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Heterogeneous Expression of Glycopeptide Resistance in Enterococci Associated with Transfer of *vanB*

MARY K. HAYDEN,[1] ROGER N. PICKEN,[1] AND DANIEL F. SAHM[2]*

Section of Infectious Disease, Department of Medicine, Rush Medical College, Chicago, Illinois 60612,[1] and Department of Pathology and Medicine, Washington University School of Medicine, St. Louis, Missouri 63110[2]

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In mating experiments using a clinical strain that constitutively expresses *vanB*-encoded glycopeptide resistance, resistance transfer was detectable at a frequency of $<10^{-7}$ transconjugants/donor. Vancomycin MICs for transconjugants were 2- to 10-fold lower than those for the donor; both inducibly and constitutively resistant transconjugants were obtained. These findings demonstrate that the transfer of *vanB* among enterococci can be associated with substantial alterations in the level and control of glycopeptide resistance expression.

Enterococci that contain *vanB* can express various levels of resistance to vancomycin and teicoplanin, with a wide range of MICs (7, 14). The *vanB* gene cluster is transferable from some enterococcal strains at a low frequency (2, 6, 14, 16). Transfer has been shown to be mediated by large chromosomal elements (13) or plasmids (2, 16), and transconjugants have usually displayed glycopeptide resistance phenotypes similar to those of the donor strains. In this study, we demonstrate that transfer of the *vanB* gene can be associated with substantial alterations in the expression of glycopeptide resistance by transconjugants.

We used *Enterococcus faecium* JB7, a previously characterized VanB-type clinical strain that constitutively expresses resistance to glycopeptides (7), as the donor in mating experiments. *E. faecium* UC1R and *E. faecium* EFRF1, which carry rifampin and fusidic acid resistance as markers, were used as recipients. Filter matings were carried out according to the method of Jacob and Hobbs, with a donor-to-recipient ratio of 1:1 (8). The antibiotic concentrations for selection of transconjugants were as follows: 8 μg of vancomycin/ml, 50 μg of rifampin/ml, and 50 μg of fusidic acid/ml. Transconjugants were verified if they demonstrated the following characteristics: a vancomycin MIC of ≥8 μg/ml; a *Sma*I-digested total genomic DNA pattern, observed by pulsed-field gel electrophoresis (PFGE), identical or very similar to that of the recipient (9); and the *vanB* gene, detected by PCR (5, 11).

In 20 separate mating experiments, JB7 transferred vancomycin resistance at frequencies ranging from 1×10^{-9} to 8×10^{-8} transconjugants/donor (mean threshold of transfer detection, 4×10^{-9} transconjugants/donor [range, 1×10^{-9} to 5.3×10^{-9} transconjugants/donor]). Two to five transconjugants from each experiment were randomly selected for further evaluation. When measured by recommended antimicrobial dilution testing methods (10), vancomycin MICs for transconjugants were reproducibly 2- to 10-fold lower than those for the donor (Table 1). Heterogeneity in the level of vancomycin resistance was apparent among transconjugants from a single mating experiment and among those from different experiments. Both teicoplanin-resistant and teicoplanin-susceptible transconjugants were seen.

* Corresponding author. Mailing address: Microbiology Laboratory, Barnes-Jewish Hospital, 216 S. Kingshighway, St. Louis, MO 63110. Phone: (314) 454-7074. Fax: (314) 454-5505. E-mail: dansahm@pathology.wustl.edu.

Teicoplanin resistance in VanB strains may indicate either constitutive expression of resistance or induction of resistance by this glycopeptide. In the donor strain, JB7, teicoplanin resistance is constitutively expressed and is associated with constitutive production of a normally inducible 41-kDa membrane protein (7). Therefore, the teicoplanin susceptibility that we observed in some transconjugants suggested inducible expression of glycopeptide resistance. To analyze this possibility, two transconjugants were selected for study: *E. faecium* T14 (vancomycin MIC, 32 μg/ml; teicoplanin MIC, 0.5 μg/ml) and *E. faecium* T15 (vancomycin MIC, 64 μg/ml; teicoplanin MIC, 8 μg/ml). T14 and T15 were products of the same mating experiment (JB7 × EFRF1).

Analysis of membrane proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, performed as described previously (17), revealed constitutive production by T15 and JB7 of the 41-kDa membrane protein (Fig. 1). In contrast, a protein migrating at this position was detected in T14 only after this strain was incubated with an inducing concentration of vancomycin. Growth curve results were consistent with the protein profiles; the growth of T14 in vancomycin-containing broth lagged 90 to 360 min behind the growth of this strain in brain heart infusion (BHI) broth alone, while growth of T15 was not affected by the presence of vancomycin. Incubation of T14 in BHI broth containing a subinhibitory concentration of vancomycin prior to growth curve analysis resulted in suppression of the lag phase of growth. These data confirm that T14 and T15 express inducible and constitutive glycopeptide resistance, respectively. In addition, inducible and constitutive expression of glycopeptide resistance was also seen in transconjugants that resulted from the mating of JB7 and the recipient UC1R, suggesting that heterogeneous expression of glycopeptide resistance is not unique to the JB7-EFRF1 mating pair (Table 1). Of interest, we have not obtained constitutively resistant transconjugants in matings using the donor *E. faecium* JB1, the inducibly resistant isolate from which JB7 is postulated to be derived (7).

