D-Ala:D-Ala Ligase Gene Flanking the *vanC* Cluster: Evidence for Presence of Three Ligase Genes in Vancomycin-Resistant *Enterococcus gallinarum* BM4174

Ole-Herman Ambúr,[1] Peter E. Reynolds,[1] and Cesar A. Arias[1,2]*

Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom,[1] and Bacterial Molecular Genetics Unit, Centro de Investigaciones, Universidad El Bosque, Bogotá, Colombia[2]

An open reading frame located 230 nucleotides downstream from the stop codon of *vanS_c* and in the opposite direction relative to the other genes of the *vanC* cluster was identified in *Enterococcus gallinarum* BM4174. This gene (designated *ddl2*) encoded a protein of 343 amino acids that had significant predicted structural similarity to D-Ala:D-Ala ligases and displayed 33 and 35% amino acid identity to VanC-1 and the previously reported partial sequence of Ddl from *E. gallinarum*, respectively. Biochemical characterization by thin-layer chromatography confirmed that Ddl2 is a D-Ala:D-Ala ligase with no detectable D-Ala:D-Ser ligase activity. The vancomycin dependence of *Enterococcus faecalis* BM4320 (*ddl* mutant) was lost on electroporation of a plasmid construct expressing *ddl2* constitutively. The latter strain was able to grow in the absence of vancomycin, and peptidoglycan precursor analysis under the same conditions indicated the synthesis of pentapeptide[D-Ala] as the main precursor, confirming the activity of Ddl2 in vivo. Expression of *ddl* and *ddl2* in BM4174 was tested by reverse transcription-PCR: results suggested that both D-Ala:D-Ala ligases were expressed concomitantly. Our findings indicate that vancomycin-resistant *E. gallinarum* BM4174 is likely to express one D-Ala:D-Ser and two D-Ala:D-Ala ligase genes.

Resistance to glycopeptide antibiotics in enterococci is due to the synthesis of modified peptidoglycan precursors that display decreased affinity for vancomycin and teicoplanin (4). The alteration of the peptidoglycan biosynthetic pathway is the result of the expression of specific gene clusters. In VanA, VanB, and VanD isolates, UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Lac is synthesized as the main peptidoglycan precursor (4). In VanC and VanE isolates, UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ser (4, 15, 30) is used by the transglycosylase and transpeptidase enzymes to synthesize the mature peptidoglycan (20). The resistant biochemical pathway is completed with the destruction of "glycopeptide-susceptible" precursors (ending in D-Ala-D-Ala): all vancomycin-resistant strains encode enzymes with D,D-dipeptidase and/or D,D-carboxypeptidase activities that ensure that UDP-MurNAc-pentapeptide [D-Ala] is unavailable for peptidoglycan synthesis (29, 31).

The D-Ala:D-Ala ligases are ATP-dependent enzymes that operate by using two chemically identical substrate molecules of D-Ala in distinct ways. The enzyme activates the N-terminal D-Ala as an electrophilic acyl phosphate, by transfer of the γ-PO$_3^{2-}$ from ATP, and then activates the C-terminal D-Ala as a nucleophile to attack the bound acyl-P; this gives rise to a tetrahedral adduct as intermediate that then decomposes to yield the dipeptide product (35). Vancomycin-resistant enterococci have acquired genes that encode D-Ala:D-X ligase activities with altered substrate specificities: VanA, VanB, and

VanD function as D-Ala:D-Lac ligases using a chemical mechanism that rejects the protonated form of D-Ala at subsite 2 of the active site (23). VanC and VanE synthesize the dipeptide D-Ala-D-Ser (6, 15). Using the three-dimensional structure of DdlB of *Escherichia coli* (13) and VanA from *Enterococcus faecium* (32), site-directed mutagenesis studies have defined the residues responsible for substrate specificity (13, 21, 23, 24, 35).

