DIGITAL ACCESS TO SCHOLARSHIP AT HARVARD
DASH.HARVARD.EDU

HARVARD LIBRARY

Effects of amino acids on expression of enterococcal vancomycin resistance

Citation

Zarlenga LJ, Gilmore MS, Sahm DF. 1992. Effects of amino acids on expression of enterococcal vancomycin resistance. Antimicrob Agents Chemother. 36, no. 4: 902-5.

Link

http://nrs.harvard.edu/urn-3:HUL.InstRepos:35141066

Terms of use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material (LAA), as set forth at
https://harvardwiki.atlassian.net/wiki/external/NGY5NDE4ZjgzNTc5NDQzMGIzZWZhMGFlOWI2M2EwYTg

Accessibility

https://accessibility.huit.harvard.edu/digital-accessibility-policy

Share Your Story

The Harvard community has made this article openly available.
Please share how this access benefits you. Submit a story

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1992, p. 902-905
0066-4804/92/040902-04$02.00/0
Copyright © 1992, American Society for Microbiology
Vol. 36, No. 4

Effects of Amino Acids on Expression of Enterococcal Vancomycin Resistance

LANA J. ZARLENGA,[1] MICHAEL S. GILMORE,[2] AND DANIEL F. SAHM[1,3]†*

Department of Pathology,[1] the Clinical Microbiology Laboratories,[3] The University of Chicago Medical Center, Chicago, Illinois 60637, and the Department of Microbiology and Immunology, Oklahoma University Health Sciences Center, Oklahoma City, Oklahoma 73190[2]

Received 15 October 1991/Accepted 27 January 1992

The effects of various amino acids on vancomycin MICs obtained with resistant enterococci was investigated by using broth dilution testing. For both the type A (i.e., possessing transferable resistance to teicoplanin and vancomycin) and the type B (i.e., possessing teicoplanin susceptibility and nontransferable vancomycin resistance) resistant strains, vancomycin MICs in the presence of glycine were substantially lower than those in unsupplemented broth (range of MIC decrease, 8- to 128-fold). No such effect was seen with *Enterococcus gallinarum* AIB-39 or with the susceptible control *Enterococcus faecalis* ATCC 29212. Further testing of two type B strains (*E. faecalis* V583 and V583-2) showed that certain other amino acids (i.e., D-methionine, D-serine, D-alanine, and D-phenylalanine) had effects similar to that of glycine. Results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis with membrane preparations from these strains revealed the production of vancomycin-inducible proteins of sizes comparable to those described for other enterococcal isolates that exhibit acquired vancomycin resistance. Even in the presence of 0.2 M glycine, the inducible proteins were produced. These results indicate that certain amino acids specifically interfere with the mechanism(s) of acquired vancomycin resistance in enterococci and that the nature of interference probably involves inhibition or circumvention of the inducible proteins' functions.

As stated by Reynolds, "The structure of glycopeptides, as well as their unique site and mechanism of action, is likely to ensure that any resistance mechanisms acquired by a gram-positive bacterium will be unusual" (18). Several recent reports (14, 19, 22, 25) are evidence that some such resistance mechanisms have been acquired by enterococci, and there is every indication that the incidence of this resistance will become more widespread (5, 8, 13). While investigating the plasmid content of *Enterococcus faecalis* V583, a strain previously described as the first reported vancomycin-resistant *E. faecalis* strain isolated in the United States (19), we noted the inability of V583 to grow in usually subinhibitory concentrations of vancomycin when glycine was present in the growth medium. This finding prompted us to investigate whether this effect was specific for vancomycin-resistant enterococci and to determine whether other amino acids could produce similar effects. The results of these studies are the subject of this report. Additionally, how these findings pertain to current hypotheses concerning glycopeptide resistance mechanisms in enterococci is discussed.

