ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1992, p. 867–869
0066-4804/92/040867-03$02.00/0

Vol. 36, No. 4

Evidence for In Vivo Incorporation of D-Lactate into Peptidoglycan Precursors of Vancomycin-Resistant Enterococci

MICHEL ARTHUR,[1]* CATHERINE MOLINAS,[1] TIMOTHY D. H. BUGG,[2] GERARD D. WRIGHT,[2] CHRISTOPHER T. WALSH,[2] AND PATRICE COURVALIN[1]

Unité des Agents Antibactériens, Institut Pasteur, 75724 Paris Cedex 15, France,[1] and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115[2]

Received 3 September 1991/Accepted 2 January 1992

The VanA ligase encoded by the vancomycin resistance plasmid pIP816 of *Enterococcus faecium* BM4147 condenses D-alanine with various D-2-hydroxy and D-2-amino acids in vitro. D-Lactate added to the culture medium restored the vancomycin resistance of a strain that does not produce the VanH dehydrogenase and therefore appears to be a substrate of VanA in vivo.

Inducible resistance to high levels of the glycopeptide antibiotics vancomycin and teicoplanin in *Enterococcus faecium* BM4147 is mediated by the 34-kb plasmid pIP816 (8). The *van* gene cluster of pIP816 comprises five genes, *vanR*, *vanS*, *vanH*, *vanA*, and *vanX*, that are necessary and sufficient for regulated expression of glycopeptide resistance (1). The *vanR* and *vanS* genes encode a two-component regulatory system that activates transcription of *vanH*, *vanA*, and *vanX* in response to the presence of vancomycin in the culture medium (1). The VanH dehydrogenase encoded by *vanH* (2) catalyzes the stereospecific reduction of various 2-keto acids to form the corresponding D-2-hydroxy acids (4). The *vanA* gene encodes an enzyme, VanA, that is related to D-alanine:D-alanine ligases from *Escherichia coli* (5) but that displays a broader substrate specificity (3, 4). In addition to synthesis of dipeptides, VanA catalyzes ester bond formation between D-alanine (D-Ala) and D-2-hydroxy acids (3, 4). The enzyme favors incorporation of substrates with an ethyl side chain at the C-terminal position of dipeptides and depsipeptides, whereas D-Ala is incorporated at the N terminus. The *vanX* gene encodes a protein, VanX, of unknown function that is required for resistance (1).

In crude extracts of *E. faecium* BM4147, VanA catalyzes ester bond formation between D-Ala and the D-2-hydroxy acid products of VanH; the resulting depsipeptides are incorporated onto the UDP-muramyl tripeptide by the D-Ala-D-Ala adding enzyme (4). Thus, the catalytic properties of VanH and VanA suggest that these enzymes can mediate in vivo incorporation of a D-2-hydroxy acid at the C-terminal position of peptidoglycan precursors by the putative biosynthetic pathway described in Fig. 1A. However, VanA condenses D-Ala with D-2-hydroxybutyrate or D-2-aminobutyrate with similar catalytic efficiencies (4), suggesting an alternative pathway for the synthesis of peptidoglycan precursors (Fig. 1B). In fact, NAD^+-dependent dehydrogenases related to VanH are known to produce 2-keto acids that are subsequently converted to the corresponding D-amino acids by a transaminase (2). VanH may therefore be involved in the synthesis of a D-amino acid that is a substrate for VanA, provided that the host chromosome or VanX encodes a transaminase. Since the dipeptide D-Ala-D-2-aminobutyrate is a substrate of the D-Ala-D-Ala adding enzyme (4), D-2-aminobutyrate might be incorporated into peptidoglycan precursors.

Vancomycin inhibits cell wall synthesis by formation of a complex with the D-Ala-D-Ala termini of peptidoglycan precursors in the outer surface of the bacterial membrane (6, 9). Measurements of vancomycin affinity for peptidoglycan precursor analogs indicated that the D-Ala residue at the C-terminal position is essential for the stability of the complex (10). Substitution of the NH group of the amide D-Ala-D-Ala linkage by an oxygen in a D-Ala-D-lactate ester linkage leads to >1,000-fold lower binding by vancomycin (4). Similarly, substitution of a methyl by an ethyl side chain (D-Ala versus D-2-aminobutyrate) reduced more than 300-fold the binding of the antibiotic to precursor analogs (4).