The existence of two D-Ala:D-Ala ligase genes has been previously reported in *E. coli* and *Salmonella enterica* serovar Typhimurium (41). In this work we provide evidence for the first time that vancomycin-resistant *Enterococcus gallinarum* possess at least three dipeptide ligase genes, two encoding D-Ala:D-Ala ligases and the third encoding a D-Ala:D-Ser ligase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this work are described in Table 1. Enterococci were grown in brain heart yeast extract (BHY) (Difco Laboratories, Detroit, Mich.) broth or on BHY agar. Vancomycin-dependent *E. faecalis* BM4320 (*ddl* mutant) (5) used for complementation studies was grown in BHY supplemented with vancomycin (10 μg/ml); 150 γg of gentamicin (Sigma, Steinheim, Germany) per ml was added to the medium for pAT392-containing derivatives of this strain. *E. coli* XL1-Blue (7) was grown in Luria-Bertani (LB) (Difco Laboratories) broth or agar supplemented with gentamicin (8 μg/ml) *E. coli* derivatives containing pAT392 (3). *E. coli* M15(pREP4) (Qiagen) was grown in LB agar or broth supplemented with kanamycin (50 μg/ml). Ampicillin (100 μg/ml) was added for pQE-30 (Qiagen)-containing derivatives.

DNA manipulations, and plasmid construction and sequencing. *E. gallinarum* BM4174 total DNA was extracted as described previously (28). Cloning, digestion with restriction endonucleases (Boehringer GmbH, Mannheim, Germany), isolation of plasmid DNA (Wizard Plus SV Minipreps; Promega), ligation, and transformation were carried out by standard methods (33). The sequence of *ddl2* was obtained from an insert in plasmid pCA8 that has been described previously

* Corresponding author. Mailing address: Bacterial Molecular Genetics Unit, Universidad El Bosque, Transv 9ª Bis No. 133-25, Bogotá, Colombia. Phone: (571) 523-4879. Fax: (571) 216-5116. E-mail: caa22@cable.net.co.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype and relevant characteristics	Source or reference
Strains		
E. coli XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac* F [*proAB⁺ lacIq lacZ*ΔM15 Tn*10*(Tetʳ)]	7
E. coli M15(pREP4)	Nalʳ Strˢ Rifˢ *lac ara gal mtl* F⁻ *recA⁺ uvr⁺ lon⁺* (pREP4)	Qiagen
E. faecalis BM4320	VanB, vancomycin-dependent strain harboring an S319→I mutation in Ddl	5
E. gallinarum BM4174	Vanʳ strain, clinical isolate	10
Plasmids		
pCA8	2.5-kb *Sac*I-*Xba*I inverse PCR product containing the 3′ end of *vanSc* and *ddl2* cloned into pUC18	1
pAT392	*oriR*pAMβ1 *oriR*pUC *oriT*RK2 *spc lacZα* P2 *aac(6′)-aph(2″)*	3
pQE-30	Plasmid expression vector with T5 promoter and 6His tag encoding sequence 5′ to the cloning region	Qiagen
pHA1	1,081-bp fragment containing *ddl2*, including the RBS, cloned into pAT392	This work
pHA2	1,032-bp fragment containing the coding region of *ddl2*, cloned into pQE-30	This work

(1). Plasmid pHA1 was obtained after cloning a *Sac*I-*Xba*I fragment containing the *ddl2* gene including the putative ribosome-binding site (RBS) into the shuttle vector pAT392 under the control of the constitutive P2 promoter (3). This fragment was obtained after amplification with *Pwo* polymerase (Boehringer) using total DNA of *E. gallinarum* BM4174 with primers A (5′-GACT<u>GAGCTC</u> GCTAGCTATTTCTAAACCAAAAC) and B (5′-ACGC<u>TCTAGA</u>CTATTTG TACCAGCTTTC) (*Sac*I and *Xba*I sites underlined, respectively). pHA1 was purified from *E. coli* and electroporated into the vancomycin-dependent *E. faecalis* BM4320 (*ddl*) (5), as described previously (9). Transformants from electroporation experiments were isolated on BHY plates in the absence of vancomycin. No transformants were obtained when the vector (lacking the fragment encoding *ddl2*) was used or in the absence of plasmid DNA under the same conditions.

