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Novel Vancomycin Resistance System in *Streptomyces coelicolor*

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Abstract

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 *van* genes are organised into four transcription units, *vanRS*, *vanJ*, *vanK* and *vanHAX*, and these transcripts are induced by vancomycin in a *vanR*-dependent manner. *vanHAX* are orthologuous to genes found in vancomycin resistant enterococci that encode enzymes predicted to reprogramme peptidoglycan biosynthesis such that cell wall precursors terminate in D-Ala-D-Lac, rather than D-Ala-D-Ala. *vanR* and *vanS* encode a two-component signal transduction system that mediates transcriptional induction of the seven *van* genes. *vanJ* and *vanK* are novel genes that have no counterpart in previously characterised vancomycin-resistance clusters from pathogens. VanK is essential for vancomycin resistance in *S. coelicolor* and it is required for adding Gly branch to stem peptides terminating D-Ala-D-Lac. Because VanK can recognise D-Lac-containing precursors but the constitutively expressed FemX enzyme, encoded elsewhere on the chromosome, cannot recognize D-Lac-containing precursors as a substrate, vancomycin-induced expression of VanHAX in a *vanK* mutant is lethal. Further, *femX* null mutants are viable in the presence of glycopeptide antibiotics but die in their absence. Bioassay using *vanJp-neo* fusion reporter system also showed that all identified inducers for *van* genes expression were glycopeptide antibiotics, but teicoplanin, a membrane-anchored glycopeptide, failed to act as an inducer.

Introduction

The spread of vancomycin resistance among pathogenic bacteria is an important public health concern. Since vancomycin-resistant strains of pathogenic *Enterococcus faecalis* and *Enterococcus faecium* (VRE) first emerged in the late 1980s, the inter-generic transfer of vancomycin resistance from these strains to methicillin-resistant *Staphylococcus aureus* (MRSA), 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 USA (1-2). 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-Ala-D-Ala terminus of lipid-attached peptidoglycan precursors and this interaction blocks the formation of mature peptidoglycan, preventing formation of the peptide crosslinks between polysaccharide strands that give the cell wall its structural rigidity (3). In the late 1980s, however, the first clinical isolates of VRE appeared and were found to reprogramme cell wall biosynthesis such that the pendant pentapeptide of peptidoglycan precursors terminated in D-Ala-D-Lac, rather than in D-Ala-D-Ala. The affinity of vancomycin for precursors terminating in D-Ala-D-Lac is ~1000-fold lower than for precursors terminating D-Ala-D-Ala, rendering the modified bacterial resistance. This remodelling 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 (4). Here, we show the characterization of novel vancomycin system in the non-pathogen, non-glydopeptide-producing actinomycete *Streptomyces coelicolor*.

Results and Discussion

Transcription analysis of *vanSRJKHAX*

Using high-resolution S1 nuclease protection analysis, we identified four separate promoters in front of the *vanR*, *vanJ*, *vanK* and *vanH* and these transcripts are induced by vancomycin in a *vanR*-dependent manner. Unusually, the *vanR*, *vanK* and *vanH* transcripts each start at the 'A' of the AUG translation initiation codon, and these mRNAs must therefore be translated in the absence of a conventional 5' mRNA leader and ribosome-binding site. Leaderless messages are rare in most bacteria and the biological significance of leaderless messages is not understood, although a disproportionately large number of leaderless mRNAs have been identified in *Streptomyces*, often encoding antibiotic resistance. Expression of the seven *van* genes is regulated at the level of transcription by the VanR/VanS two-component signal transduction system. The *vanRS* transcript is undetectable in the absence of vancomycin induction (5). Given that transcription of the *van* genes is completely dependent on *vanR*, it presumably follows that the *vanRS* operon must be transcribed at some level (albeit too low to be detected) in the absence of vancomycin such that VanR and VanS are present to mediate induction. Alternatively, in the total absence of VanR and VanS, perhaps crosstalk from another two-component system could prime initial expression of *vanRS*.