Species identification of all strains was based on the conventional scheme reported by Facklam and Collins (7). *E. faecalis* ATCC 29212 was the vancomycin-susceptible control, and *Enterococcus gallinarum* AIB-39, provided by the Antimicrobics Investigation Branch, Centers for Disease Control, Atlanta, Ga. (20), represented the enterococcal species known to exhibit intrinsic, noninducible, low-level vancomycin resistance (26). The previously described *Enterococcus faecium* 228 (11), also provided by the Antimicrobics Investigation Branch, demonstrates type A vancomycin resistance (i.e., transferable vancomycin and teicoplanin resistance) and is known to hybridize with a *vanA* probe developed by Dutka-Malen et al. (6, 23). *E. faecalis* KS-2 is a vancomycin-resistant transconjugant obtained by filter mating *E. faecium* 228 and *E. faecalis* JH2-2 (12). *E. faecium* UCVR-1 is a recent blood culture isolate from the University of Chicago Clinical Microbiology Laboratories. *E. faecalis* V583 has been described previously (19), and *E. faecalis* V583-2 is a high-level, vancomycin-resistant derivative of V583 obtained by serial passage on media containing increasing concentrations of vancomycin. *E. faecium* UCVR-1, *E. faecalis* V583, and *E. faecalis* V583-2 each exhibit type B vancomycin resistance (i.e., teicoplanin susceptibility and nontransferable vancomycin resistance).

Broth macrodilution was performed in 1-ml volumes of unsupplemented brain heart infusion broth (BHIB) (Difco Laboratories, Detroit, Mich.) or broth supplemented with 0.2 M amino acid (Sigma Biochemical Co., St. Louis, Mo.) according to the recommended testing procedures (15). The chosen concentration of amino acid approximated that being used for the plasmid preparation procedure when the "glycine effect" was first noted. Testing was performed in triplicate for each drug-amino acid-organism combination studied. In every instance, all three MICs were within 1 twofold dilution, and in most cases they were the same. When all three MICs were not identical, the value obtained twice was used.

In the presence of glycine, the decrease in vancomycin MICs for the five most resistant enterococci ranged from 8- to 128-fold after 24 h of incubation (Table 1). The most substantial MIC decrease occurred with *E. faecalis* V583 and V583-2, while the effect of glycine on vancomycin MICs for the susceptible control *E. faecalis* ATCC 29212 and for *E. gallinarum* AIB-39 was negligible. Although MICs for some strains increased 1 dilution with incubation prolonged

* Corresponding author.
† Present address: Division of Microbiology and Serology, Jewish Hospital at Washington University Medical Center, St. Louis, MO 63110.

TABLE 1. Effects of glycine on vancomycin MICs[a]

Strain	Vancomycin MIC (μg/ml)		Decrease (fold)[b]
	No glycine	0.2 M glycine	
E. faecalis ATCC 29212	4	2	2
E. gallinarum AIB-39	16	16	NC
E. faecium 228	1,024	128	8
E. faecalis KS-2	512	64	8
E. faecium UCVR-1	64	4	16
E. faecalis V583	128	2	64
E. faecalis V583-2	1,024	8	128

[a] All MICs were determined after 24 h of incubation.
[b] MIC without glycine divided by MIC with 0.2 M glycine. NC, no change in MIC.

to 48 h, there was no notable difference in the extent to which MICs decreased in the presence of glycine. Vancomycin MICs for *E. faecalis* V583 were noted to decrease with increasing concentrations of glycine; vancomycin MICs were 256, 128, 64, 64, 16, 8, and 4 μg/ml in the presence of 0, 0.006, 0.0125, 0.025, 0.05, 0.1, and 0.2 M glycine, respectively.

To investigate the effect of glycine on other cell wall-active agents, ampicillin and teicoplanin MICs for *E. faecalis* ATCC 29212 and *E. faecalis* V583 were determined in the presence and absence of glycine. Although ampicillin and teicoplanin MICs decreased four- and eightfold, respectively, in the presence of glycine, these decreases were not nearly as substantial as the decreases in vancomycin MICs for *E. faecalis* V583 shown in Table 1.

