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Vancomycin-Resistant *Leuconostoc mesenteroides* and *Lactobacillus casei* Synthesize Cytoplasmic Peptidoglycan Precursors That Terminate in Lactate

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The emergence of acquired high-level resistance among *Enterococcus* species has renewed interest in mechanisms of resistance to glycopeptide antibiotics in gram-positive bacteria. In *Enterococcus faecalis* and *Enterococcus faecium*, resistance is encoded by the *van* gene cluster and is due to the production of a peptidoglycan precursor terminating in D-alanyl-D-lactate, to which vancomycin does not bind. Most *Leuconostoc* and many *Lactobacillus* species are intrinsically resistant to high levels of glycopeptide antibiotics, but the mechanism of resistance has not been elucidated. To determine whether the mechanisms of resistance are similar in intrinsically resistant bacteria, cytoplasmic peptidoglycan precursors were isolated from *Leuconostoc mesenteroides* and *Lactobacillus casei* and analyzed by mass spectrometry, revealing structures consistent with UDP-*N*-acetylmuramyl-L-Ala–D-Glu–L-Lys–(L-Ala)–D-Ala–D-lactate and UDP-*N*-acetylmuramyl–L-Ala–D-Glu–L-Lys–D-Ala–D-lactate, respectively.

Glycopeptide antibiotics such as vancomycin act by binding to the D-alanyl-D-alanine terminus of stem pentapeptides present in bacterial peptidoglycan (16, 21). It had been widely assumed that this terminus was ubiquitous among eubacterial species producing peptidoglycan and that vancomycin resistance was therefore unlikely to emerge in the absence of a barrier to vancomycin binding such as the outer membrane of gram-negative bacteria. However, high-level transmissible resistance to glycopeptides has recently been recognized among several gram-positive species, particularly enterococci, in which the incidence of resistance has been rising among clinical isolates (2).

Most *Leuconostoc* and *Pediococcus* and some *Lactobacillus* species have been described as being intrinsically resistant to glycopeptides (9, 15, 17, 20), but the mechanism of resistance has not been systematically investigated, perhaps because these organisms were considered to be of little clinical significance. However, the widespread use of vancomycin has led to more frequent recognition of these species as opportunistic pathogens (9, 20). This trend, as well as the emergence of acquired vancomycin resistance in *Enterococcus* species, has renewed interest in the mechanism of resistance to glycopeptides.

In *Enterococcus faecalis* and *Enterococcus faecium*, high-level resistance is inducible and mediated by plasmids harboring the *van* cluster of genes (2, 3). This cluster includes *vanH*, which encodes an enzyme catalyzing the reduction of 2-keto acids to D-2-hydroxy acids (6), and *vanA*, which encodes a ligase capable of adding either amino acids or hydroxy acids to D-alanine (5, 6). In vitro evidence had shown that vancomycin binding to a depsipeptide was reduced up to 1,000-fold compared with binding to D-alanyl-D-alanine (5, 16). It was therefore postulated that VanA and VanH act in concert to form a depsipeptide (D-alanyl-D-hydroxy acid) which is added in place of D-alanyl-D-alanine to the UDP-muramyl-tripeptide precursor, resulting in a peptidoglycan precursor that is not bound by vancomycin (6). This hypothesis was supported by the observation that the MIC for resistant enterococci was diminished when tested with high concentrations of D-amino acids that can be incorporated into pentapeptide and that are bound by vancomycin to a greater extent than D-2-hydroxy acids (23). Subsequently, the cytoplasmic peptidoglycan precursor in resistant *E. faecium* and *E. faecalis* was identified as UDP-*N*-acetyl-muramyl-L-Ala–D-Glu–L-Lys–D-Ala–D-lactate (1, 10, 14).

Leuconostoc and *Pediococcus* and some *Lactobacillus* species display the same high level of resistance to glycopeptides as do resistant enterococci harboring the *van* cluster. However, this resistance appears to be constitutive and chromosomal in origin. We therefore aimed to determine whether these intrinsically resistant gram-positive bacteria also produce a peptidoglycan precursor with an altered stem peptide terminus. The strains used were the clinical isolate *Leuconostoc mesenteroides* subsp. *mesenteroides* VR1 (9) and *Lactobacillus casei* subsp. *rhamnosus* ATCC 7469. Cultures were grown in deMan, Rogosa, and Sharpe medium (Oxoid, Basingstoke, United Kingdom) at 37°C for strain ATCC 7469 and at 30°C for strain VR1.