Results of these in vitro investigations have established that the vancomycin resistance of *E. faecium* BM4147 is due to the production of peptidoglycan precursors that bind the antibiotic with reduced affinity (4). However, the actual D-Ala substituent incorporated in vivo could not be identified because of the relatively broad substrate specificity of VanA, VanH, and the D-Ala-D-Ala adding enzyme. Synthesis of a depsipeptide precursor (Fig. 1A) was considered to be the most likely pathway mainly because, in contrast to VanA, the D-Ala:D-Ala ligase from susceptible enterococci or *E. coli* does not catalyze ester bond formation (4). The aim of the present study was to confirm that depsipeptide synthesis is the actual pathway for the peptidoglycan precursor assembly in resistant cells and to identify in vivo the VanA substrates that allow cell wall synthesis in the presence of vancomycin. The pathway in Fig. 1A implies that VanH synthesizes a 2-keto acid from a 2-hydroxy acid, whereas in the pathway in Fig. 1B, VanH catalyzes the reverse reaction and produces a 2-hydroxy acid. To determine whether VanH functions in vivo by producing a 2-keto or a 2-hydroxy acid, we performed an assay to determine whether either of these compounds, when added to the culture medium, could compensate for the insertional inactivation of the *vanH* gene.

Plasmid pAT80, which confers vancomycin resistance, was constructed by cloning the *van* gene cluster of pIP816 into the shuttle vector pAT29 (1). Derivatives of pAT80 carrying the *aphA-1* kanamycin resistance gene inserted into *vanH* (pAT83), *vanA* (pAT84), or *vanX* (pAT85) do not confer resistance to the antibiotic (1). The MICs of vancomycin for *E. faecalis* JH2-2 (7) and derivatives harboring plasmids pAT80, pAT83, pAT84, and pAT85 were deter-

* Corresponding author.

