTC	TCG	1771
GGTATTTTCTCCCACTTCAGTGTCAGTGACTGTCTGGAGGAAAATTGTGCCTTCACCAATCACCAGCACCAGCAGSATTGTGCCTTCACCAATCACCACCACCACCACCACCACCACCACCACCAGSATTTCAATACACCAGCACSATTCCAATCCADLRPEGLTPVQRLCS69AQLWNFKLSEPALDYVRPGLLWM592AGACAGCTATTATTCGAAATTTTCGAACCTGAATTAGATGATGATCATTAAATTTAAGAACATCGTCAGTTAGATGATGATTAATAAATTTACGAACATCGTCAGCATGAAGATTATGAAGATTATGA	G	I	F	S	Н	F	S	V	S	D	С	L	D	Е	Е	N	С	А	F	т	N	Н	Q	546
T A L F E R V L A D L R P E G L T P V Q R I C 5569 ATT GCG TTG TTG GCG GCG GCT TTG GCG GCT CCG GCA GGA GGA TTG ACC CCG GTA CCG GTG TTG TGC TGC 1909 R Q L W N F K L S E P A L D Y V R P G L L W M 592 AGA CT TGG AAT TTT ATT CGA CCT GCA TTG GCA TTG GCA TTG GCA TTG GCA TTG GCA TTG GCA CT GCA GCA GCA CT GCA GCA GCA CT GCA GCA GCA CGC GGA GCA	GGT	ATT	TTC	TCC	CAC	TTC	AGT	GTC	AGT	GAC	TGT	CTG	GAT	GAG	GAA	AAT	TGT	GCC	TTC	ACC	AAT	CAC	CAG	1840
ATT GCG TTG TTT GAA CGT GTT CTG GCG GAT TTG CGT CCA GAA GGA TTG ACC CCG GTA CAA CGC AIC TGC 1909 R Q L W N F K L S E P A L D Y V R P G L L W M 592 AGA CAG CTA TGG AAT TT <u>T AA</u> TTA TCC GAA CCG GCA TTA GAT TAT GTA CGT CCG GGT CTA TTA TGG ATG 1978 G V P A M M H W Q F A R H R S F S P H G M G M E G 615 GGA GTT CCA GCG ATG ATG CAC TGG CAA TTC GCA CGG CAC CGC AGT TTC AGC CCT CAT GGA ATG GAA GGC 2047 K C F P G K G Y S A R C Y G Q L R P S F Q A E 638 AAA TGT TTC CCT GGT AAA GGA TAT TCA GCC CGG TGT TAC GGT CAG CTA CGG CCG TCA TTT CAA GCG GAA 2116 V C T R V A T L A V G Y A D G F P R S V S N Q 661 GTC TGT ACG AGG GTG GCA ACA CTG GCT GTC GGA TAT GCG GAT GGT TTT CCG CGC AGT GTA TCC AAT CAG G A C V L L H G R R V P I I G N I C M D Q M M 684 GGA GCC TGT GTG CTG CTG CAT GGC AGG CGT GTT CCC ATT ATC GGC AAT ATC TGT ATG GAT CAG ATG ATG 2254 V D I T G L D G V S E G D V A T L I G C D G D 707 GTG GAT ATC ACC GGA CTT GAT GGT GTG AGT GAA GGC GAT GTC TCT ATG GAA CAC CTG GAT GAT 2323 E V L S V D E L S R L A H T I N N E T L C W I 730 GAG GTT CTT TCC GTT GAT GAA CTC AGC AGA CTT GCA CAT ACC ATA AAC AAT GAA ACA CTC TGC TGG ATT 2323 S Q R V P R I Y K * 740	Ĩ	A	L	F	E	R	V	L	A	D	L	R	P	E	G	L	T	P	V	Q	R	I	С	1000
AGA CAG CTA TGG AAT TT AAA TTA TTA CCT GCA TTA GAT TTA TGG AAT TTA TGG AAA TTA TGC GAA CCT GCA TTA GAT TTA TGG ATA TTA TGC GAA CCT GCA TTA GAT CTA TTA TGG GAT TTA TGG AAA TGA ATA TTA TCC GAA CCT GCA CGT GCA CGA CGA CGG CAC TGG GAA GC Y S A R H R S F S P H G M E G 1978 GGA GTC CAG GTG CAA GAA CAA TT CAA R R R P S F Q A E 638 AAA TGT TC GGA AT L A V G Y A D G F	ATT	GCG	TTG	TTT	GAA	CGT	GTT	CTG	GCG	GAT	TTG	CGT	CCA T	GAA	GGA V	TTG	ACC	UUG D	GTA	CAA T.	LGC T.	MIC W	M	1909