Cytoplasmic pools of UDP-linked peptidoglycan precursors were extracted by a modification of a previously described method (10). Cultures were grown to mid-logarithmic phase, bacitracin was added to a final concentration of 100 μg/ml to accumulate precursors, and the cultures were incubated for one additional hour and then chilled rapidly. Cells were harvested by centrifugation and extracted with cold trichloroacetic acid (final concentration of 5%) for 30 min. The supernatant fluid was separated by gel filtration (Sephadex G-25; Pharmacia, Alameda, Calif.) with water elution. Hexosamine-containing fractions were identified by the assay of Ghuysen et al. (8), pooled, and lyophilized.

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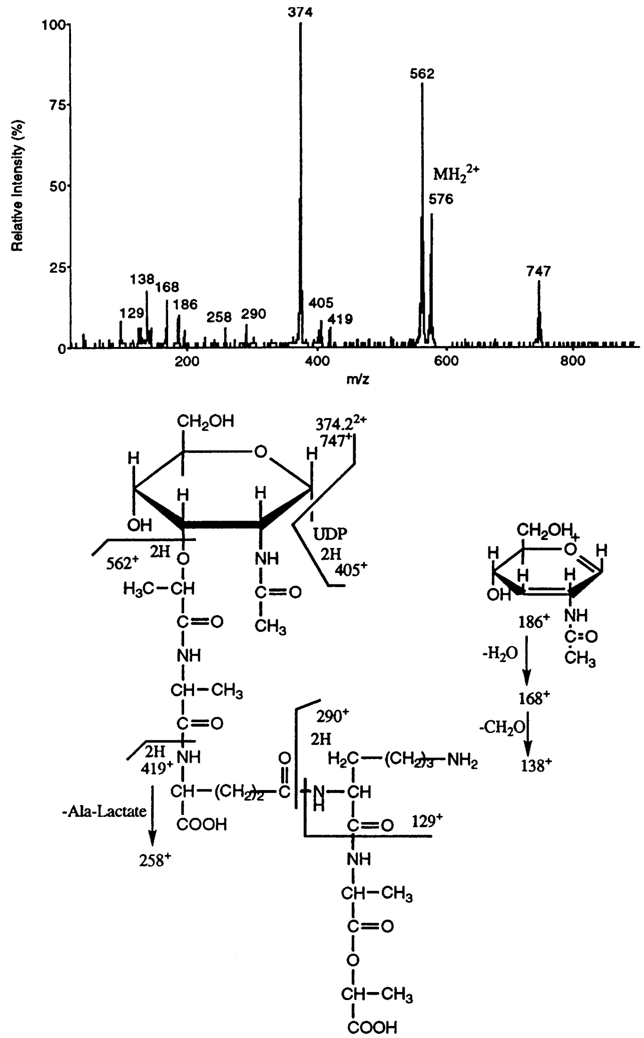


FIG. 1. Product ion mass spectrum of the MH_2^{2+} ion at *m/z* 576 of the modified tetrapeptide precursor observed in the extract of *L. casei* ATCC 7469 (top) and resulting proposed structure of the precursor UDP-*N*-acetyl–L-Ala–D-Glu–L-Lys–D-Ala–D-lactate and fragments (bottom).