The effects of amino acids besides glycine on vancomycin MICs were investigated by testing *E. faecalis* V583, *E. faecalis* V583-2, *E. faecalis* ATCC 29212, and *E. gallinarum* AIB-39. For *E. faecalis* V583, testing in the presence of D-methionine, D-serine, D-alanine, and D-phenylalanine resulted in the greatest decreases in vancomycin MICs (Table 2). More modest decreases in MICs were seen with D-valine, D-threonine, and L-alanine. No change was noted with either L-serine or L-threonine. Comparable results were obtained with *E. faecalis* V583-2, but the presence of D-methionine, D-serine, and D-alanine did not substantially alter the vancomycin MICs for the susceptible strain *E. faecalis* ATCC 29212 or *E. gallinarum* AIB-39 (data not shown).

For the analysis of cytoplasmic membrane proteins, enterococcal strains were grown overnight at 35°C in 200 ml of

TABLE 2. Effects of D- and L-amino acids on vancomycin MICs for *E. faecalis* V583[a]

Amino acid	MIC (μg/ml)		Decrease (fold)[b]
	Control	0.2 M	
D-Methionine	256	2	128
D-Serine	256	2	128
D-Alanine	256	8	32
D-Phenylalanine	256	4	64
D-Valine	256	32	8
D-Threonine	256	64	4
L-Alanine	256	64	4
L-Serine	256	256	NC
L-Threonine	256	256	NC

[a] All MICs were determined after 48 h of incubation. Control MICs were determined with BHIB alone; 0.2 M MICs were determined with BHIB supplemented with 0.2 M each indicated amino acid.
[b] Control MIC divided by 0.2 M MIC. NC, no change in MIC.

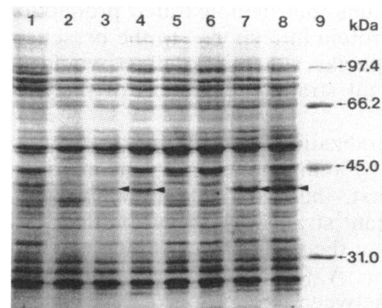

FIG. 1. SDS-polyacrylamide gel electrophoresis of *E. faecalis* V583 and V583-2 membrane preparations from various growth conditions. Lanes: 1, *E. faecalis* V583 in BHIB only; 2, *E. faecalis* V583 in BHIB plus 0.2 M glycine; 3, *E. faecalis* V583 in BHIB plus 4 μg of vancomycin per ml; 4, *E. faecalis* V583 in BHIB plus 0.2 M glycine plus 1 μg of vancomycin per ml; 5, *E. faecalis* V583-2 in BHIB only; 6, *E. faecalis* V583-2 in BHIB plus 0.2 M glycine; 7, *E. faecalis* V583-2 in BHIB plus 4 μg of vancomycin per ml; 8, *E. faecalis* V583-2 in BHIB plus 0.2 M glycine plus 4 μg of vancomycin per ml; 9, molecular size markers as indicated. Arrowheads indicate the 41-kDa protein produced in response to the presence of vancomycin.

unsupplemented BHIB or broth supplemented with appropriate concentrations of vancomycin and/or 0.2 M glycine. The subsequent steps for the preparation of membrane proteins were performed according to the protocol described by Dutka-Malen et al. (6). The protein concentration of each membrane sample was estimated by using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.). Equal amounts of each sample (ca. 50 μg of protein per lane) were loaded onto 20-cm 10% sodium dodecyl sulfate (SDS)-polyacrylamide resolving gels with a 5% stacking gel. Samples were electrophoresed at 18 mA of constant current for 5 h or until the tracking dye reached the bottom of the gel. Gels were stained with Coomassie blue. For both *E. faecalis* V583 and V583-2, a 41-kDa protein was prominently apparent only when the organisms were grown in the presence of vancomycin (Fig. 1). Notably, both strains still produced the 41-kDa protein when grown in the concentration of glycine that substantially lowers vancomycin MICs. Although not shown in Fig. 1, the amount of protein produced by both strains increased with the concentration of vancomycin used for induction, and more protein was produced by *E. faecalis* V583-2 than by the parent strain, *E. faecalis* V583, which exhibits a lower level of resistance (Table 1). An inducible protein of comparable size also was noted with *E. faecium* 228 and *E. faecium* UCVR-1 but not with either *E. faecalis* ATCC 29212 or *E. gallinarum* AIB-39 (data not shown).