G V P A M M H W Q F A R H R S F S P H G M E G 615 GGA GTT CCA GCG ATG ATG CAC TGG CAA TTC GCA CGG CAC CGC AGT TTC AGC CCT CAT GGA ATG GAA GGC 2047 K C F P G K G Y S A R C Y G Q L R P S F Q A E 638 AAA TGT TTC CCT GGT AAA GGA TAT TCA GCC CGG TGT TAC GGT CAG CTA CGG CCG TCA TTT CAA GCG GAA 2116 V C T R V A T L A V G Y A D G F P R S V S N Q 661 GTC TGT ACG AGG GTG GCA ACA CTG GCT GTC GGA TAT GCG GAT GGT TTT CCG CGC AGT GTA TCC AAT CAG G A C V L L H G R R V P I I G N I C M D Q M M 684 GGA GCC TGT GTG CTG CTG CAT GGC AGG CGT GTT CCC ATT ATC GGC AAT ATC TGT ATG GAT CAG ATG ATG V D I T G L D G V S E G D V A T L I G C D G D 707 GTG GAT ATC ACC GGA CTT GAT GGT GTG AGT GAA GGC GAT GTC TCT ATC GGG TGT GAC GGT GAT 2323 E V L S V D E L S R L A H T I N N E T L C W I 730 GAG GTT CTT TCC GTT GAT GAA CTC AGC AGA CTT GCA CAT ACC ATA AAC AAT GAA ACA CTC TGC TGG ATT 2323 S Q R V P R I Y K * TCA CAG GG GTA CCG AGA ATA TAC AAT TAC CGG GAT GTT TTC CAG ATG AGA	AGA	CAG	CTA	TGG	ААТ	ThT	AAA	TTA	тсс	GAA	ССТ	GCA	TTA	GAT	TAT	GTA	CGT	CCG	GGT	CTA	TTA	TGG	ATG	1978
GGA GTT CCA GCG ATG ATG ATG CAC TGG CAA TTC GCA CGG CAC CGC AGT TTC AGC CCT CAT GGA ATG GAA GGC2047KCFPGKGYSARCYGQLRPSFQAE638AAA TGT TTC CCT GGT AAA GGA TAT TCA GCC CGG TGT TAC GGT CAG	G	v	P	A	M	M	H	W	0	F	A	R	Н	R	s	F	S	P	Н	G	М	Е	G	615
KCFPGKGYSARCYGQLRPSFQAE638AAATTTCGGTAAAGGATATTCAGCCCGGTGTTACGGTCAGCAGCAGCGGTCATTTCAAGCGGAA2116VCTRVATLAVGYADGFPRSVSNQ661GTCTGTACGACACTGGCTGTTGGAGGATATGCGGATGGTTTTCCGCGAATTCCAATCAACAG2116GTCTGTACGACACTGGCTGTTGCGGATACGGGTGTTGCGGTTTTCCGCGAATTCCAATCAG2116GTCTGTACGACACTGGCTGTTGCGGATGGTTTTCCGCGAATTCCAATCAG2185GACVLLHGRRVPIIGNICMDQMM684GGAGCTGTGGTGGTGGTGGTGGTGGTGCTGATTATCGGCATTATTCAG2254V <t< td=""><td>GGA</td><td>GTT</td><td>CCA</td><td>GCG</td><td>ATG</td><td>ATG</td><td>CAC</td><td>TGG</td><td>CÃA</td><td>TTC</td><td>GCA</td><td>CGG</td><td>CAC</td><td>CGC</td><td>AGT</td><td>TTC</td><td>AGC</td><td>CCT</td><td>CAT</td><td>GGA</td><td>ATG</td><td>GAA</td><td>GGC</td><td>2047</td></t<>	GGA	GTT	CCA	GCG	ATG	ATG	CAC	TGG	CÃA	TTC	GCA	CGG	CAC	CGC	AGT	TTC	AGC	CCT	CAT	GGA	ATG	GAA	GGC	2047