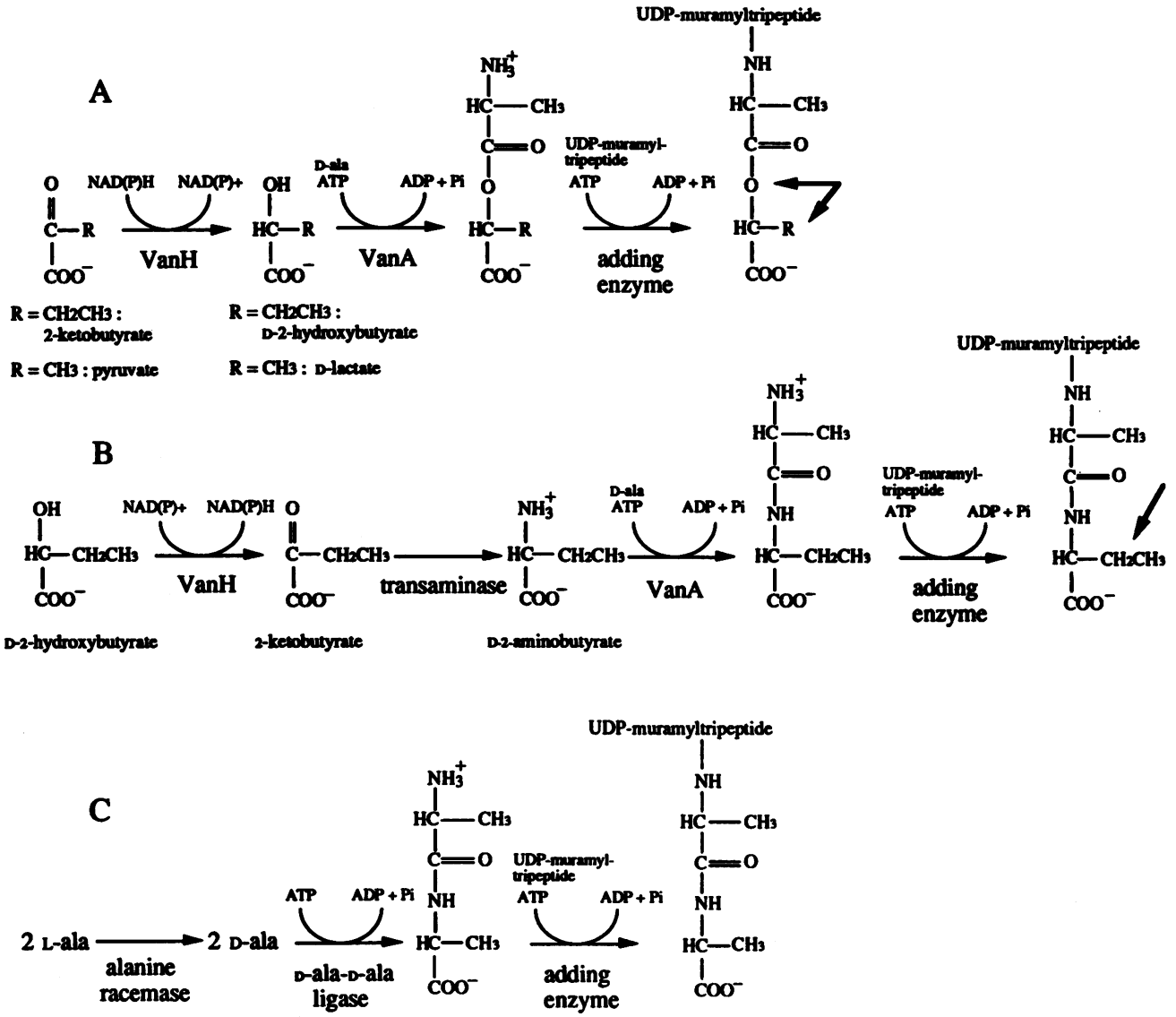

FIG. 1. Pathways for the assembly of cytoplasmic peptidoglycan precursors. (A) Incorporation of D-2-hydroxybutyrate or D-lactate at the C-terminal position of peptidoglycan precursors of enterococci resistant to glycopeptides. (B) Alternative pathway involving incorporation of D-2-aminobutyrate. (C) Synthesis of UDP-muramyl pentapeptide in enterococci susceptible to glycopeptides (12). Boldface arrows indicate chemical groups that prevent binding of vancomycin to peptidoglycan precursor analogs (4): the ethyl side chain and the oxygen of the ester linkage for D-2-hydroxybutyrate (A), the oxygen of the ester linkage for D-lactate that contains a methyl side chain as D-Ala (A), and the ethyl side chain for D-2-aminobutyrate (B).

mined in the presence or absence of 2-ketobutyrate and DL-2-hydroxybutyrate in the culture medium (Table 1). The acids did not interfere with the inhibition of cell wall synthesis by vancomycin in the susceptible host *E. faecalis* JH2-2. The MIC of vancomycin for JH2-2/pAT83 (*vanHΩaphA-1*) was not modified by 2-ketobutyrate, but DL-2-hydroxybutyrate (100 mM) raised the MIC from 1 to 1,024 μg/ml. This effect was not observed with JH2-2/pAT84 (*vanAΩaphA-1*). These results indicate that in vivo the role of VanH is the production of a D-2-hydroxy acid and that VanA-mediated incorporation of D-2-hydroxybutyrate at the C-terminal position of peptidoglycan precursors allows cell wall synthesis in the presence of vancomycin (Fig. 1A). In agreement with this notion, 100 mM D-2-aminobutyrate did not permit the growth of JH2-2/pAT83 (*vanHΩaphA-1*) in the presence of vancomycin (data not shown). In addition, purified VanX did not bind pyridoxal phosphate and did not possess transaminase activity in the presence of all 20 naturally occurring amino acids (14).

Since D-2-hydroxybutyrate did not compensate for the insertional inactivation of the *vanX* gene (Table 1), VanX is not involved in the synthesis of the 2-keto acid substrate for the VanH dehydrogenase. Previous studies have shown that the chromosome of *E. faecium* BM4147 encodes an adding enzyme for incorporation of depsipeptides onto UDP-muramyl tripeptide (4). Thus, VanX probably acts at a later stage of cell wall assembly.

