The vancomycin resistance VanRS two-component signal transduction system of *Streptomyces coelicolor*

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Summary

We took advantage of the vancomycin-dependent phenotype of *Streptomyces coelicolor* femX null mutants to isolate a collection of spontaneous, drug-independent femX suppressor mutants that expressed the vancomycin-resistance (van) genes constitutively. All of the suppressor mutations were in vanS but, unexpectedly, many were predicted to be loss-of-function mutations. Confirming this interpretation, a constructed vanS deletion mutation also resulted in constitutive expression of the van genes, suggesting that VanS negatively regulated VanR function in the absence of drug. In contrast, a vanS pta ackA triple mutant, which should not be synthesiz size acetyl phosphate, failed to express the van genes, whereas a pta ackA double mutant showed wild-type, regulated induction of the van genes. These results suggest that in the absence of vancomycin, acetyl phosphate phosphorlylates VanR, and VanS acts as a phosphatase to suppress the levels of VanR-P. On exposure to vancomycin, VanS activity switches from a phosphatase to a kinase and vancomycin resistance is induced. In *S. coelicolor*, the van genes are induced by both vancomycin and the glycopeptide A47934, whereas in *Streptomyces toyocaensis* (the A47934 producer) resistance is induced by A47934 but not by vancomycin. We exploited this distinction to replace the *S. coelicolor* vanRS genes with the vanRS genes from *S. toyocaensis*. The resulting strain acquired the inducer profile of *S. toyocaensis*, providing circumstantial evidence that the VanS effector ligand is the drug itself, and not an intermediate in cell wall biosynthesis that accumulates as result of drug action. Consistent with this suggestion, we

found that non-glycopeptide inhibitors of the late steps in cell wall biosynthesis such as monomycin A, bacitracin and ramoplanin were not inducers of the *S. coelicolor* VanRS system, in contrast to results obtained in enterococcal VanRS systems.

Introduction

Vancomycin is clinically important for treating enterococcal infections arising after abdominal surgery and is vital as the only widely effective treatment for infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA), a major killer in hospital-acquired infections. Vancomycin and other glycopeptide antibiotics inhibit cell wall biosynthesis by binding to the D-alanyl-D-alanine (D-Ala-D-Ala) terminus of lipid-attached peptidoglycan precursors on the outside of the cytoplasmic membrane (Williams et al., 1983; Barna and Williams, 1984). This interaction blocks formation of mature peptidoglycan, principally by denying transpeptidase access to its substrate, thereby preventing formation of the peptide crosslinks between polysaccharide strands that give the cell wall its rigidity. The first clinical isolates of vancomycin-resistant strains of pathogenic *Enterococcus faecalis* and *Enterococcus faecium* (VRE) appeared in the late 1980s, and were shown to reprogramme cell wall biosynthesis such that the stem pentapeptide of peptidoglycan precursors terminated in D-alanyl-D-lactate (D-Ala-D-Lac), rather than in D-Ala-D-Ala (Bugg et al., 1991; Walsh et al., 1996; Healy et al., 2000; Pootoolal et al., 2002a). The affinity of vancomycin for precursors terminating in D-Ala-D-Lac is ∼1000-fold lower than for precursors terminating D-Ala-D-Ala (Bugg et al., 1991), rendering the modified bacteria resistant. 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. Because vancomycin is the front-line therapy for treating problematic infections caused by MRSA, the spread of vancomycin resistance through bacterial populations is an acute public health issue, highlighted by the recent emergence of vancomycin-resistant, methicillin-resistant *Staphylococcus aureus* (VRSA) in hospitals (Pearson, 2002; Chang et al., 2003; Weigel et al., 2003; Tenover et al., 2004).
We previously showed that the non-pathogen, *Streptomyces coelicolor*, carries a gene cluster conferring inducible, high-level resistance to vancomycin (Hong et al., 2004). *S. coelicolor* is the model species of a genus of Gram-positive, mycelial soil bacteria responsible for 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 bacterial order to which the streptomycetes belong. As 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. The *S. coelicolor* resistance genes are clearly associated with a laterally acquired DNA element (H.-J. Hong, unpublished); it therefore seems likely that *S. coelicolor* ultimately acquired the *van* genes from an actinomycete glycopeptide producer, which must carry these genes to avoid auto-toxicity.