DNA containing the coding region of *ddl2* was amplified by PCR with *Pwo* polymerase (Boehringer) using chromosomal DNA from BM4174 as a template with primers C (5′-GCTA<u>GGATCC</u>ATGAATATTGTGGTATTA), which includes a *Bam*HI site (underlined), and D (5′-CGGT<u>AAGCTT</u>*CTA*TTTGTAC CAGCTTTC), which includes a *Hind*III site (underlined) and the stop codon of *ddl2* (italicized). The product was purified, digested, and cloned into pQE-30 under the control of the T5 promoter using *Bam*HI and *Hind*III. The resultant plasmid was designated pHA2 and allowed the expression of a gene encoding a protein with the incorporation of six histidine residues (6His tag) at the N terminus to enable affinity chromatography to be carried out. Sequencing of the inserts in pCA8 (two independent clones), pHA1, and pHA2 was performed on both strands by the dideoxy-chain terminator method (34) using fluorescent cycle sequencing with dye-labeled terminators (ABI Prism Dye Terminator cycle-sequencing ready reaction kit; Perkin-Elmer) on a 373A automated DNA sequencer (Perkin-Elmer).

Purification of Ddl2. *E. coli* M15 (pREP4) containing plasmid pHA2 encoding the His-tagged Ddl2 was grown at 37°C in LB broth (300 ml) until the absorbance at 600 nm was 0.5. The culture was cooled and incubated at 18°C for 20 h in the presence of 0.05 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Bacteria were harvested at 4°C, washed in lysis buffer (50 mM Bis-Tris propane [pH 7.5], 300 mM NaCl, 10 mM imidazole), resuspended in 5 ml of the same buffer, and sonicated. The broken-cell preparation was centrifuged at 40,000 × *g* for 20 min at 4°C, and the supernatant was collected. All the following steps were carried out at 4°C. The supernatant was applied to a 2-ml nickel-containing agarose column (Agarose Ni-NTA; Qiagen) equilibrated with the same buffer. The column was washed with at least 100 ml of 50 mM Bis-Tris propane (pH 7.5)–300 mM NaCl–30 mM imidazole buffer, and Ddl2 was eluted with 50 mM Bis-Tris propane (pH 7.5)–300 mM NaCl–250 mM imidazole buffer. The purified protein was dialyzed against the assay buffer (2× concentrated) containing 200 mM Tris-HCl (pH 8.6), 20 mM KCl, 20 mM MgCl₂, and 1 mM dithiothreitol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% polyacrylamide gel was carried out under denaturing conditions using the Laemmli buffer system (22) to determine the purity of Ddl2. For calibration, standard proteins in the range M_r 9,000 to 175,000 (New England Biolabs, Hitchin, United Kingdom) were used. Proteins were stained with 0.1% (wt/vol) Coomassie blue in 50% (vol/vol) methanol–10% (vol/vol) acetic acid for 30 min at 37°C and destained with 10% (vol/vol) methanol–10% (vol/vol) acetic acid at room temperature overnight.

TLC assays of Ddl2. Assay mixtures (20 μl) contained 100 mM Tris-HCl (pH 8.6), 10 mM KCl, 10 mM MgCl₂, 0.5 mM dithiothreitol, 6 mM ATP, 2 to 5 μg of enzyme, and combinations of different concentrations of [¹⁴C]D-Ala and D-Ser (see Fig. 2B). The samples were incubated at 37°C for 2 h, and the reaction was stopped by boiling the sample for 2 min. A 10-μl portion of the reaction mixture was spotted on a 0.1-mm microcrystaline cellulose thin-layer chromatography (TLC) plate (Polygram Cel 400; Macherey-Nagel) and developed for 4.5 h in butanol-glacial acetic acid-water (12:3:5 vol/vol/vol). The plate was dried for 5 min at 70°C, wrapped, and exposed to a Storage Phosphor-Screen (Molecular Dynamics, Sunnyvale, Calif.). Dipeptide formation was identified by comparing the spots (see Fig. 2B) with standards of D-Ala-D-Ala, D-Ala, D-Ala-D-Ser, and D-Ser after staining in 0.25% ninhydrin in acetone followed by drying for 5 min at 70°C. The radioactive spots were quantitated using image software (Image-Quant; Molecular Dynamics). The enzyme activity results were expressed in moles of product per minute per mole of enzyme.