AAA TGT TTC CCT GGT AAA GGA TAT TCA GCC CGG TGT TAC GGT CAG CTA CGG CCG TCA TTT CAA GCG GAA 2116 V C T R V A T L A V G Y A D G F P R S V S N Q 661 GTC TGT ACG AGG GTG GCA ACA CTG GCT GTC GGA TAT GCG GAT ATC GCG TTT CCG CGC AGT GTA TCC AAT CAG A C V L L H G R R V P I I G N I C M D Q M M 684 GGA AC V L H G R R V P I I G N I C M D Q M M 684 GGA GCC TGT GTG GTG GTG CTG CTG CAT GGC AGG CGT GTT CCC ATT ATC GGC AAT ATC TGT ATG GAT CAG ATG ATG 2254 V D I T G L D G V S E G D V A T L I G C D G 707 707 <td>K</td> <td>С</td> <td>F</td> <td>Ρ</td> <td>G</td> <td>K</td> <td>G</td> <td>Y</td> <td>S</td> <td>А</td> <td>R</td> <td>С</td> <td>Y</td> <td>G</td> <td>Q</td> <td>L</td> <td>R</td> <td>Ρ</td> <td>S</td> <td>F</td> <td>Q</td> <td>А</td> <td>Ε</td> <td>638</td>	K	С	F	Ρ	G	K	G	Y	S	А	R	С	Y	G	Q	L	R	Ρ	S	F	Q	А	Ε	638
V C T R V A T L A V G Y A D G F P R S V S N Q 661 GTC TGT ACG AGG GTG GCA ACA CTG GCT GTC GGA TAT GCG GAT GGT TTT CCG CGC AGT GTA TCC AAT CAG 2185 G A C V L L H G R R V P I I G N I C M D Q M M 684 GGA GCC TGT GTG CTG CTG CAT GGC AGG CGT GTT CCC ATT ATC GGC AAT ATC TGT ATG GAT CAG ATG ATG 2254 V D I T G L D G V S E G D V A T L I G C D G D 707 GTG GAT ATC ACC GGA CTT GAT GGT GTG AGT GAA GGC GAT GTC GCT ACT CTG ATC GGG TGT GAC GGT GAT 2323 E V L S V D E L S R L A H T I N N E T L C W I 730 GAG GTT CTT TCC GTT GAT GAA CTC AGC AGA CTT GCA CAT ACC ATA AAC AAT GAA ACA CTC TGC AGT 2392 S Q R V P R I Y K * 740	AAA	TGT	TTC	CCT	GGT	AAA	GGA	TAT	TCA	GCC	CGG	TGT	TAC	GGT	CAG	CTA	CGG	CCG	TCA	TTT	CAA	GCG	GAA	2116
GAR GAR ACC V L L H G R R V P I I G N I C M D Q M M 684 GGA GCC TGT GTG CTG CTG CAT GGC AGG CGT GTT CCC ATT ATC GGC AAT ATC TGT ATG GAT CAG ATG ATG 2254 V D I T G L D G V S E G D V A T L I G C D G D 707 GTG GAT ATC ACC GGA CTT GAT GGT GTG AGT GAG GGC GAT GAC GGT ACT CTG ATC GGG TGT GAC GGT GAT 2323 E V L S V D E L S R L A H T I N N E T L C W I 730 GAG GTT CTT TCC GTT GAT GAA CTC AGC AGA CTT GCA CAT ACC ATA AAC AAT GAA ACA CTC TGC TGG ATT 2392 S Q R V P R I Y K * 740	V	C	T	R	V	A	T	L	A	V CTTC	G	Y m a m	A	D C M T	G	ե. ա.ա.ա.	P	R	5 лст	CTTA	ъ тсс	א דימ מ	CAG	2185
GGA GCC TGT GTG CTG CTG CAT GGC AGG CGT GTT CCC ATT ATC GGC AAT ATC TGT ATG GAT CAG ATG ATG 2254 V D I T G L D G V S E G D V A T L I G C D G D 707 GTG GAT ATC ACC GGA CTT GAT GGT GTG AGT GAG GGC GAT GTC GCT ACT CTG ATC GGG TGT GAC GGT GAT 2323 E V L S V D E L S R L A H T I N N E T L C W I 730 GAG GTT CTT TCC GTT GAT GAA CTC AGC AGA CTT GCA CAT ACC ATA AAC AAT GAA ACA CTC TGC AGT 2392 2392 S Q R V P R I Y K * 740 TCA CAG GGG GTA CCG AGA ATA TAG AATA AAC AAT GAA ACA CTC TGC AGA TAGA 2465	GIU C	TGT D	ACG C	AGG V	GTG T	GCA T.	ACA H	CTG G	GCT R	R	U V	P	UUU T	GAT	G G G G G G	N	I	C	M	D	0	M	M	684