The *S. coelicolor* cluster consists of seven genes, *vanSRJKHAX*, divided into four transcription units: *vanRS*, *vanJ*, *vanK* and *vanHAX* (Fig. 1; Hong et al., 2004). The *vanRS* two-component signal transduction system, the principal focus of the work reported here, activates transcription from the four *van* promoters in response to extracellular vancomycin. *VanJ* is a membrane protein of unknown function, and *VanHAX*, which are orthologous to the VRE enzymes, alter the cell wall precursors to terminate D-Ala-D-Lac (Hong et al., 2004; 2005).

*VanK* is a particularly interesting gene because it is essential for vancomycin resistance in *S. coelicolor*, despite the fact that it has no orthologues in the vancomycin-resistance gene clusters of pathogenic enterococci (Hong et al., 2004). *VanK* is a member of the *Fem* family of enzymes, which add the branch amino acid(s) to the stem pentapeptide of PG precursors. In *S. coelicolor*, the branch is a single glycine residue and, in the absence of vancomycin, this glycine is added by an enzyme called FemX (Hong et al., 2005). However, the constitutive FemX activity of *S. coelicolor* can recognize only precursors that terminate in D-Ala-D-Ala as a substrate, and *VanK* is therefore required for vancomycin resistance because it is the only enzyme that can add the Gly branch to precursors terminating in D-Ala-D-Lac (production of precursors lacking the Gly branch is lethal in *Streptomyces* because it prevents cross-linking of the peptidoglycan by transpeptidase, leading to cell lysis) (Hong et al., 2005).

FemX is essential under normal growth conditions (Hong et al., 2005). However, because *VanK* replaces the function of FemX during vancomycin-induced cell wall precursor remodelling, *femX* null mutants are viable in the presence of vancomycin, but die in its absence, when *VanK* is not expressed (Hong et al., 2005). We took advantage of this drug-dependent phenotype to isolate a collection of spontaneous, drug-independent *femX* suppressor mutants that expressed the vancomycin-resistance genes constitutively, all of which mapped to the *vanS* sensor kinase gene. Here we report the resulting genetic and biochemical dissection of the *vanRS* two-component signal transduction system.

### Results

**Suppressors of *femX* map to *vanS***

When the vancomycin-dependent *femX* null mutant strain of *S. coelicolor* was plated for confluent lawns on medium lacking vancomycin, it gave rise to spontaneous suppressor mutants that grew in the absence of the antibiotic (Fig. 2). Using S1 nuclease protection analysis we found that, in two suppressor mutants chosen at random, the *vanH* promoter was transcribed in the absence of vancomycin, and that transcription was not induced to higher levels by addition of the drug, contrasting with the inducible transcription of the *van* genes seen in the wild type (Fig. 3). Based on a classical model of two-component systems (Hakenbeck and Stock, 1996), we expected these suppressor mutants to carry gain-of-function mutations in either *vanR* or *vanS*, causing constitutive activity of the signal transduction system. Accordingly, we isolated 23 independent spontaneous suppressor mutants and from each sequenced the *vanRS* operon plus 100 bp of flanking DNA on either side. In every case, the *vanR* sequence was wild type but a mutation was found in *vanS* (Table 1). Significantly, most of the sequenced changes

![Fig. 1. Organization and regulation of the vancomycin resistance (*van*) gene cluster of *S. coelicolor*. The *van* genes are organized into four transcription units, *vanRS*, *vanJ*, *vanK* and *vanHAX*, and these transcripts are induced by vancomycin in a *vanR*-dependent manner (Hong et al., 2004). The SCO designations for the *van* genes in the genome annotation are *vanS* (SCO3589), *vanR* (SCO3590), *vanJ* (SCO3592), *vanK* (SCO3593), *vanH* (SCO3594), *vanA* (SCO3595) and *vanX* (SCO3596).](image-url)
were not compatible with gain-of-function mutations. For example, two suppressors (J3135 and J3137) had IS element insertions in vanS, and many contained frame-shift alleles that could not conceivably give rise to a functional VanS protein (Table 1).