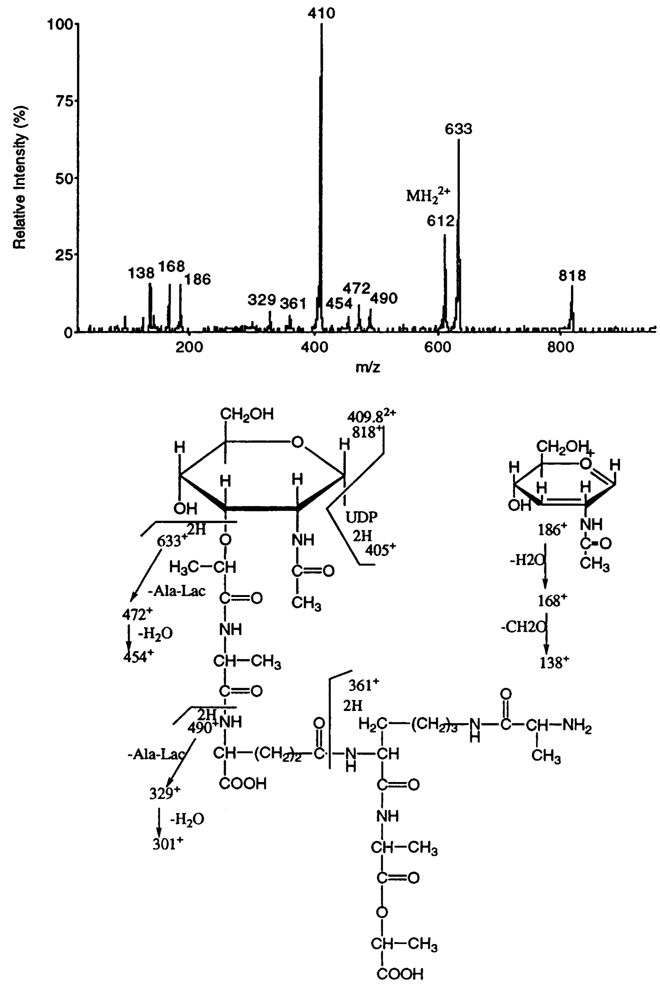


FIG. 2. Product ion mass spectrum of the MH_2^{2+} ion at *m/z* 612 of the modified pentapeptide precursor observed in the extract of *L. mesenteroides* VR1 (top) and resulting proposed structure of the precursor UDP-*N*-acetyl–L-Ala–D-Glu–L-Lys–(L-Ala)–D-Ala–D-lactate and fragments (bottom).

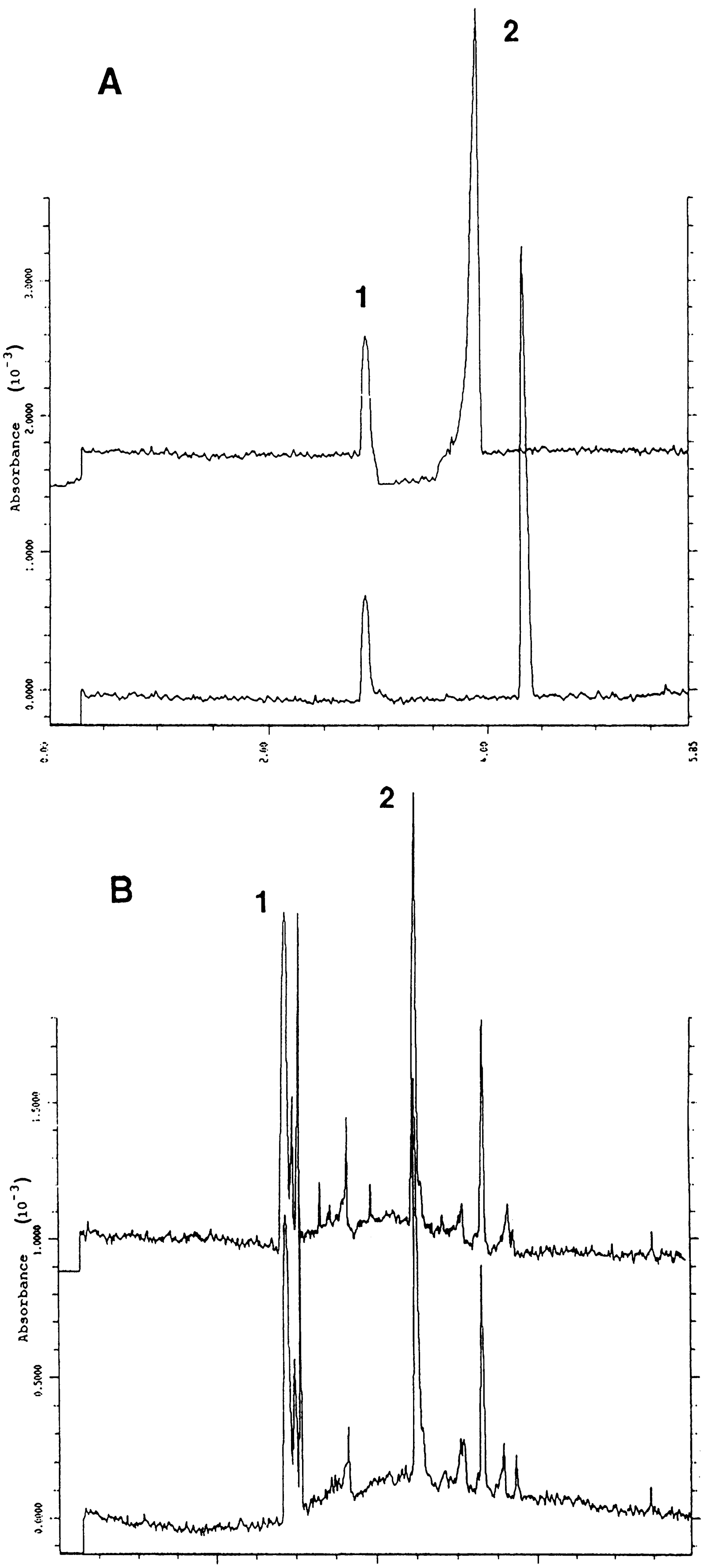
The precursor extracts were chromatographically separated on-line with a Beckman System Gold high-performance liquid chromatograph (HPLC) and a Sciex API triple-quadrupole mass spectrometer equipped with an Ionspray interface, which can resolve 1 Da at up to 2,000 Da. The normal pentapeptide precursor, UDP-*N*-acetyl–muramyl–L-Ala–D-Glu–L-Lys–D-Ala–D-Ala, isolated from *Staphylococcus aureus* 209P, was used as a control.

