The Role of the Novel Fem Protein VanK in Vancomycin Resistance in *Streptomyces coelicolor* 

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The non-pathogenic, non-glycopeptide-producing actinomycete *Streptomyces coelicolor* carries a cluster of seven genes (*vanSRJKHAX*) that confers inducible, high level resistance to vancomycin. The vanK gene has no counterpart in previously characterized vancomycin resistance clusters, yet vanK is required for vancomycin resistance in *S. coelicolor*. VanK belongs to the Fem family of enzymes, which add the branch amino acid(s) to the stem pentapeptide of peptidoglycan precursors. Upon exposure to vancomycin, the VanRS two-component system switches on expression of all seven *van* genes, and the VanHAX enzymes reprogram the cell wall such that precursors terminate D-Ala-D-lactate (Lac) rather than D-Ala-D-Ala, thus conferring resistance to vancomycin, which only binds D-Ala-D-Ala-containing precursors. Here we provide biochemical and genetic evidence that VanK is required for vancomycin resistance because the constitutively expressed FemX enzyme, encoded elsewhere on the chromosome, cannot recognize D-Lac-containing precursors as a substrate, whereas VanK can. Consistent with this view, D-Lac-containing precursors carrying the Gly branch are present in the wild type transiently exposed to vancomycin but are undetectable in a *vanK* mutant treated in the same way. Further, *femX* null mutants are viable in the presence of vancomycin but die in its absence. Because only VanK can recognize D-Lac-containing precursors, vancomycin-induced expression of VanHAX in a *vanK* mutant is lethal, and so *vanK* is required for vancomycin resistance.

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The spread of vancomycin resistance among pathogenic bacteria is an important public health concern. Ever since vancomycin-resistant strains of pathogenic *Enterococcus faecalis* and *Enterococcus faecium* (VRE) first emerged in the late 1980s, the intergeneric transfer of vancomycin resistance from these strains to methicillin-resistant *Staphylococcus aureus*, a major killer in hospital-acquired infections, has been widely anticipated. This recently became a reality with the first reports of clinical isolates of vancomycin-resistant *Staphylococcus aureus* (VRSA) from hospitals in the United States (1–4).

Vancomycin and other glycopeptide antibiotics inhibit cell wall biosynthesis in Gram-positive bacteria but not in Gram-negative bacteria because they cannot penetrate the outer membrane permeability barrier. They bind the D-alanyl-D-alanine (D-Ala-D-Ala) terminus of lipid-attached peptidoglycan precursors on the outside of the cytoplasmic membrane (5, 6), and this interaction blocks the formation of mature peptidoglycan, principally by denying transpeptidase access to its substrate, thus preventing formation of the peptide cross-links between polysaccharide strands that give the cell wall its structural rigidity. Because of the distinctive mode of action of vancomycin, mutations in transpeptidase cannot give rise to drug resistance. For this reason, it was originally suggested that pathogens might never acquire resistance to vancomycin because it would require them to remodel the peptidoglycan biosynthetic pathway itself. In the late 1980s, however, the first clinical isolates of VRE appeared and were found to reprogram cell wall biosynthesis such that the pendant pentapeptide of peptidoglycan precursors terminated in D-alanyl-D-lactate (D-Ala-D-Lac), rather than in D-Ala-D-Ala (7–12). The affinity of vancomycin for precursors terminating in D-Ala-D-Lac is ~1000-fold lower than for precursors terminating D-Ala-D-Ala (7), rendering the modified bacteria resistant. This remodeling of cell wall precursors requires three enzymes: VanH, which converts pyruvate into D-lactate; VanA, a D-Ala-D-Lac ligase; and VanX, a D-Ala-D-Ala dipeptidase that cleaves any residual circulating D-Ala-D-Ala dipeptide, ensuring that peptidoglycan precursors terminate uniformly in D-Ala-D-Lac.