The van genes are transcribed constitutively in a vanS null mutant

Sequence analysis suggested that most (and therefore conceivably all) of the 23 characterized spontaneous mutations were not compatible with gain-of-function mutations. For example, two suppressors (J3135 and J3137) had IS element insertions in vanS, and many contained frame-shift alleles that could not conceivably give rise to a functional VanS protein (Table 1).
femX suppressor mutations in vanS were loss-of-function mutations. To confirm and extend this interpretation, we constructed vanS and vanRS deletion mutants. While the vanRS mutant failed to grow on 10 µg ml⁻¹ vancomycin, the minimum inhibitory concentration (MIC) for the vanS mutant was 160 µg ml⁻¹ (Figs 2 and 3). Furthermore, in the vanS null mutant the vanH promoter was transcribed in the absence of vancomycin and transcription was not induced to higher levels by addition of the drug (Fig. 3). Thus, while in the wild-type expression of the van genes was inducible, it is constitutive in the vanS null mutant. The level of constitutive van gene transcription was higher in the two △femX suppressor strains examined than in the △vanS mutant (Fig. 3); the molecular basis of this observation is currently unclear.

A vanR D51A allele is inactive

As an explanation for the constitutive expression of the van genes in a vanS null mutant, we first considered the possibility that unphosphorylated VanR might be the active form of the protein. To test this hypothesis, aspartate 51, the predicted site of phosphorylation in VanR, was changed to alanine. The D51A and wild-type vanR alleles were used to generate two otherwise identical constructs, based on the integrative vector pMS82 (Gregory et al., 2003), and each was introduced into the vanRS double mutant. The vancomycin-sensitive phenotype of the vanRS deletion strain was complemented in trans by wild-type vanR but not by the D51A allele (Fig. 2), suggesting that phosphorylation of D51 is required for van gene activation.

Construction of a vanS pta ackA triple mutant suggests that VanR can be activated by acetyl phosphate in vivo

An alternative explanation for constitutive expression of the van genes in a vanS null mutant would be that VanR–P can be generated in a VanS-independent manner and that VanS acts as a VanR–P phosphatase in the absence of vancomycin. VanS-independent synthesis of VanR–P might arise through crosstalk with one of the other 83 sensor-kinases in S. coelicolor (Hutchings et al., 2004). Alternatively, VanR could be activated by a small molecule phosphodonor such as acetyl phosphate. Acetyl phosphate is routinely used to phosphorylate response regulators in vitro in the absence of their cognate sensor kinases (Hakenbeck and Stock, 1996) and, in Escherichia coli at least, there is genetic evidence that acetyl phosphate can act as a phosphodonor in vivo, for example to the response regulator RssB, the σ⁵ regulator (Bouché et al., 1998). Synthesis and degradation of acetyl phosphate is controlled by acetate kinase (Ack) and phosphotransacetylase (Pta); in E. coli, an ackA mutant over-produces acetyl phosphate, a pta mutant has very low levels of acetyl phosphate, and an ackA pta double mutant cannot produce acetyl phosphate from acetate or acetyl CoA (Pruss and Wolfe, 1994). To see if acetyl phosphate might be responsible for generating VanR–P in a vanS null mutant, we introduced a further lesion into the vanS null mutant background, removing the pta and ackA genes, which are adjacent to each other in S. coelicolor. In contrast to the vanS mutant that grew readily on vancomycin, the vanS pta ackA triple mutant was vancomycin-sensitive (Fig. 2) and transcription from the vanH promoter was virtually undetectable in the presence or absence of vancomycin (Fig. 3), suggesting that acetyl phosphate can act as a phosphodonor to VanR in vivo.