Purified peptidoglycan was prepared from mid-logarithmic-phase cultures of *L. mesenteroides* VR1. Cells were harvested by centrifugation, boiled with sodium dodecyl sulfate (SDS) (4%), washed extensively with water, and disrupted by vortexing with glass beads for 10 1-min pulses. Unbroken cells were removed by centrifugation, and the cell wall fraction was harvested by centrifugation and treated successively with DNase (10 μg/ml), RNase (50 μg/ml), trypsin (100 μg/ml), boiling SDS (1%), LiCl (8 M), and EDTA (100 mM).

Lyophilized cell wall material was incubated in 6 N HCl for 16 h at 110°C. Acid was removed from an aliquot by evaporation, and the preparation was suspended in 10 mM triethylamine buffer and incubated overnight with D-amino acid oxidase (20 μg/ml) at 37°C. Treated and untreated aliquots were then subjected to amino acid analysis.

For both *L. casei* ATCC 7469 and *L. mesenteroides* VR1 the MIC of vancomycin was 1,024 μg/ml. When testing was performed with DL-alanine (200 mM), the MIC was reduced to 64 μg/ml for both strains. The diminution in MIC upon the addition of high concentrations of alanine suggested that these strains were resistant to vancomycin via a mechanism similar to that of acquired resistance in enterococci.

HPLC separation of precursor extracts from *L. mesenteroides* VR1 and *L. casei* ATCC 7469 demonstrated a peak for each which was eluted later than the normal pentapeptide precursor from *S. aureus*. These peaks were collected and analyzed by mass spectrometry. Examination of the full-scan mass spectrum obtained during on-line microcolumn liquid chromatography-mass spectrometry analysis of the collected peaks indicated the molecular masses of the precursors. A component with a molecular mass of 1,150 Da was detected in the precursor extract from *L. casei* ATCC 7469. Microcolumn liquid chromatography-mass spectrometry analysis of the extract from *L. mesenteroides* VR1 indicated a precursor with a



A
1
2
Absorbance (10^-3)
3.0000
2.0000
1.0000
0.0000
0.00
2.00
4.00
5.85
B
1
2
Absorbance (10^-3)
1.5000
1.0000
0.5000
0.0000
0.00
2.00
4.00
6.00
8.00