RNA extraction. *E. gallinarum* BM4174 was grown in 50 ml of BHY broth to an absorbance at 600 nm of 0.7 to 0.8. The bacteria were harvested, resuspended in 100 μl of TE (10 mM Tris HCl [pH 8.0], 1 mM EDTA) containing lysozyme (5 mg/ml), and incubated at 37°C for 30 min. Total RNA was extracted using a commercial kit (RNeasy mini kit; Qiagen). RNA was treated with RNase-free DNase I (DNA-free; Ambion, Austin, Tex.) to remove contaminating genomic DNA.

RT-PCR of *ddl* and *ddl2*. Total RNA was reverse transcribed using a commercial kit (Omniscript reverse transcriptase; Qiagen) in a total volume of 20 μl containing 0.5 mM each dATP, dCTP, dGTP, and dTTP, 1 μM reverse primers (5′- TCAGGAATAATTCGTTTTTGTTCG for *ddl*; 5′-CTTTGTTCACTAAT ACGGTC for *ddl2*), 10 U of RNase inhibitor (SUPERase-IN; Ambion), 4 U of reverse transcriptase in corresponding buffer, and 2 μg of template RNA. The reverse transcription (RT) reaction was carried out at 37°C for 60 min. PCR was performed in a final volume of 25 μl which contained 2 μl of cDNA template, using *Taq* DNA polymerase (Pharmacia) and 0.5 μM primers specific for *ddl* (forward, 5′-TTGAAAATTATTTTATTATATGGCGG; reverse as above) and *ddl2* (forward, 5′-CACCGAGCATTATTGTTGG; reverse as above). Amplification was carried out for 30 cycles (95°C for 1 min, 58°C for 1 min, and 72°C for 1 min). The sample was subjected to electrophoresis on a 1% agarose gel containing ethidium bromide. Non-reverse-transcribed PCR controls were used to indicate the absence of contaminating genomic DNA. To test for the specificity of the PCR targeting the *ddl* gene, primers used for *ddl* were included in a PCR assay with purified plasmid pHA1 as the template under the same conditions.