V D I T G L D G V S E G D V A T L I G C D G D 707 GTG GAT ATC ACC GGA CTT GAT GTG	GGA	GCC	ТGT	GTG	CTG	CTG	CAT	GGC	AGG	CGT	GTT	ccc	ATT	ATC	GGC	AAT	ATC	TGT	ATG	GAT	CĂG	ATG	ATG	2254
GTG GAT ATC ACC GGA CTT GAT GGT GAT GAT GAT GTC GCT ACT CTG ATC GGG GAT GAT GTC GCT ATC GGG GTT GAT GAT GTC GCT ATC GGG GTT GAT GAT GTC GAT GTC GTC GAT GAT GTC GTC GAT GTC G	V	D	I	T	G	L	D	G	V	S	E	G	D	v	A	Т	L	I	G	С	D	G	D	70 7
E V L S V D E L S R L A H T I N N E T L C W I 730 GAG GTT CTT TCC GTT GAA CTC AGC AGC AGC AGC TGC TGC TGC AGC AGC AGC CTC TGC TGC AGC AGC AGC AGC TGC TGC AGC AGC AGC TGC AGC AGC AGC TGC TGC AGC AGC AGC TGC AGC AGC AGC TGC AGC AGC AGC TGC TGC AGC AGC TGC AGC AGC AGC TGC TGC AGC AGC AGC AGC	GTG	GAT	ATC	ACC	GGA	CTT	GAT	GGT	GTG	AGT	GAA	GGC	GAT	GTC	GCT	ACT	CTG	ATC	GGG	TGT	GAC	GGT	GAT	2323
GAG GTT CTT TCC GTT GAT GAA CTC AGC AGA CTT GCA CAT ACC ATA AAC AAT GAA ACA CTC TGC TGG ATT 2392 S Q R V P R I Y K * 740 TCA CAG CGG GTA CCG AGA ATA TAC AAA TAG CAGagagagagagagagagagagagagagagagagagagag	Е	V	L	S	V	D	E	L	S	R	L	A	H	Т	I	N	N	E	Т	L	С	W	I	730
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	S TCT	Q Cac	R	v د ۳۰	2 CCC	K ACA	רית ב גיית ב	Y TAC	К ддл	т».С	~~	acar	cadd	taaa	ctat	++++	caar	adaa	aaaa	gata	ataa	с		2465

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. faecalis* MM4174 [VanA] (AAA65956), *E. faecium* [Ddl] (ZP_00036460), *E. gallinarum* BM4174 [Ddl1] (AAN62561), *E. gallinarum* BM4174 [Ddl1] (AAN62561), *E. gallinarum* BM4174 [Ddl1] (CAB043439), *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] (CAB04467), *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

Diamid	MIC of vancomycin	Type of peptidoglycan precursors $(\%)^a$					
riasiniu	(µg/ml)	Tetra	Penta[Ser]	Penta[Ala]			
pJIM2246	2	b	_	100			
pJIM2246 Ω ddl _c innocuum	2	_	_	100			
pJIM2246 Ω C. innocuum racemase gene	2	_	_	100			
pJIM2246 Ω ddl _{c, innocuum} , C. innocuum racemase gene	2		9	91			
pJIM2246 Ω vanXY _c	2	90.8	_	9.2			
pJIM2246 Ω ddl _{c.innocuum} , vanXY _c	2	ND^{c}	ND	ND			
pJIM2246 Ω C. innocuum racemase gene, vanXY _c	2	92.5	_	7.5			