We have shown previously that the non-pathogenic *Streptomyces coelicolor* carries a gene cluster conferring inducible, high-level resistance to vancomycin (13). *S. coelicolor* is the model species of a genus of Gram-positive, mycelial soil bacteria responsible for the production of two-thirds of the commercially important antibiotics. *S. coelicolor* itself does not make a glycopeptide, but all of the known glycopeptide antibiotics are produced by actinomycetes, the family to which the streptomycetes belong. Because most non-pathogenic actinomycetes live in the soil, it seems likely that *S. coelicolor* encounters glycopeptide producers and that the *van* gene cluster therefore confers a selective advantage. Further, it is widely believed that all glycopeptide resistance genes are ultimately derived from actinomycete glycopeptide producers (14), which must carry these genes to avoid autotoxicity. Consistent with this idea, the *S. coelicolor* resistance genes are clearly associated with a laterally acquired DNA element.

The *S. coelicolor* cluster consists of seven genes, *vanSR-JKHAX* (Fig. 1) (13). VanHAX are orthologous to the genes found in VRE strains. *vanR* and *vanS* encode a two-component signal transduction system that mediates transcriptional ethanesulfonic acid; MS, mass spectrometry; HPLC, high pressure liquid chromatography.

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† The abbreviations used are: VRSA, vancomycin-resistant *S. aureus*; Lac, lactate; TES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethylamino-
duction of the seven van genes in response to extracellular vancomycin. vanJ and vanK are particularly interesting because they are novel genes with no counterpart in previously characterized vancomycin-resistance clusters from pathogens. vanJ, which encodes a predicted membrane protein, is not required for vancomycin resistance, but vanK was found to be essential for vancomycin resistance (13).

VanK is a member of the Fem family of enzymes, which are non-ribosomal peptidyltransferases that add the branch amino acid(s) to the stem pentapeptide of peptidoglycan precursors. For example, S. aureus has a pentaglycine branch and three Fem proteins, FemA, -B, and -X, are involved in its synthesis (15–18). Rohrer et al. (19) showed that femX (also called fmhB) is an essential gene in S. aureus and that controlled deletion of its expression results in the disappearance of glycine-substituted peptidoglycan precursors. In contrast, femA, femB, and femAB mutants are viable; disruption of femB leads to shortening of the staphylococcal branch from five to three glycines, whereas loss of femA or femAB reduces the branch to a single glycine. From these results it was deduced that FemA adds the first glycine, FemA adds the second and third, and FemB adds the fourth and fifth (17, 19–22). This has now been demonstrated in a purified in vitro system, and staphylococcal FemX has been shown to use lipid II exclusively as acceptor for the first glycine (18). Interestingly, in Streptomyces the branch is a single glycine (23–25), yet S. coelicolor has three fem genes: vanK, SCO3904 (here designated femX), and SCO00602 (here designated femY).

The importance of the peptidoglycan remodeling enzymes VanHAX and the VanRS two-component signal transduction system was expected. However, the discovery that VanK was an essential gene in S. coelicolor was surprising given the absence of vanK homologues in the vancomycin-resistance clusters of pathogens. Because VanK is a member of the Fem family of proteins, we proposed two alternative hypotheses to explain why vanK is required for vancomycin resistance (13). One was that S. coelicolor needs to change the nature of the peptidoglycan precursor branch to attain resistance. Changes in the sequence of the branch have been shown to result in increased levels of glycopeptide resistance in Staphylococcus haemolyticus (26). The second hypothesis was that the constitutive FemX activity of S. coelicolor can recognize only precursors that terminate d-Ala-d-Ala as a substrate, and VanK might therefore be required for vancomycin resistance because it is the only enzyme that can add the Gly branch to precursors terminating d-Ala-d-Lac (production of precursors lacking a branch is lethal in many bacteria because it prevents cross-linking of the peptidoglycan by transpeptidase, leading to cell lysis). Here we describe experiments that establish the second explanation.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, Oligonucleotides, and Growth Conditions—Strains, plasmids, and oligonucleotides are listed in Table I. For cell wall precursor analysis, S. coelicolor spores were germinated in heat-shock treatment in 5 ml of TES buffer (0.05 M, pH 8) at 50 °C for 10 min and then diluted with an equal volume of double-strength germination medium (1% (w/v) Difco yeast extract, 1% (w/v) Difco casaminoacids, 0.01 M CaCl2) and incubated with shaking at 37 °C for 2–3 h (27). New minimal medium with phosphate (27) was inoculated with germinated spores, and the cultures were grown to mid-log phase (A600 of 0.3–0.6) at 30 °C. For the preparation of vancomycin-induced peptidoglycan precursors, cultures were grown to mid-log phase in NMMP and then exposed to vancomycin (50 μg/ml) for 3–4 h before harvesting. Other media and culture conditions for bacterial growth were as described previously (13, 28).