Because vancomycin inhibits cell growth primarily by interfering with transglycosylation (18) and glycine and certain other D-amino acids interfere with transpeptidation (10, 16, 21, 24), the "amino acid effect" could be due to a synergy between vancomycin and various D-analogs that is unrelated to the underlying vancomycin resistance mechanism. However, results of this study indicate that the effect is due to the specific interference of acquired vancomycin-resistant mechanisms. The amino acid effect was observed for each vancomycin-resistant *E. faecalis* and *E. faecium* strain, but it was not observed with the susceptible control *E. faecalis* ATCC 29212 or with *E. gallinarum* AIB-39, an enterococcal species known to intrinsically exhibit low-level vancomycin resistance (26). That is, the effect was specific

for those strains that demonstrated production of a unique membrane protein in response to the presence of vancomycin. A nonspecific synergistic effect would have been observed with all strains, regardless of their levels of resistance.

Two generalizations concerning the nature of specific interference by amino acids with vancomycin resistance can be made. First, the extent of the amino acid effect varies among resistant strains. This may be due to distinct differences between the types of resistance mechanisms, that is, between type A (6) and type B (2) resistance, due to differences between expression of resistance in *E. faecalis* and that in *E. faecium*, or it may be a combination of these factors. The eightfold decrease in vancomycin MICs for *E. faecium* 228, a strain known to hybridize with *vanA* (23), was the same as the decrease seen with *E. faecalis* KS-2, a transconjugant of *E. faecium* 228. These findings indicate that the extent of interference with resistance is dictated more by the type of resistance than by the species in which resistance is expressed. On the other hand, both *E. faecium* UCVR-1 and *E. faecalis* V583 phenotypically exhibit type B resistance, but the extent of the glycine effect on vancomycin MICs for V583 was notably greater than that for *E. faecium* UCVR-1 (Table 1). A definitive explanation for these species-to-species variations will require further studies involving a greater variety of resistant isolates. Secondly, glycine does not inhibit production of the inducible 41-kDa protein, which is comparable to, if not the same as, the inducible proteins described by others as the primary mediators of vancomycin resistance in *E. faecalis* and *E. faecium* (1, 2, 6, 11). Because the protein continues to be produced, amino acid interference probably involves inhibition or circumvention of the protein's function.

Because our results indicate that the amino acid effect is specific for the underlying vancomycin resistance mechanisms, some discussion of these findings in the context of current hypotheses concerning vancomycin resistance is warranted. Reports by Bugg et al. (3, 4) have shown that in a type A strain, resistance is mediated by the interactions of a modified D-alanine:D-alanine ligase (Van A) and a D-specific α-keto acid dehydrogenase (Van H) that results in the synthesis of an altered D-alanyl-X dipeptide (3) or depsipeptide (4). When these products are incorporated into the terminus of the pentapeptide, they are not bound by vancomycin. Regarding the amino acid effect reported in our study, when certain amino acids are present in excess, they could compete with product X and be incorporated into the dipeptide. Dipeptides containing these amino acids are known to incorporate into the pentapeptide and bind vancomycin (17, 21, 24). Such termini then may bind vancomycin more effectively than termini containing product X, resulting in a decrease in the level of vancomycin resistance. Another possibility is that an excess of certain amino acids causes a shutdown of the modified ligase-dehydrogenase system and allows normal ligase to synthesize conventional peptidoglycan termini that are susceptible to vancomycin action. Our finding that D-alanine, the normal component of the pentapeptide terminus, decreased the level of vancomycin resistance seems most consistent with this latter possibility.