pJIM2246 Ω 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-Ser; Penta[Ala], UDP-MurNac-L-Ala-γ-D-Glu-Lys-D-Ala-D-Ser; Penta[Ala], UDP-MurNac-Lys-D-Ala-D-Ser; Penta[Ala], UDP-MurNac-Lys-D-Ser; Penta[Ala], Penta[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 ddlc. 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.

ACKNOWLEDGMENTS

V. David was the recipient of a grant from the Fondation pour la Recherche Médicale. This work was supported in part by a grant from INSERM.

REFERENCES

- Abadia Patiño, L., P. Courvalin, and B. Perichon. 2002. vanE gene cluster of vancomycin-resistant Enterococcus faecalis BM4405. J. Bacteriol. 184:6457– 6464.
- Ambur, O. H., P. E. Reynolds, and C. A. Arias. 2002. D-Ala:D-Ala ligase gene flanking the vanC cluster: evidence for presence of three ligase genes in vancomycin-resistant *Enterococcus gallinarum* BM4174. Antimicrob. Agents Chemother. 46:95–100.
- Arias, C. A., M. Martin-Martinez, T. L. Blundell, M. Arthur, P. Courvalin, and P. E. Reynolds. 1999. Characterization and modelling of VanT: a novel, membrane-bound, serine racemase from vancomycin-resistant *Enterococcus* gallinarum BM4174. Mol. Microbiol. 31:1653–1664.
- Arias, C. A., P. Courvalin, and P. E. Reynolds. 2000. vanC cluster of vancomycin-resistant *Enterococcus gallinarum* BM4174. Antimicrob. Agents Chemother. 44:1660–1666.
- Arthur, M., P. Reynolds, and P. Courvalin. 1996. Glycopeptide resistance in enterococci. Trends Microbiol. 4:401–407.
- Billot-Klein, D., L. Gutmann, E. Collatz, and J. van Heijenoort. 1992. Analysis of peptidoglycan precursors in vancomycin-resistant enterococci. Antimicrob. Agents Chemother. 36:1487–1490.
- 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-resistant VANB-type *Enterococcus* D366 and of the naturally glycopeptide-resistant species *Lactobacillus casei*, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, and *Enterococcus gallinarum*. J. Bacteriol. 176:2398–2405.
- Billot-Klein, D., R. Legrand, B. Schoot, J. van Heijenoort, and L. Gutmann. 1997. Peptidoglycan structure of *Lactobacillus casei*, a species highly resistant to glycopeptide antibiotics. J. Bacteriol. **179**:6208–6212.
- Billot-Klein, D., D. Shlaes, D. Bryant, D. Bell, R. Legrand, L. Gutmann, and J. van Heijenoort. 1997. Presence of UDP-N-acetylmuramyl-hexapeptides and -heptapeptides in enterococci and staphylococci after treatment with ramoplanin, tunicamycin, or vancomycin. J. Bacteriol. 179:4684–4688.