Extraction and analysis of peptidoglycan precursors from *E. gallinarum* and *E. faecalis* BM4320 and derivatives. Extraction and analysis of peptidoglycan precursors was performed as described previously (30). In brief, *E. gallinarum* BM4174, *E. faecalis* BM4320, and derivatives were grown in BHY medium supplemented with vancomycin if needed. Ramoplanin (3 μg/ml) was added to inhibit peptidoglycan synthesis, and incubation was continued for 0.5 mean generation time (*ca.* 19 min) to allow the accumulation of peptidoglycan precursors. Bacteria were harvested, and cytoplasmic precursors were extracted, desalted on G10-Sephadex and analyzed by high-performance liquid chromatography.
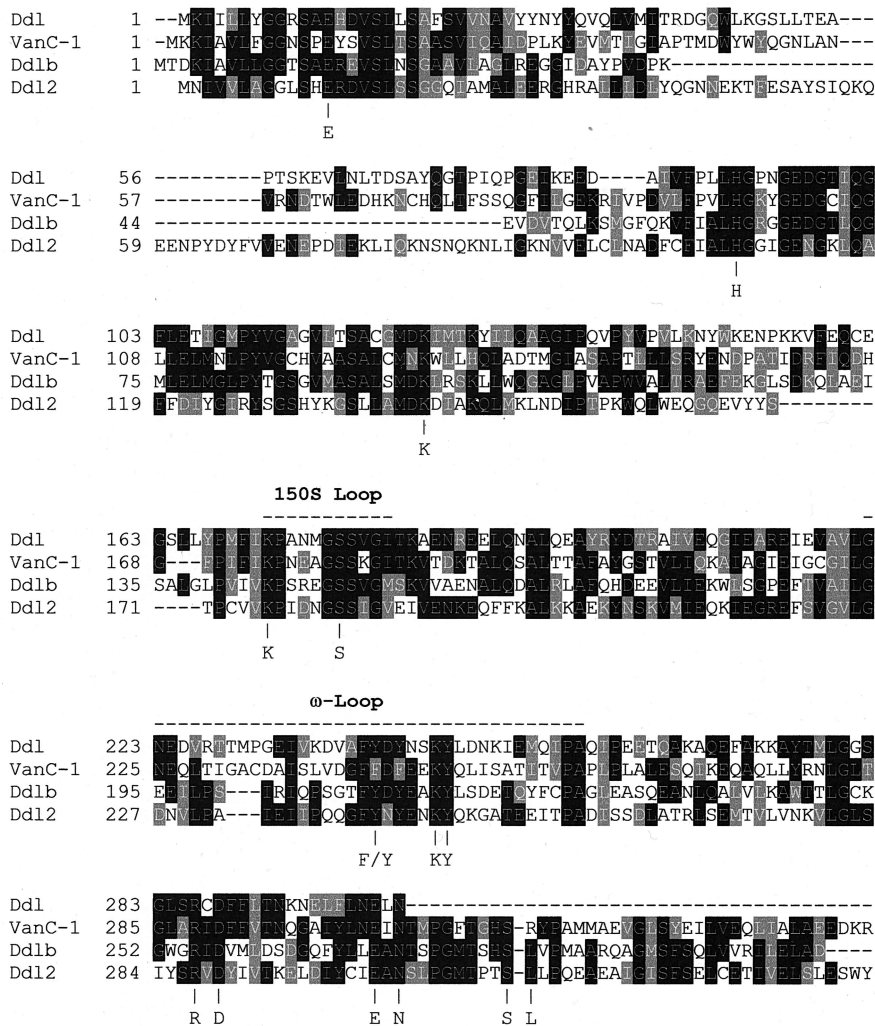
FIG. 1. Alignment of the deduced amino acid sequence of Ddl2 with that of Ddl from *E. gallinarum* (303 amino acids) (12), Ddlb from *E. coli* (13), and VanC-1 (10). The alignment was performed with Clustalw (37). Predicted amino acids that interact with ATP, D-Ala$_1$ and D-Ala$_2$, are indicated below the alignment. The putative ω and 150S loops are shown above the alignment (13). Black and shaded boxes indicate amino acid identity and similarity, respectively.

RESULTS AND DISCUSSION

Nucleotide sequence of *ddl2* and amino acid comparisons. The sequence of *ddl2* was obtained from sequencing the 3′ end of the insert of plasmid pCA8 (1). The putative open reading frame was located 230 nucleotides from the stop codon of *vanS$_c$* (1) and in the opposite direction to the other genes of the *vanC* cluster. The gene was preceded by a putative RBS (5′-AGAAGGGGATAN$_6$ATG) that displayed similarity (underlined) to the 3-OH terminus of *Bacillus subtilis* 16S rRNA (3′-OH UCUUUCCUCC) (27). The putative product of *ddl2* was a soluble protein of 343 amino acids with a calculated M_r of 38,430. Screening of the OWL protein sequence database showed similarity to D-Ala:D-X ligases from a variety of organisms. Ddl2 had 31 and 37% amino acid identity to DdlA and DdlB ligases, respectively, from *E. coli*. Compared with other D-Ala:D-X ligases previously described in *E. gallinarum* (10, 12)