Construction of femX, femY, and vanK Mutants—femX (SCO3904) and femY (SCO00602) null mutants, in which the entire coding sequence was replaced with a cassette carrying the apramycin resistance gene (apr) and oriT of RK2, were constructed by PCR targeting of cosmids H24 (femX) and F55 (femY) (29, 30). The appropriate cosmid was introduced into Escherichia coli BW25113 (31) carrying plpJ790 (29), and the target gene was disrupted by electroporation of the cells with the PCR-amplified apr-oriT cassette generated using the primers femX KO I and femX KO II for femX, and femY KO I and femY KO II for femY (Table I). The resulting cosmids (H24ΔfemX::apr and F55ΔfemY::apr) were introduced into E. coli ET12567 carrying pUZ8002 (32) and transferred into S. coelicolor M600 by conjugation, selecting for apramycin resistance. In the case of femY, apramycin-resistant (Apr+) kanamycin-sensitive (Kan+) exconjugants were identified and purified; the disruption was confirmed by PCR and Southern analysis, and a representative femY mutant was designated J3131 (Table I). In the case of femX, despite extensive screening, no Apr+ Kan+ colonies were detected on medium containing apramycin alone, suggesting that disruption of SCO3904 under these conditions was lethal. However, when spores isolated from Apr+ Kan+ colonies were restreaked on medium containing vancomycin in addition to apramycin, Apr+ Kan+ colonies were readily detected, and the successful disruption of femX was confirmed by PCR and Southern blotting. A representative femX mutant was designated J3130 (Table I). The vanK in-frame deletion mutant J3230 was obtained from the ΔvanK::apr strain J3221 by the method of Gust et al. (29).

Constitutive Expression of vanK in the femX Mutant—To drive constitutive expression of vanK, the gene was cloned under the control of
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**RESULTS**

The Stem Peptide Branch Does Not Change in Response to Vancomycin.—To test the effect of vancomycin on the stem peptide branch, peptidoglycan was isolated from *S. coelicolor* grown in the presence or absence of vancomycin, and the 25 most abundant muropeptides derived from each of these two samples were separated by HPLC and characterized by mass spectrometry. The branch associated with each muropeptide was a single glycine in all cases, both before and after exposure to vancomycin. Therefore VanK does not change the stem peptide branch.

Precursor Analysis Suggests that VanK Is Required to Add the Gly Branch to Stem Peptides Terminating \(\alpha\)-Ala-\(\alpha\)-Lac—Because the \(\Delta vanK\) mutant is sensitive to vancomycin, it was not possible to examine the effect of vancomycin induction on its cell wall structure. However, because cytoplasmic cell wall precursor pools change rapidly in response to vancomycin, it was possible to examine the effect of vancomycin induction on these precursor pools in the \(\Delta vanK\) mutant.

Wild-type *S. coelicolor* and the congenic \(\Delta vanK\) mutant were grown to mid-log phase in NMMP medium (27). For the preparation of vancomycin-induced peptidoglycan precursors, cultures were then exposed to vancomycin (50 \(\mu\)g/ml) for 3–4 h before harvesting. Peptidoglycan precursors were extracted, separated by HPLC, and analyzed by mass spectrometry (Fig. 2). The structures of the four relevant molecules are given in Fig. 3. As expected, in the absence of vancomycin, when the \(van\) genes were not expressed, no significant differences were seen between the wild-type and the \(\Delta vanK\) mutant (Fig. 2A). Under the conditions used, precursors with and without the glycine branch co-eluted. Thus, peak 1, with a retention time of 13.5 min, contained two molecules: a 1194-Da species that matched the predicted mass of Molecule I, and a second species of 1251 Da that matched the predicted mass of Molecule III (Figs. 2 and 3).
3). The identity of Molecule I was confirmed by its MS/MS fragmentation pattern (Fig. 4).