In addition to the ligase-dehydrogenase system reported by Bugg et al. (3, 4), Al-Obeid et al. (1) and Gutmann et al. (9) suggest that vancomycin resistance is also partially mediated by D,D-carboxypeptidase activity. This enzyme is thought to decrease the number of D-alanyl-D-alanine termini essential for vancomycin binding by cleaving the terminal D-alanine from the UDP-MurNac-pentapeptide unit or by hydrolyzing the D-alanyl-D-alanine dipeptide before adding it to the UDP-MurNac-tripeptide. Relevant to our findings, the amino acids that decreased vancomycin MICs are known to act as D-alanine analog and to substitute for D-alanine in the pentapeptide terminus (21, 24). While such modified termini can still bind vancomycin (17), they may not bind the DD-carboxypeptidase thought to mediate resistance and thus preserve some level of vancomycin susceptibility. What is not consistent with this possible mechanism of interference with vancomycin resistance is that vancomycin MICs decreased in the presence of D-alanine and, to a lesser extent, L-alanine (Table 2). Because both of these amino acids are normal components of pentapeptide synthesis and structure, their presence would be expected to result in the production of the usual dipeptide termini. Such termini should bind to and be acted upon by the D,D-carboxypeptidase without any notable decrease in vancomycin resistance.

In summary, the findings of this study indicate that certain amino acids specifically interfere with the levels of acquired vancomycin resistance expressed in *E. faecalis* and *E. faecium*. Although these results are consistent with major aspects of both the modified ligase-dehydrogenase mechanism (3, 4) and the D,D-carboxypeptidase mechanism (1, 9) of resistance, more studies are needed to establish the phenomena underlying the amino acid effect described in this report. Such information should be useful for planning further studies designed to elucidate the complex phenomena underlying enterococcal glycopeptide resistance.

We sincerely appreciate the editorial expertise provided by Josephine Morello, the University of Chicago Medical Center, and the helpful discussions and suggestions concerning peptidoglycan biochemistry offered by Francis Neuhaus and Michael Heaton of Northwestern University.

REFERENCES

1. **Al-Obeid, S., E. Collatz, and L. Gutmann.** 1990. Mechanism of resistance to vancomycin in *Enterococcus faecium* D366 and *Enterococcus faecalis* A256. Antimicrob. Agents Chemother. **34:**252–256.
2. **Al-Obeid, S., L. Gutmann, D. M. Shlaes, R. Williamson, and E. Collatz.** 1990. Comparison of vancomycin-inducible proteins from four strains of enterococci. FEMS Microbiol. Lett. **70:** 101–106.
3. **Bugg, T. D. H., S. Dutka-Malen, M. Arthur, P. Courvalin, and C. T. Walsh.** 1991. Identification of vancomycin resistance protein Van A 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* BM 4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins Van H and Van A. Biochemistry **30:**10408–10415.
5. **Clark, N., B. Hill, J. Swenson, and R. Cooksey.** 1991. Molecular analysis of a cluster of glycopeptide-resistant *Enterococcus faecium*, p. 372, C-183. Abstr. 91st Gen. Meet. Am. Soc. Microbiol. 1991. American Society for Microbiology, Washington, D.C.
6. **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.
7. **Facklam, R. R., and M. D. Collins.** 1989. Identification of *Enterococcus* species isolated from human infections by a conventional test scheme. J. Clin. Microbiol. **27:**731–734.
8. **Fowler, C. L., M. E. Willy, D. E. Koziol, D. K. Henderson, and V. J. Gill.** 1991. Vancomycin-resistant enterococci: prevalence of colonization in oncology patients, p. 425, L-14. Abstr. 91st

Gen. Meet. Am. Soc. Microbiol. 1991. American Society for Microbiology, Washington, D.C.