(Fig. 1), Ddl2 exhibited 33 and 35% identity to VanC-1 (a D-Ala:D-Ser ligase) and to a 303-amino-acid sequence of Ddl respectively, indicating the presence of three different ligase genes in this organism. Amino acid identity to the depsipeptide ligases VanA and VanB was 31 and 32%, respectively. Based on the crystal structure of DdlB from *E. coli* (13), all the important residues involved in binding ATP (K141, K176, K247, E302, and N304), D-Ala$_1$ (E13, H107, S182, and R287), and D-Ala$_2$ (Y248, D289, S313, and L314) were identified (Fig. 1). Additionally, by analogy to DdlB, residue 242 in Ddl2 should be involved in holding a loop of the protein over the nucleotide and the substrate-binding cavity: in D-Ala:D-Ala ligases this residue is invariably Tyr whereas in D-Ala:D-Ser ligases it is Phe (10, 15, 21, 26). The structure of DdlB also revealed two important amino acid loops: the first loop (the 150S loop) is part of an X-Gly-Ser-Ser-X-Gly motif that is conserved throughout all D-Ala:D-X ligases and contains the Ser150 residue, which is part of a hydrogen-bonding triad that orients Glu15 for binding the first D-Ala and which is also
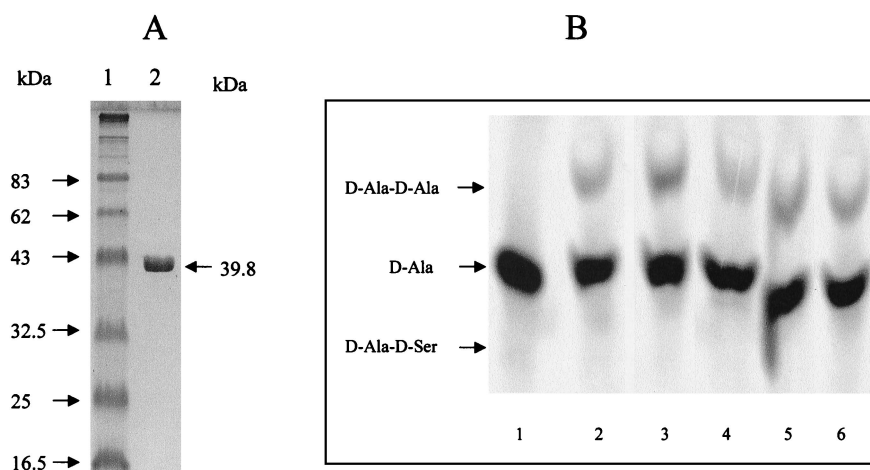
FIG. 2. (A) SDS-PAGE of purified His-tagged Ddl2. Lanes: 1, standard proteins; 2, sample containing His-tagged Ddl2 (M_r 39,828). (B) TLC of the products formed by Ddl2. Lanes: 1, 0.125 mM D-[^{14}C]Ala standard; 2, 1 mM D-[^{14}C]Ala incubated with purified His-tagged Ddl2; 3, as lane 2 but incubated with 10 mM D[^{14}C]Ala; 4, Purified His-tagged Ddl2 incubated with 1 mM D[^{14}C]Ala and 10 mM D-Ser; 5, as lane 4 but incubated with 10 mM D-[^{14}C]Ala and 10 mM D-Ser; 6, as lane 4 but incubated with 1 mM D-[^{14}C]Ala and 1 mM D-Ser.

believed to hold the second loop in place (24). The second loop, called the ω-loop, forms a short helix, spanning residues 210 to 217 in DdlB, which closes over the bound ADP and reaction intermediates during catalysis, but it is believed to be flexible and exposed in the absence of substrates (13). All these residues were also conserved in Ddl2 (Fig. 1).

Purification of Ddl2 and D-Ala:D-Ala ligase activity. N-terminal His-tagged Ddl2 was purified from *E. coli* strain M15(pREP4) containing pHA2 (Fig. 2A). The ability of Ddl2 to synthesize a mixed dipeptide containing D-alanine was tested by incubation of the enzyme with different combinations of D-[^{14}C]alanine (concentration range, 0.125 to 10 mM) and D-serine, followed by thin-layer chromatography and visualization in a Phosphor Imager. The results indicated that Ddl2 was able to synthesize D-Ala-D-Ala but not D-Ala-D-Ser (Fig. 2B). Maximum D-Ala:D-Ala ligase activity was 2.8 mol/min/mol of enzyme.