Exposure to vancomycin changed the precursor profiles of both the wild-type and the ΔvanK mutant. Peak 1 decreased in abundance, and a new peak (peak 2) appeared with a retention time of 21 min (Fig. 2B). In both the wild-type and the ΔvanK mutant, peak 2 contained a 1195-Da species corresponding to Molecules I, II, III, and IV (Fig. 3). In the wild type, peak 2 also contained a second species of 1252 Da, corresponding to Molecule IV (Figs. 2 and 3). Importantly, however, Molecule IV, the D-Lac-containing precursor with the Gly branch, was not detectable in the vancomycin-treated ΔvanK mutant (Fig. 2B).

The identity of Molecule II was confirmed by its MS/MS fragmentation pattern (Fig. 4).

A femX Mutant Is Vancomycin-dependent—Given that Molecule IV (Fig. 3) was present in the wild type exposed to vancomycin but was undetectable in the ΔvanK mutant treated in the same way, we reasoned that the role of VanK might be to add the Gly branch to D-Lac-containing precursors and that the constitutively expressed FemX enzyme probably cannot recognize these precursors as a substrate. Because the Gly branch is likely to be essential for peptidoglycan cross-linking, and hence cell viability, VanK would therefore be required for vancomycin resistance. If this hypothesis is true, it follows that femX, an essential gene in *S. aureus* (19), would instead be conditionally essential in *S. coelicolor*. In other words, if VanK substitutes for FemX when the cell accumulates D-Ala-D-Lac-containing precursors, FemX would be redundant when expression of the van genes is activated by vancomycin (Fig. 5A). To test this hypothesis, we attempted to disrupt femX (SCO3904) in the presence and absence of vancomycin, using the PCR-directed approach of Gust et al. (29). In the first step of this method, *S. coelicolor* genes carried on cosmids in *E. coli* are replaced with a selectable marker generated by PCR. For our purposes, this method had the advantage that it permits the ready analysis of essential genes because the initial gene disruption is performed in *E. coli*, rather than in *S. coelicolor*.

A cosmid carrying a ΔfemX:apr gene replacement in the insert and a kanamycin resistance (KanR) gene in the vector was introduced into *S. coelicolor* by conjugation, selecting for apramycin resistance (AprR). All the AprR primary exconjugants were KanR, and despite extensive screening, no AprR
Kan^S^ isolates were detected on subculture, implying that femX might be essential under these conditions. However, when vancomycin was added to the plates, Apr^R Kan^S^ colonies (double crossovers) were readily isolated, and these were confirmed as femX mutants by PCR and Southern blot analyses. Significantly, when the femX mutants were plated back on medium lacking vancomycin, they failed to grow, confirming that femX is essential in the absence of vancomycin but dispensable in its presence (Fig. 5B).

**DISCUSSION**

Our interpretation of the data given in this paper is as follows. FemX adds the single branch glycine to the stem peptide of S. coelicolor cell wall precursors terminating d-Ala-d-Ala (Fig. 7). Because transpeptidation of this branch is essential for mature cell wall formation, FemX is essential under “normal” conditions. However, induction of the vanHAX genes remodels cell wall precursors such that the stem peptidoglycan precursors, separated them by HPLC, and analyzed them by mass spectrometry (Fig. 6B). As expected, a peak with a retention time of 13.5 min (equivalent to peak 1 in Fig. 2) was observed, containing Molecule I and Molecule II (Figs. 3 and 6B), showing that VanK can add the glycine branch to d-Ala-d-Ala-containing precursors.

**FIG. 4. Characterization by electrospray ionization/MS/MS fragmentation of (A) Molecule I and (B) Molecule II.** Each molecule was trapped in a DecaXP^Plus^ ion trap with an isolation width of 4.0 atomic mass units and fragmented at 40% collision energy to produce an MS^2^ spectrum. The predominant products, with a loss of 404 atomic mass units, were trapped and fragmented further under the same conditions to yield MS^3^ spectra. The probable identity of fragments found is shown below; as expected, most are breakages of peptide bonds. Note that breakage of the d-Ala-d-Ala peptide bond in Molecule I is observed but that breakage of the d-Ala-d-Lac ester bond in Molecule II is not detected. The masses of the observed fragment ions are 1 Da greater than those of the neutral structures because of the addition of H^+. 