VanS is both a kinase and phosphatase

The data given above were consistent with VanS acting as a VanR phosphatase in the absence of vancomycin, but they did not show whether exposure to vancomycin converted VanS into an active VanR kinase, or simply turned off its phosphatase activity. We addressed this question genetically and biochemically. First, we constructed a pta ackA double mutant in a vanS background and found that this strain was vancomycin resistant (Fig. 2) and that transcription of the van genes was induced in response to vancomycin (Fig. 3), suggesting
that VanS can generate VanR-P in vivo. Second, we over-expressed VanR and the cytosolic domain of VanS (c-VanS, corresponding to residues 85–364) in E. coli and purified them to homogeneity as his-tagged proteins. Incubation of c-VanS with [γ-32P]ATP resulted in auto-phosphorylation, with maximum phosphorylation occurring after 60 min (Fig. 4A). When radiolabelled c-VanS was mixed with VanR in the presence of ATP, rapid phototransfer was observed (Fig. 4B, lane 2), confirming that c-VanS has kinase activity. Purified VanR protein could also be phosphorylated using radiolabelled acetyl phosphate (Fig. 4B, lane 3). Incubation of VanR-P generated in this way with c-VanS resulted in removal of the phosphate group from VanR, confirming that c-VanS has phosphatase activity. This dephosphorylation is unlikely to involve reverse phosphotransfer to VanS because no radiolabelled c-VanS was detected in this reaction.

The femX mutant provides a novel bioassay for inducers of VanS

In enterococci, the nature of the effector ligand that binds the sensor domain of VanS to activate the signal transduction pathway is controversial. Although glycopeptides activate the enterococcal van genes, several structurally unrelated compounds that inhibit late steps in cell wall biosynthesis, such as moenomycin A, bacitracin and ramoplanin, have also been reported to induce van gene expression (Handwerger and Kolokathis, 1990; Allen and Hobbs, 1995; Baptista et al., 1996; 1999; Lai and Kirsch, 1996; Ulijasz et al., 1996; Grissom-Arnold et al., 1997;

The origin of the VanRS signal transduction system, not the host, determines inducer specificity

Streptomyces toyocaensis produces the glycopeptide antibiotic A47934, and the sequence of the entire A47934 biosynthetic gene cluster has been determined (Pootoolal et al., 2002b). Associated with the cluster are homologues of the S. coelicolor vanSRJKHAX genes. In S. coelicolor, the van genes are induced by both A47934 and vancomycin (Fig. 5; Hong et al., 2004). In contrast, in S. toyocaensis, resistance is induced by A47934 but not by vancomycin (Neu and Wright, 2001), perhaps implying that the ligands recognized by the VanS proteins from these two Streptomyces species are different. To test this hypothesis, we swapped the signal transduction systems of the two species by introducing the S. toyocaensis vanRS genes (vanRSst) into the S. coelicolor vanRS dele-
tion mutant, using the integrative vector pRT801 (Gregory et al., 2003). Significantly, the resulting strain was resistant to A47934, but sensitive to vancomycin (Fig. 6A). Consistent with this result, vancomycin did not induce transcription from the vanH promoter in this strain, but A47934 did (Fig. 6B). Thus, switching the VanRS signal transduction system from one species to the other also switched inducer specificity.