In vivo activity of Ddl2. *E. faecalis* BM4320 is a vancomycin-dependent strain carrying the *vanB* cluster, with an S139→I mutation in Ddl which abolishes D-Ala:D-Ala ligase activity (5). The strain cannot grow in the absence of vancomycin, because the antibiotic is required for induction of the *vanB* gene cluster and consequent synthesis of D-Ala-D-Lac for peptidoglycan assembly (5). Analysis of peptidoglycan precursors of *E. faecalis* BM4320 grown in the presence of vancomycin (10 µg/ml)

confirmed that the strain synthesized pentadepsipeptide as the main precursor (Table 2). No pentapeptide[D-Ala] was detected under these conditions.

Growth of *E. faecalis* BM4320 in the absence of vancomycin was restored by electroporation of plasmid pHA1, which encodes Ddl2 under the control of the constitutive P2 promoter. Control electroporation experiments carried out in the absence of plasmid DNA or with pAT392 lacking the *ddl2* fragment yielded no colonies on BHY plates without vancomycin, confirming that growth restoration was not due to a spontaneous reversion of the dependent phenotype. The peptidoglycan precursor profile of this strain (analyzed in the absence of glycopeptide in the growth medium) indicated the presence of pentapeptide[D-Ala] and no pentadepsipeptide (Table 2). The amount of UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys (tripeptide) was found to be significant (33%) when *E. faecalis* BM4320/pHA1(*ddl2*) was grown in the absence of glycopeptides, suggesting that the rate of synthesis of pentapeptide[D-Ala] (and hence the synthesis of D-Ala-D-Ala) was low compared to the availability of the tripeptide precursor. When the same strain was grown in the presence of vancomycin, the amount of tripeptide was almost undetectable and pentadepsipeptide was virtually the sole peptidoglycan precursor synthesized (Table 2): the absence of pentapeptide indicates that VanX$_B$ (D,D-dipeptidase) is efficient in hydrolyzing D-Ala-D-Ala synthesized

TABLE 2. Peptidoglycan precursors of *E. faecalis* BM4320 and derivatives and *E. gallinarum* BM4174 in the presence or absence of vancomycin

Strain	Vancomycin (10 µg/ml) present	Presence of peptidoglycan precursor (%)[a]				
		UDP-MurNAc-tripeptide	UDP-MurNAc-tetrapeptide	UDP-MurNAc-pentapeptide[Ser]	UDP-MurNAc-pentapeptide[Ala]	UDP-MurNAc-pentadepsipeptide
E. faecalis BM4320	Yes	0.5	0.5	0	0	99
E. faecalis BM4320/pAT392	Yes	2.5	0.5	0	0	97
E. faecalis BM4320/pHA1(*ddl2*)	Yes	0.5	0.5	0	0.5	98.5
E. faecalis BM4320/pHA1(*ddl2*)	No	33	0	0	67	0
E. gallinarum BM4174	No	0	76	24	0	0

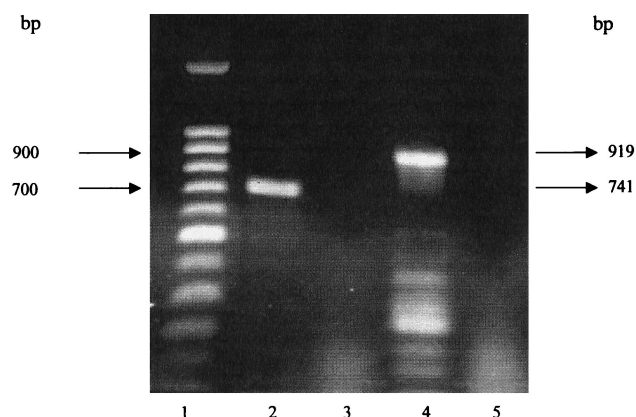[a] Determined by HPLC from the integrated peak areas.