To determine whether the differences between T14 and T15 were due to switching between constitutive and inducible resistance expression unrelated to transfer, cultures were inoculated onto BHI agar containing an inducing concentration of vancomycin (4 μg/ml) and incubated overnight at 35°C. Following incubation 1,000 colonies of each strain were replica plated onto BHI agar with and without 4 μg of teicoplanin/ml. Emergence of teicoplanin resistance among the T14 colonies

TABLE 1. Glycopeptide resistance profiles of *E. faecium* JB7 and transconjugants[a]

Mating pair	Donor and recipeint profiles[b]		Transconjugant profile[b]	
	Vancomycin	Teicoplanin	Vancomycin	Teicoplanin
E. faecium JB7	512	32	64	8
×			32	8
E. faecium EFRF1	0.5	0.25	32	1
			32	0.5
E. faecium JB7	512	32	256	8
×			256	4
E. faecium UC1R	1	0.25	256	2
			128	4
			64	4
			64	2
			64	1
			32	16
			32	0.5
			16	2

[a] Representative transconjugants from multiple mating experiments.
[b] Presented as antimicrobial MICs in micrograms per milliliter.

would be a marker for a switch to constitutive resistance expression, while teicoplanin susceptibility among T15 colonies would indicate a switch to inducible resistance expression. However, none of the 1,000 colonies each of T14, T15, and JB7 changed its teicoplanin profile, indicating that both inducible and constitutive expression of VanB resistance in these strains is relatively stable. Therefore, heterogeneity observed among transconjugants of JB7 is likely associated with some aspect of resistance transfer.

Localization of the *vanB* gene in JB7 and transconjugants by PFGE and Southern hybridization, using a PCR-amplified, digoxigenin-11-dUTP-labeled (Boehringer-Mannheim, Indianapolis, Ind.), 600-bp *Eco*RI-*Hin*dIII DNA fragment internal to the *vanB* gene, was performed as previously described (5, 9). In the donor, JB7, and in transconjugants T14 and T15, the *vanB* gene was localized to a single 82-kb *Sma*I digestion fragment (Fig. 2, lanes B″, C″, and D″). These electrophoretic profiles suggest that a genetic element of at least 82 kb was transferred from JB7 and was associated with the inducible transconjugant T14 and the constitutive transconjugant T15.

To investigate if the 82-kb DNA fragment that carried *vanB* represented plasmid or chromosomal DNA, a *vanB* probe and a probe for total JB7 plasmid DNA, prepared as previously described (7, 15), were hybridized to *Sma*I PFGE profiles of total genomic DNA and plasmid DNA from JB7, T14, and T15

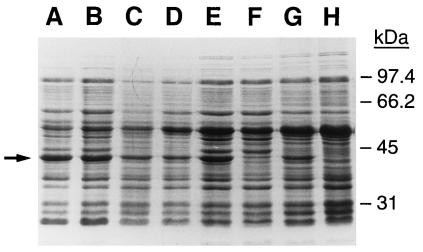

FIG. 1. Analysis of enterococcal membrane preparations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Shown are results for *E. faecium* T15 (lanes A and B), JB7 (constitutive control) (lanes C and D), T14 (lanes E and F), and JB1 (inducible control) (lanes G and H) grown in BHI broth containing 8 μg of vancomycin/ml (lanes A, C, E, and G) or in BHI broth alone (lanes B, D, F, and H). The arrow identifies 41-kDa VanB ligase protein.

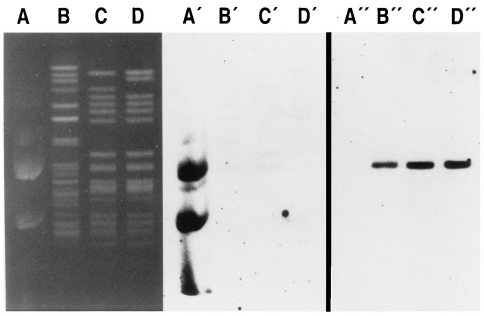

FIG. 2. Confirmation of chromosomal location of *vanB* in *E. faecium* JB7, T14, and T15 by ethidium bromide staining of *Sma*I digests (lanes A through D). Southern hybridization with a digoxigenin-labeled plasmid probe from *E. faecium* JB7 (lanes A′ through D′), and Southern hybridization with a *vanB* probe (lanes A″ through D″). Lanes A, A′, and A″, *E. faecium* JB7 plasmid DNA; lanes B, B′, and B″, *E. faecium* JB7 total DNA; lanes C, C′, and C″, *E. faecium* T14 genomic DNA; lanes D, D′, and D″, *E. faecium* T15 total DNA.

(Fig. 2). Plasmid DNA was detected in the *Sma*I digests of JB7 plasmid DNA but not in the digests of genomic DNA of JB7, T14, or T15 (Fig. 2, lanes A′ through D′). Also, while *vanB* was detected in genomic preparations of JB7, T14, and T15, there was no evidence of *vanB* in the JB7 plasmid preparation (Fig. 2, lanes A″ through D″). These hybridization patterns are consistent with a chromosomal location of *vanB* in JB7 and in the transconjugants T14 and T15, and they provide evidence that resistance transfer occurred from chromosome to chromosome via a genetic element of at least 82 kb. These results are consistent with those of Quintiliani and Courvalin (12, 13), who described transfer of *vanB* on DNA fragments of 90 to 250 kb.

This study demonstrates that transfer of *vanB* can be associated with marked changes in the control of glycopeptide resistance expression. Furthermore, while heterogeneity in *vanA* expression has been associated with transfer of the *vanA* determinant to *Bacillus thuringiensis* (3), the association between resistance transfer among enterococci and heterogeneity in the level of *vanB*-mediated glycopeptide resistance demonstrated in this study has not been described before. Such heterogeneity suggests that the wide variations in vancomycin resistance levels typical among VanB strains isolated in clinical settings may be partially due to alterations that occur because of gene transfer events in nature. While explanations for these phenomena are not yet available, the donor strains and transconjugants obtained provide valuable tools for studies designed to further elucidate the complex mechanisms regulating expression of VanB-type resistance and its transfer.

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