FIG. 3. Comparison of vancomycin binding to precursors by affinity capillary electrophoresis. In each panel, the lower tracing contained buffer alone (0.2 M glycine, 0.03 M Tris [pH 8.3]) and the upper tracing contained buffer with 25 μM vancomycin. (A) *S. aureus* 209P. Peak 1, neutral marker; peak 2, UDP-*N*-acetyl–muramyl–L-Ala–D-Glu–L-Lys–D-Ala–D-Ala. (B) *L. mesenteroides* VR1. Peak 1, neutral marker; peak 2, UDP-*N*-acetyl–L-Ala–D-Glu–L-Lys–(L-Ala)–D-Ala–D-lactate.

molecular mass of 1,221 Da. The standard pentapeptide precursor isolated from *S. aureus* has a molecular mass of 1,149 Da.

Fragmentation patterns of the precursors from *L. mesenteroides* VR1 and *L. casei* ATCC 7469 were consistent with substructures of modified pentapeptide and modified tetrapeptide precursors, respectively, as shown in Fig. 1 and 2. Tandem mass spectrometry was utilized to obtain structural information for the doubly charged ion (MH_2^{2+}) of each precursor. A comparison of the tandem mass spectrometry fragmentation patterns of the precursors from *L. mesenteroides* VR1 and *L. casei* ATCC 7469 with the fragmentation template of the normal pentapeptide obtained from *S. aureus* indicated an alteration of an amino acid near the C terminus of each precursor. A key fragmentation route common to each precursor involved cleavage of the Glu-Lys amide bond. Fragment ions were observed to be consistent with the Lys-Ala-Ala (*m/z* 289), Lys-Ala-lactate (*m/z* 290), and Lys-(Ala)-Ala-lactate (*m/z* 361) substructures of the standard pentapeptide precursor (*S. aureus*), modified tetrapeptide precursor (*L. casei*), and modified pentapeptide precursor (*L. mesenteroides*), respectively. Consecutive tandem mass spectrometry fragmentations resulting in the neutral loss of the Ala-lactate substructure from the Glu-Lys-Ala-lactate and Glu-Lys-(Ala)-Ala-lactate fragment ions provide additional evidence for the proposed structural modification.

Previous investigators have identified a cytoplasmic hexapeptide precursor in bacterial species with interpeptide bridges of the structure L-Ala, L-Ala–L-Ser, or L-Ser–L-Ala which is present in some *Lactobacillus* and *Leuconostoc* spp. (13, 18). In these organisms, the first amino acid of the interpeptide bridge is added to the ε-amino group of L-Lys at the level of the UDP-linked cytoplasmic precursor rather than at the lipid intermediate (18). We therefore performed an amino acid analysis of peptidoglycan of strain VR1 to determine whether the additional alanine noted was of the L configuration consistent with such a precursor.

Amino acid analysis of peptidoglycan from strain VR1 yielded a Glu/Lys/Ala ratio of 1:1:3. After treatment of hydrolyzed cell wall with D-amino acid oxidase, amino acid analysis revealed a Glu/Lys/Ala ratio of 1:1:2, suggesting that two of the alanine residues were of the L configuration. Taken together, these results suggest the structure UDP-*N*-acetyl–muramyl–L-Ala–D-Glu–L-Lys–(L-Ala)–D-Ala–D-lactate for the cytoplasmic precursor of *L. mesenteroides* VR1.

The ability of vancomycin to bind the three precursors was studied by affinity capillary electrophoresis. This technique is based on the migration shifting before and after the formation of an affinity complex (4). The normal pentapeptide precursor, UDP-*N*-acetyl–muramyl–L-Ala–D-Glu–L-Lys–D-Ala–D-Ala, from strain 209P, bound tightly to vancomycin, as indicated by migration shifting (Fig. 3A). No shifting was observed under the same conditions for the precursor from *L. mesenteroides* VR1 (Fig. 3B); this is consistent with the proposed modification at the stem peptide terminus. A similar lack of shift was observed for *L. casei* ATCC 7469 (data not shown).