FIG. 3. RT-PCR of *ddl* and *ddl2* using total RNA from *E. gallinarum* BM4174 as a template. Lanes: 1, Molecular size markers; 2, *ddl2* RT-PCR; lane 3, as lane 2 but without reverse transcriptase; 4, *ddl* RT-PCR; 5, as lane 4 but without reverse transcriptase.

by Ddl2. Similarly, the high proportion of accumulated tetrapeptide in *E. gallinarum* BM4174 (76%) suggests that the bifunctional D,D-carboxypeptidase/D,D-dipeptidase (VanXY$_c$) (29) prevents the utilization of pentapeptide[D-Ala] as the main precursor for wall synthesis: VanXY$_c$ effectively hydrolyzes pentapeptide that is likely to be made available by both *ddl* and *ddl2*. These findings confirmed that Ddl2 functioned as a D-Ala:D-Ala ligase in vivo.

Expression of *ddl* and *ddl2* in *E. gallinarum* BM4174. To investigate if both *ddl* and *ddl2* were expressed at the same time, RT-PCR targeting both genes was performed with primers specific for each gene. To test such specificity, a PCR assay was performed with primers obtained from the *ddl* sequence but using plasmid pHA1(*ddl2*) as a template. No amplification product was observed under these conditions (data not shown), confirming the specificity of the assay. Expression of both genes was detected by RT-PCR (Fig. 3), indicating that both *ddl1* and *ddl2*, encoding D-Ala:D-Ala ligases, are likely to be functional in *E. gallinarum* BM4174. The existence of two D-Ala:D-Ala ligase genes has been previously reported in *E. coli* and *S. enterica* serovar typhimurium (40), and our findings indicate for the first time that a gram-positive bacterium also possesses two *ddl* genes. The physiological relevance of this phenomenon is not clear. There are examples in bacteria and fungi of two genes encoding apparently similar enzymes: usually they are used for two separate metabolic pathways, e.g., two anthranilate synthases, *trpE* and *phnA*, in *Pseudomonas aeruginosa* (11) or two dehydroquinase activities of *Neurospora crassa* (18). On the other hand, both proteins are isoenzymes that are mostly regulated by feedback inhibition from different end products: the *E. coli* isoenzymes of acetolactate synthase, DAHP (3-deoxy-D-arabino-heptulosonate-7-phosphate) synthase, and aspartate kinase (38) are further examples of this phenomenon. In other situations the reasons are unclear, such as the two *E. coli* lysyl-tRNA synthetases (8). Two genes encoding similar enzymes involved in the synthesis of peptidoglycan precursors have also been reported: the alanine racemase *alr* and *dadB* genes (40). The constitutively expressed alanine racemase (Alr) is likely to be involved in the synthesis of peptidoglycan (17), and the inducible DadB racemase func-

tions in the utilization of D-Ala as a sole carbon source. *E. gallinarum* BM4174 also harbors at least two racemases: an alanine racemase and a serine racemase (2). Due to the importance of the peptidoglycan synthetic pathway for bacteria, one could speculate that the two D-Ala:D-Ala ligases might be involved in different systems as well. Interestingly, gene homologues encoding the D-Ala-D-Ala-hydrolyzing enzyme VanX have been found in *E. coli* (*ecovanX*) associated with a putative dipeptide permease gene cluster. The genes form an operon under the control of the stationary-phase transcription factor RpoS. The consecutive action of EcoVanX and the membrane-bound D-amino acid dehydrogenase and pyruvate oxidase would allow the cell to utilize D-Ala-D-Ala released during the turnover of cell wall peptidoglycan as an energy source for cell survival during starvation (25).

The presence of additional ligase genes in enterococci could also affect the emergence of the vancomycin-dependent phenotype (14, 16, 19, 36, 39) in two ways: (i) the presence of more than one functional *ddl* gene would decrease the probability of emergence of such a phenotype, or (ii) the presence of additional unexpressed *ddl* genes in vancomycin-dependant strains (due to specific regulatory mechanisms or repressors) would increase the possibility of restoring a previously mutated ligase gene by, for example, homologous recombination.

In conclusion, the characterization of a second D-Ala:D-Ala ligase gene indicates that vancomycin-resistant *E. gallinarum* BM4174 is likely to express three ligase genes: two encoding D-Ala:D-Ala ligases and one encoding a D-Ala:D-Ser ligase.

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