- Chang, S., D. M. Sievert, J. C. Hageman, M. L. Boulton, F. C. Tenover, F. P. Downes, S. Shah, J. T. Rudrik, G. R. Pupp, W. J. Brown, D. Cardo, and S. K. Fridkin. 2003. Infection with vancomycin-resistant *Staphylococcus aureus* containing the *vanA* resistance gene. N. Engl. J. Med. 348:1342–1347.
- Cutrona, A. F., C. Watanakunakorn, C. R. Schaub, and A. Jagetia. 1995. Clostridium innocuum endocarditis. Clin. Infect. Dis. 21:1306–1307.
- De Jonge, B. L., Y. S. Chang, D. Gage, and A. Tomasz. 1992. Peptidoglycan composition of a highly methicillin-resistant *Staphylococcus aureus* strain. The role of penicillin binding protein 2A. J. Biol. Chem. 267:11248–11254.
- De Jonge, B. L., S. Handwerger, and D. Gage. 1996. Altered peptidoglycan composition in vancomycin-resistant *Enterococcus faecalis*. Antimicrob. Agents Chemother. 40:863–869.
- 14. Dutka-Malen, S., S. Evers, and P. Courvalin. 1995. Detection of glycopep-

tide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. **33**:24–27.

- 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.
- 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.
- Felsenstein, J. 1988. Phylogenies from molecular sequences: inference and reliability. Annu. Rev. Genet. 22:521–565.
- Grohs, P., L. Gutmann, R. Legrand, B. Schoot, and J. L. Mainardi. 2000. Vancomycin resistance is associated with serine-containing peptidoglycan in *Enterococcus gallinarum*. J. Bacteriol. 182:6228–6232.
- Mainardi, J. L., D. Billot-Klein, A. Coutrot, R. Legrand, B. Schoot, and L. Gutmann. 1998. Resistance to cefotaxime and peptidoglycan composition in *Enterococcus faecalis* are influenced by exogenous sodium chloride. Microbiology 144:2679–2685.
- Mainardi, J. L., R. Legrand, M. Arthur, B. Schoot, J. van Heijenoort, and L. Gutmann. 2000. Novel mechanism of beta-lactam resistance due to bypass of DD-transpeptidation in *Enterococcus faecium*. J. Biol. Chem. 275:16490–16496.
- McKessar, S. J., A. M. Berry, J. M. Bell, J. D. Turnidge, and J. C. Paton. 2000. Genetic characterization of *vanG*, a novel vancomycin resistance locus of *Enterococcus faecalis*. Antimicrob. Agents Chemother. 44:3224–3228.
- Moran, C. P., Jr., N. Lang, S. F. LeGrice, G. Lee, M. Stephens, A. L. 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.
- Mory, F., A. Lozniewski, V. David, J. P. Carlier, L. Dubreuil, and R. Leclercq. 1998. Low-level vancomycin resistance in *Clostridium innocuum*. J. Clin. Microbiol. 36:1767–1768.
- Ochman, H., A. S. Gerber, and D. L. Hartl. 1988. Genetic applications of an inverse polymerase chain reaction. Genetics 120:621–623.
- Onderdonk, A. B., and S. D. Allen. 1995. *Clostridium*, p. 574–586. *In P. R. Murray*, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 6th ed. American Society for Microbiology, Washington, D.C.
- Renault, P., G. Corthier, N. Goupil, C. Delorme, and S. D. Ehrlich. 1996. Plasmid vectors for Gram-positive bacteria switching from high and to low copy number. Gene 183:175–182.
- Reynolds, P. E., F. Depardieu, S. Dutka-Malen, M. Arthur, and P. Courvalin. 1994. Glycopeptide resistance mediated by enterococcal transposon Tn1546 requires production of VanX for hydrolysis of D-alanyl-D-alanine. Mol. Microbiol. 13:1065–1070.
- Reynolds, P. E., C. A. 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.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.