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staphylococci may lie somewhere between the two extremes represented by the enterococcal and the streptomycete enzymes. When VRSA strains were grown in the absence of vancomycin, they made the typical pentaglycine branch characteristic of staphylococci (35). However, muropeptides isolated from the same strains grown in the presence of vancomycin frequently had shorter branches or lacked a branch altogether (35). These results strongly suggest that D-Lac-containing precursors are poor substrates for FemX, FemA, and FemB, the three enzymes that build the pentaglycine branch in S. aureus, but not so poor as to block the synthesis of a branch altogether (35). Thus, enterococci, staphylococci, and streptomycetes seem to present a spectrum of FemX substrate specificities in which enterococcal FemX can recognize D-Lac-containing precursors efficiently, streptomycete FemX cannot (necessitating the presence of vanK under VanRS control), with the staphylococcal enzyme lying somewhere in between, able to contribute to the production of a viable cell wall, but one that is significantly defective in comparison to the wall produced in the absence of vancomycin. The VRSA isolates arose from intergeneric transfer of the vancomycin-resistance transposon Tn1546 from E. faecalis (3, 4).

There is circumstantial evidence that the need to cope with D-Lac-containing substrates may present problems for members of other genera in addition to Streptomyces and Staphylococcus. Actinoplanes teicomyceticus, an actinomycete relative of S. coelicolor, produces the glycopeptide teicoplanin. The chromosome of A. teicomyceticus encodes (at least) two MurF proteins, one of them (MurF2) associated with the teicoplanin resistance genes and a second (MurF1) encoded elsewhere on Vancomycin Resistance in S. coelicolor. VanK adds the single branch glycine to the stem pentapeptide of S. coelicolor cell wall precursors terminating d-Ala-d-Ala (Fig. 7). Because transpeptidation of this branch is essential for cell wall formation, femX is an essential gene under normal conditions. However, the van cluster encodes a FemX homologue, VanK, which can recognize precursors terminating d-Ala-d-Lac as a substrate (Fig. 7). As a consequence, femX is non-essential provided that the van genes are expressed. Therefore, femX null mutants are viable in the presence of vancomycin but die in its absence. B, vancomycin-dependent growth of the ΔfemX mutant. Wild type (M600) and ΔvanR and ΔfemX mutant derivatives of M600 were streaked on soy flour mannitol agar in the absence (left) or presence (right) of vancomycin (10 μg/ml). Plates were photographed after incubation at 30 °C for 4 days.

FIG. 5. A, rationale for the attempted disruption of femX. femX is constitutively expressed, but vanK expression is induced by vancomycin. FemX adds the single branch glycine to the stem pentapeptide of S. coelicolor cell wall precursors terminating d-Ala-d-Ala (Fig. 7). Because transpeptidation of this branch is essential for cell wall formation, femX is an essential gene under normal conditions. However, the van cluster encodes a FemX homologue, VanK, which can recognize precursors terminating d-Ala-d-Lac as a substrate (Fig. 7). As a consequence, femX is non-essential provided that the van genes are expressed. Therefore, femX null mutants are viable in the presence of vancomycin but die in its absence. B, vancomycin-dependent growth of the ΔfemX mutant. Wild type (M600) and ΔvanR and ΔfemX mutant derivatives of M600 were streaked on soy flour mannitol agar in the absence (left) or presence (right) of vancomycin (10 μg/ml). Plates were photographed after incubation at 30 °C for 4 days.

S. coelicolor, produces the glycopeptide teicoplanin. The chromosome of A. teicomyceticus encodes (at least) two MurF proteins, one of them (MurF2) associated with the teicoplanin resistance genes and a second (MurF1) encoded elsewhere on
the chromosome (39). MurF catalyzes condensation of the dipeptide d-Ala-d-Ala with UDP-MurNAc-tripeptide to form the UDP-MurNAc-pentapeptide (40). By analogy with FemX/VanK in S. coelicolor, one possible explanation for the presence of murF2 in the teicoplanin resistance gene cluster is that MurF1 cannot efficiently recognize d-Ala-d-Lac as a substrate. Unfortunately, A. teicomyceticus cannot be disrupted to see if it is required for teicoplanin resistance (39).

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