Characterization of the novel Fem protein, VanK

vanJ and *vanK* are novel genes that have no counterpart in previously characterized vancomycin resistance clusters from pathogens. The *vanJ* mutant showed slightly increased sensitivity to vancomycin but had no significant phenotype. However, VanK was essential for vancomycin resistance in *S. coelicolor*. Because VanK belongs to the Fem family of enzymes, which add the branch amino acids(s) to the stem pentapeptide of peptidoglycan precursors, we proposed two alternative hypotheses to explain why *vanK* is required for vancomycin resistance. One was that *S. coelicolor* needs to change the nature of the peptidoglycan precursor branch to attain resistance. 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. Muropeptides analysis showed that VanK does not change the stem peptide branch in response to vancomycin. However peptidoglycan precursors analysis showed that Gly attached D-Lac-containing precursors was only present in the wild type exposed to vancomycin but was undetectable in the *vanK* mutant treated in the same say, suggesting that VanK is required to add the Gly branch to stem peptide terminating D-Ala-D-Lac. Because the constitutively expressed FemX enzymes, encoded elsewhere on the chromosome, cannot recognize D-Lac-containing precursors as a substrate, *femX* null mutants are only viable in the presence of vancomycin (or other glycopeptides except teicoplanin) which induces the expression of *vanK*. Consistent with this result, vancomycin-induced expression of VanHAX in a *vanK* mutant is lethal, and so *vanK* is required for vancomycin resistance (6).

The *femX* mutant creates a novel bioassay for inducers of expression of *van* genes

We previously established a bioassay for inducers of the *S. coelicolor* VanRS signal transduction system by making a multicopy construct in which the *vanJ* promoter drove expression of the *neo* gene, which confers resistance to neomycin and kanamycin (5). Using this bioassay, we showed that a variety of glycopeptides induced *van* genes expression. In constructing the drug-dependet *femX* null mutant, we created an optimal bioassay for inducers in *S. coelicolor*: this strain is only viable in the presence of compounds that activate the VanRS signal transduction system, the readout is simple growth, and there are no multicopy plasmids or reporter genes involved. As we previously found using the *vanJp-neo* bioassay, structurally closely related glytopeptide antibiotics including vancomycin were strong inducers. Strikingly, the glycopeptide teicoplanin did not act as an inducer of the *van* signal transduction system, and further investigation showed that *S. coelicolor* is sensitive to this

antibiotic. Expression of the *van* genes confers resistance to the glycopeptide teicoplanin, but *S. coelicolor* is sensitive to teicoplanin in isolation because it is not an inducer of the VanRS signal transduction system.

Conclusion

We have identified and characterized a novel vancomycin resistance cluster in the non-pathogenic, non-glycopeptide-producing actinomycete S. coelicolor. Expression of the seven van genes is regulated at the level of transcription by the VanR/VanS two-component signal transduction system. Induction of the vanHAX genes remodels cell wall precursors such that the stem pentapeptide terminates D-Ala-D-Lac instead of D-Ala-D-Ala, and FemX cannot recognize D-Lac-containing precursors. The constitutively expressed FemX adds the single branch glycine to the stem pentapeptide of cell wall precursors terminating D-Ala-D-Ala and is essential under normal conditions. Instead, the FemX homologue, VanK, recognizes D-Lac-containing precursors, therefore, femX gene is non-essential produced the *van* genes are expressed. Because only VanK can recognize D-Lac-containing precursors, expression of VanHAX in a vanK mutant is lethal, and so vanK is required for vancomycin resistance. We also reported that the inducers of *van* genes expression were all structurally closely related glycopeptide antibiotics, except teicoplanin. Teicoplanin is important as the only glycopeptie antibiotic apart from vancomycin in current clinical use. The most conspicuous difference between teicoplanin and the other glycopeptides is a long fatty acid chain attached to the vancosamine sugar of teicoplanin. This hydrophobic moiety serves to anchor teicoplanin in the membrane, and it is possible that membrane anchoring prevents teicoplanin from interacting with the VanS sensor domain.

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