Although D-2-hydroxybutyrate was the preferential substrate for the VanA ligase in in vitro experiments, the enzyme was also capable of catalyzing ester bond formation

TABLE 1. Effect of the addition of keto and hydroxy acids in the culture medium on the MIC of vancomycin for *E. faecalis* JH2-2 harboring various plasmids

Plasmid (relevant characteristics)	MIC (μg/ml) in Mueller-Hinton agar supplemented with[a]:									
	No addition	2-Keto-butyrate (20 mM)	DL-2-Hydroxybutyrate at:				DL-Lactate at:			
			1 mM	5 mM	20 mM	100 mM	1 mM	5 mM	20 mM	100 mM
None	1	1	1	1	1	1	1	1	1	1
pAT80 (*vanR vanS vanH vanA vanX*)	1,024	1,024	1,024	1,024	1,024	2,048	1,024	1,024	1,024	2,048
pAT83 (*vanR vanS vanHΩaphA-1 vanA vanX*)	1	1	4	64	512	1,024	64	512	1,024	2,048
pAT84 (*vanR vanS vanH vanAΩaphA-1 vanX*)	1	1	1	1	1	1	1	1	1	1
pAT85 (*vanR vanS vanH vanXΩaphA-1*)	1	1	1	1	1	1	1	1	1	1

[a] The method of Steers et al. (11), with 10^4 CFU per spot, was used to determine the MICs of vancomycin in Mueller-Hinton agar (Diagnostics Pasteur, Marnes-la-Coquette, France) and in the same medium supplemented with 2-ketobutyrate, DL-2-hydroxybutyrate, DL-lactate (Sigma, S.A.R.L., La Verpillière, France). The carboxylic acids were added to the culture medium as 1 M neutralized (pH 7.0) solutions.

between D-Ala and D-lactate at about a 14-fold lower catalytic efficiency (4). This hydroxy acid restored the vancomycin resistance of JH2-2/pAT83 (*vanHΩaphA-1*) at about a fivefold lower concentration than that required by D-2-hydroxybutyrate (Table 1). In *E. faecalis*, the homofermentative pathway for hexose dissimilation leads to the production of L-lactate from pyruvate (13). Thus, pyruvate is expected to be available for the synthesis of D-lactate by VanH. Reduction of pyruvate is efficiently catalyzed by the purified enzyme (4). These observations suggest that D-lactate is probably the D-Ala substituent present at the C-terminal position of peptidoglycan precursors of enterococci that are resistant to high levels of glycopeptides.

We thank J.-P. Carlier, O. Bouvet, and K. H. Schleifer for helpful discussions.

This work was supported by grant DMB 8917290 from the National Science Foundation.

REFERENCES

1. **Arthur, M., C. Molinas, and P. Courvalin.** The VanS-VanR two-component regulatory system controls synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J. Bacteriol., in press.
2. **Arthur, M., C. Molinas, S. Dutka-Malen, and P. Courvalin.** 1991. Structural relationship between the vancomycin resistance protein VanH and 2-hydroxycarboxylic acid dehydrogenases. Gene **103**:133–134.
3. **Bugg, T. D. H., S. Dutka-Malen, M. Arthur, P. Courvalin, and C. T. Walsh.** 1991. Identification of vancomycin resistance protein VanA as a D-alanine:D-alanine ligase of altered substrate specificity. Biochemistry **30**:2017–2021.
4. **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.
5. **Dutka-Malen, S., C. Molinas, M. Arthur, and P. Courvalin.** 1990. The VANA glycopeptide resistance protein is related to D-alanyl-D-alanine ligase cell wall biosynthesis enzymes. Mol. Gen. Genet. **224**:364–372.
6. **Gale, E. F., E. Cundliffe, P. E. Reynolds, M. H. Richmond, and M. J. Waring.** 1981. The molecular basis of antibiotic action, p. 144–174. John Wiley & Sons, London.
7. **Jacob, A. E., and S. J. Hobbs.** 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes*. J. Bacteriol. **117**:360–372.
8. **Leclercq, R., E. Derlot, J. Duval, and P. Courvalin.** 1988. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. N. Engl. J. Med. **319**:157–161.
9. **Nagarajan, R.** 1991. Antibacterial activities and modes of action of vancomycin and related glycopeptides. Antimicrob. Agents Chemother. **35**:605–609.
10. **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.
11. **Steers, E., E. L. Foltz, B. S. Gravies, and J. Rindel.** 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. (Basel) **9**:307–311.
12. **Walsh, C. T.** 1989. Enzymes in the D-alanine branch of bacterial cell-wall peptidoglycan assembly. J. Biol. Chem. **264**:2393–2396.
13. **Wolin, M. J.** 1964. Fructose-1,6 diphosphate requirement of streptococcal lactic dehydrogenases. Science (Washington, D.C.) **146**:775–777.
14. **Wright, G. D., and C. T. Walsh.** Unpublished data.