Discussion

Taken together, our data suggest the following model for the regulation of the VanRS signal transduction system in S. coelicolor (Fig. 7). In the absence of vancomycin, acetyl phosphate phosphorolyses D51 of VanR, and VanS acts as a phosphatase to suppress the levels of VanR\(^{\sim}P\). In the presence of vancomycin, VanS is converted from a phosphatase to a kinase, leading to accumulation of VanR-P and activation of the four promoters of the van gene cluster. Transcription of the vanRS operon is itself under VanRS control (Hong et al., 2004) and so there will be very little VanR or VanS protein in S. coelicolor growing constantly in the absence of vancomycin. Thus, the ‘futile cycle’ of VanR phosphorylation and dephosphorylation shown occurring in the absence of vancomycin in Fig. 7 will occur at a high level only after the organism has been transiently exposed to the antibiotic. The duration of high level futile cycling will therefore depend on the half-lives of VanR and VanS in vivo.

An unresolved question is how much, if at all, acetyl phosphate contributes to VanR phosphorylation when the kinase activity of VanS is turned on in the presence of vancomycin. Certainly an ackA pta vanS\(^{+}\) strain is resistant to vancomycin (Fig. 2) and still shows strong induction of the van genes (Fig. 3). If acetyl phosphate does make a significant contribution and thereby influences the organism’s ability to respond to challenge by the antibiotic, then further questions arise: how widely do acetyl phosphate levels fluctuate in S. coelicolor, and what factors influence that fluctuation. It will be interesting to discover how many of the other 79 response-regulators in S. coelicolor can be phosphorylated by acetyl phosphate in vivo (Hutchings et al., 2004).

<table>
<thead>
<tr>
<th>Ristocetin (30μg)</th>
<th>Teicoplanin (30μg)</th>
<th>Vancomycin (30μg)</th>
<th>Chloroeremomycin (30μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A47934 (30μg)</td>
<td>Mencymycin A (30μg)</td>
<td>Bacinacin (5U)</td>
<td>Ramoplanin (30μg)</td>
</tr>
<tr>
<td>Tunicamycin (30μg)</td>
<td>Enduracin (30μg)</td>
<td>Polymycin (30μg)</td>
<td>D-cycloserine (30μg)</td>
</tr>
<tr>
<td>Phosphomycin (30μg)</td>
<td>Methicillin (10μg)</td>
<td>Duramycin (100μg)</td>
<td>Kanamycin (20μg)</td>
</tr>
</tbody>
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Fig. 5. Use of the vancomycin-dependent femX null mutant (J3130) as a bioassay for inducers of VanS. Approximately 10\(^7\) spores of S. coelicolor J3130 were spread on a 12 cm x 12 cm plate and antibiotics were applied on 6-mm paper discs to the freshly spread plate. Inducers of VanS led to expression of vanK and hence induced a halo of growth around the disc. Kanamycin, which targets the ribosome, served as a negative control; all of the other antibiotics used target cell wall biosynthesis. The amount of antibiotic in each disc is indicated.
Similar results have previously been obtained in pathogenetic enterococci. Depardieu et al. (2003) identified an *E. faecium* clinical isolate that expressed vancomycin resistance constitutively and showed that it carried an 18-bp in-frame deletion in *vanS* that resulted in a VanS protein deficient in phosphatase activity. Arthur et al. (1997) showed that the *van* promoters of *E. faecium* were constitutively activated by VanR in the absence of VanS, and concluded that VanS negatively controls VanR in the absence of glycopeptide inducer, presumably by dephosphorylation. Furthermore, Haldimann et al. (1997) demonstrated that heterologous expression of *E. faecium* VanR in an *E. coli ackA* strain harbouring a *vanH–lacZ* fusion stimulated high levels of β-galactosidase production, suggesting that acetyl phosphate could act as a phosphodonor to the *E. faecium* VanR protein in *E. coli in vivo*.