9. **Gutmann, L., D. Billot-Klein, S. Al-Obeid, I. Klare, S. Francoual, E. Collatz, and J. van Heijenoort.** 1992. Inducible carboxypeptidase activity in vancomycin-resistant enterococci. Antimicrob. Agents Chemother. **36:**77–80.
10. **Hammes, W., K. H. Schleifer, and O. Kandler.** 1973. Mode of action of glycine in the biosynthesis of peptidoglycan. J. Bacteriol. **116:**1029–1053.
11. **Handwerger, S., M. J. Pucci, and A. Kolokathis.** 1990. Vancomycin resistance is encoded on a pheromone response plasmid in *Enterococcus faecium* 228. Antimicrob. Agents Chemother. **34:**358–360.
12. **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.
13. **Kuritza, A., M. Oehler, M. Hayden, G. Trenholme, and A. Harris.** 1991. Vancomycin-resistant *Enterococcus* (VRE) at a large midwestern hospital, p. 20, A-119. Abstr. 91st Gen. Meet. Am. Soc. Microbiol. 1991. American Society for Microbiology, Washington, D.C.
14. **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.
15. **National Committee for Clinical Laboratory Standards.** 1990. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 2nd ed. M7-A2. National Committee for Clinical Laboratory Standards, Villanova, Pa.
16. **Neuhaus, F. C., and W. P. Hammes.** 1987. Inhibition of cell wall biosynthesis by analogues of alanine, p. 45–99. *In* D. J. Tipper (ed.), Antibiotic inhibitors of bacterial cell wall biosynthesis. Encyclopedia of pharmacology and therapeutics, no. 127. Pergamon Press, Inc., Elmsford, N.Y.
17. **Nieto, M., and H. R. Perkins.** 1971. Modification of the acyl-D-alanyl-D-alanine terminus affecting complex formation with vancomycin. Biochem. J. **123:**789–803.
18. **Reynolds, P. E.** 1989. Structure, biochemistry, and mechanisms of action of glycopeptide antibiotics. Eur. J. Clin. Microbiol. Infect. Dis. **8:**943–950.
19. **Sahm, D. F., J. Kissinger, M. S. Gilmore, P. R. Murray, R. Mulder, J. Solliday, and B. Clarke.** 1989. In vitro susceptibility studies of vancomycin-resistant *Enterococcus faecalis*. Antimicrob. Agents Chemother. **33:**1588–1591.
20. **Sahm, D. F., and L. Olsen.** 1990. In vitro detection of enterococcal vancomycin resistance. Antimicrob. Agents Chemother. **34:**1846–1848.
21. **Schleifer, K. H., W. P. Hammes, and O. Kandler.** 1976. Effect of endogenous and exogenous factors on the primary structure of bacterial peptidoglycan. Adv. Microb. Physiol. **13:**245–292.
22. **Shlaes, D. M., A. Bouvet, C. Devine, J. H. Shlaes, S. Al-Obeid, and R. Williamson.** 1989. Inducible, transferable resistance to vancomycin in *Enterococcus faecalis* A256. Antimicrob. Agents Chemother. **33:**198–203.
23. **Tenover, F.** Personal communication.
24. **Trippen, B., W. P. Hammes, K. H. Schleifer, and O. Kandler.** 1976. Die Wirkung von D-Aminosauren auf die Struktur und Biosynthese des Peptidoglycans. Arch. Microbiol. **109:**247–261.
25. **Uttley, A. H. C., C. H. Collins, J. Naidou, and R. C. George.** 1988. Vancomycin-resistant enterococci. Lancet **i:**57–58.
26. **Vincent, S., R. G. Knight, M. Green, D. F. Sahm, and D. M. Shlaes.** 1991. Vancomycin susceptibility and identification of motile enterococci. J. Clin. Microbiol. **29:**2335–2337.