Although the genetics and mechanism of intrinsic resistance to glycopeptides among *Leuconostoc* and many *Lactobacillus* species have not been extensively studied since the identification of the phenomenon in the 1980s, this trait has been exploited in the selection of these organisms for genetic research and industrial applications (17, 19). Resistance in *Leuconostoc* species appears to be chromosomally encoded. Although *Leuconostoc* isolates generally harbor a number of plasmids, transfer of resistance has not been observed; however, genetic transfer of other antibiotic resistance traits has been demonstrated (11, 19). Furthermore, plasmid-free strains of *Leuconostoc* obtained by curing with novobiocin and ethidium bromide retain resistance to glycopeptides (12). Less is known about the genetics of resistance in *Lactobacillus* species; one article has reported ethidium bromide curing of multiple plasmids and concomitant loss of vancomycin resistance in *Lactobacillus acidophilus* (22). Previous investigations had shown that vancomycin-resistant *Leuconostoc* and *Lactobacillus* isolates do not inactivate vancomycin (15, 17, 20), but little systematic investigation of the mechanism of resistance had been undertaken, perhaps because of the isolates' apparent lack of pathogenicity. However, these organisms have been recognized more frequently in recent years as opportunistic pathogens (9, 20). Furthermore, the recent emergence of enterococci with acquired glycopeptide resistance has renewed interest in organisms with intrinsic resistance, particularly as potential sources of the genetic elements present in the *van* gene cluster.

The relationship between acquired and intrinsic vancomycin resistance remains unclear. Recently, the *van* gene cluster associated with high-level resistance in enterococci has been shown to reside on a transposon, Tn*1546* (3). Multiple regions of Tn*1546* showed no relatedness with chromosomal DNA of susceptible *E. faecalis* or *E. faecium* by Southern hybridization or PCR amplification, suggesting that the *van* genes had originated from other species (3). Organisms demonstrating intrinsic resistance appear to be likely candidates for the origin of these genes, and conjugal transfer of other antibiotic resistance determinants between *E. faecalis* and both *Leuconostoc* and *Lactobacillus* species has been demonstrated (11, 19).

The present findings demonstrate that the same mechanism of resistance, utilization of a peptidoglycan precursor terminating in lactate rather than alanine, functions in acquired high-level resistance in *Enterococcus* strains and intrinsic resistance in *Leuconostoc* and *Lactobacillus* species, suggesting that the *van* genes may have originated from an intrinsically resistant bacterium. However, a 290-bp probe internal to the *vanA* gene did not hybridize with DNA prepared from a number of *Leuconostoc*, *Lactobacillus*, or *Pediococcus* strains, even under moderately stringent conditions (7). Similarly, no cross-reactivity between membranes prepared from these organisms and antiserum to the VanA protein was identified (15). Further investigation will be needed to determine the relationship between the genetic determinants of acquired and intrinsic resistance to glycopeptide antibiotics.

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REFERENCES

1. **Allen, N. E., J. N. Hobbs, J. M. Richardson, and R. M. Riggin.** 1992. Biosynthesis of modified peptidoglycan precursors by vancomycin resistant *Enterococcus faecium*. FEMS Microbiol. Lett. **98:**109–116.
2. **Arthur, M., and P. Courvalin.** 1993. Genetics and mechanisms of glycopeptide resistance in enterococci. Antimicrob. Agents Chemother. **37:**1563–1571.
3. **Arthur, M., C. Molinas, F. Depardieu, and P. Courvalin.** 1993. Characterization of Tn*1546*, a Tn*3*-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J. Bacteriol. **175:** 117–127.
4. **Avila, L. Z., Y.-H. Chu, E. C. Blossey, and G. M. Whitesides.** 1993. Use of affinity electrophoresis to determine kinetic and equilibrium constant for binding of arylsulfonamidase to bovine carbonic anhydrase. J. Med. Chem. **36:**126–133.
5. **Bugg, T. D. H., S. Dutka-Malen, M. Arthur, P. Courvalin, and C. T. Walsh.** 1991. Identification of vancomycin resistance protein VanA a D-alanine:D-alanine ligase of altered substrate specificity. Biochemistry **30:**2017–2021.
6. **Bugg, T. D. H., G. D. Wright, S. Dutka-Malen, M. Arthur, P. Courvalin, and C. T. Walsh.** 1991. Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. Biochemistry **30:**10408–10415.
7. **Dutka-Malen, S., R. Leclercq, V. Coutant, J. Duval, and P. Courvalin.** 1990. Phenotypic and genotypic heterogeneity of glycopeptide resistance determinants in gram-positive bacteria. Antimicrob. Agents Chemother. **34:**1875–1879.
8. **Ghuysen, J. M., D. J. Tipper, and J. L. Strominger.** 1966. Enzymes that degrade bacterial cell walls. Methods Enzymol. **8:**684–699.
9. **Handwerger, S., H. Horowitz, K. Coburn, A. Kolokathis, and G. P. Wormser.** 1990. Infection due to *Leuconostoc* species: six cases and review. Rev. Infect. Dis. **12:**602–610.
10. **Handwerger, S., M. J. Pucci, K. J. Volk, J. Liu, and M. S. Lee.** 1992. The cytoplasmic peptidoglycan precursor of vancomycin-resistant *Enterococcus faecalis* terminates in lactate. J. Bacteriol. **174:**5982–5984.
11. **Hill, C., C. Daly, and G. F. Fitzgerald.** 1985. Conjugative transfer of the transposon Tn*919* to lactic acid bacteria. FEMS Microbiol. Lett. **30:**115–119.
12. **Horowitz, H., A. Kolokathis, and S. Handwerger.** 1989. Genetics of vancomycin resistance in *Leuconostoc* spp., abstr. A-90. Abstr. 89th Annu. Meet. Am. Soc. Microbiol. 1989. American Society for Microbiology, Washington, D.C.
13. **Kandler, O.** 1970. Amino acid sequence of the murein and taxonomy of the genera *Lactobacillus*, *Bifidobacterium*, *Leuconostoc* and *Pediococcus*. Int. J. Syst. Bacteriol. **20:**491–507.
14. **Messer, J., and P. E. Reynolds.** 1992. Modified peptidoglycan precursors produced by glycopeptide-resistant enterococci. FEMS Microbiol. Lett. **94:**195–200.
15. **Nicas, T. I., C. T. Cole, D. A. Preston, A. A. Schabel, and R. Nagarajan.** 1989. Activity of glycopeptides against vancomycin-resistant gram-positive bacteria. Antimicrob. Agents Chemother. **33:**1477–1481.
16. **Nieto, M., and H. R. Perkins.** 1971. Modifications of the acyl-D-alanyl-D-alanine terminus affecting complex formation with vancomycin. Biochem. J. **123:**789–803.
17. **Orberg, P. K., and W. E. Sandine.** 1984. Common occurrence of plasmid DNA and vancomycin resistance in *Leuconostoc* spp. Appl. Environ. Microbiol. **48:**1129–1133.
18. **Plapp, R., and J. L. Strominger.** 1970. Biosynthesis of the peptidoglycan of bacterial cell walls. XVII. Biosynthesis of peptidoglycan and of interpeptide bridges in *Lactobacillus viridescens*. J. Biol. Chem. **245:**3667–3674.
19. **Pucci, M. J., M. E. Monteschio, and C. L. Kemker.** 1988. Intergeneric and intrageneric conjugal transfer of plasmid-encoded antibiotic resistance determinants in *Leuconostoc* spp. Appl. Environ. Microbiol. **54:**281–287.
20. **Ruoff, K. L., D. R. Kuritzkes, J. S. Wolfson, and M. J. Ferraro.** 1988. Vancomycin-resistant gram-positive bacteria isolated from human sources. J. Clin. Microbiol. **26:**2064–2068.
21. **Sheldrick, G. M., P. G. Jones, O. Kennard, H. Williams, and G. A. Smith.** 1978. Structure of vancomycin and its complex with acetyl-D-alanyl-D-alanine. Nature (London) **271:**223–225.
22. **Vescovo, M., L. Morelli, and V. Bottazzi.** 1982. Drug resistance plasmids in *Lactobacillus acidophilus* and *Lactobacillus reuteri*. Appl. Environ. Microbiol. **43:**50–56.
23. **Zarlenga, L. J., M. S. Gilmore, and D. F. Sahm.** 1992. Effects of amino acids on expression of enterococcal vancomycin resistance. Antimicrob. Agents Chemother. **36:**902–905.