In enterococci, the identity of the VanS effector ligand has been the subject of debate. Screens for inducers have been established by coupling a promoter under the control of VanRS to suitable reporter genes (Lai and Kirsch, 1996; Ulijasz et al., 1996; Grissom-Arnold et al., 1997; Mani et al., 1998), by assaying VanX activity in cell extracts (Baptista et al., 1996; 1999), by monitoring induc-
tion of Lac-containing precursors (Allen and Hobbs, 1995), or by looking for induced vancomycin resistance in pretreated cultures (Handwerger and Kolokathis, 1990; Allen and Hobbs, 1995). Most have concluded that the VanS effector ligand is an intermediate in cell wall biosynthesis, such as Lipid II, which accumulates as a result of blocking peptidoglycan biosynthesis. The key observation supporting this conclusion is that structurally unrelated, non-glycopeptide inhibitors of late steps in cell wall biosynthesis, such as moenomycin A, bacitracin and ramoplanin, have been reported to induce van gene expression in enterococci. However, it is important to note that these reports are not wholly consistent with each other: for example, some conclude that bacitracin is an inducer (Allen and Hobbs, 1995; Lai and Kirsch, 1996; Ulijasz et al., 1996; Grissom-Arnold et al., 1997), while others conclude that it is not (Baptista et al., 1996; Mani et al., 1998). Similarly, Grissom-Arnold et al. (1997) conclude that ramoplanin is an inducer, while Baptista et al. (1996) conclude that it is not. To address this issue in Streptomyces, we exploited the phenotype of our constructed femX deletion mutant.

Streptomyces coelicolor femX null mutants are viable only in the presence of compounds that activate the VanRS signal transduction system, because they rely on expression of VanK for survival. We took advantage of this discovery to create a simple bioassay for inducers of the van genes in S. coelicolor. The structurally closely related glycopeptide antibiotics vancomycin, ristocetin, chloroermomycin and A47934 all acted as inducers. However, in contrast to the enterococcal VanRS systems, moenomycin A, bacitracin and ramoplanin did not act as inducers of the VanRS system in S. coelicolor.

To address the effector ligand issue further, we carried out a ‘VanRS swap’ experiment between two glycopeptide-resistant Streptomyces species with differing spectra of inducer molecules, to see if inducer specificity was determined by VanRS itself or by the host background. In S. coelicolor, the van genes are induced by both A47934 and vancomycin, while in S. toyocaensis, resistance is induced by A47934 but not by vancomycin (Neu and Wright, 2001). Introducing the S. toyocaensis VanRS signal transduction system into an S. coelicolor vanRS null mutant switched inducer specificity to that of S. toyocaensis. Thus, inducer specificity is determined by the origin of VanRS. There are two potential explanations for this observation. If Streptomyces VanS is activated by accumulation of a cell wall intermediate, vancomycin must induce a radically different spectrum of cell wall intermediates in S. coelicolor and S. toyocaensis, which seems unlikely. The more likely alternative is that VanS is directly activated by binding antibiotic (or possibly antibiotic bound to D-Ala-D-Ala-containing cell wall precursors, such as Lipid II), and that S. toyocaensis VanS interacts productively with A47934 but not with vancomycin. This would also be consistent with the fact that structurally unrelated compounds that also inhibit late steps in cell wall biosynthesis, such as moenomycin A, bacitracin and ramoplanin, are not inducers of VanS in Streptomyces.

Whether the VanS effector ligand is a cell wall intermediate or the drug itself, the ability to respond differentially to vancomycin and A47934 must reside in differences between the sensor domains of the S. toyocaensis and S. coelicolor VanS proteins. Alignment of the VanS primary sequences from these two species shows that the proteins are very similar, with 65% identity overall (Fig. 8). It is striking, however, that this high level of identity breaks down in the 26–27-residue stretch between the two predicted transmembrane helices, corresponding to the putative VanS sensor domain (Fig. 8).
**Experimental procedures**

**Bacterial strains, plasmids, oligonucleotides and growth conditions**

Strains, plasmids and oligonucleotides are described in Table 2. MMCGT is agar minimal medium (MM; Kieser et al., 2000) supplemented with 0.6% (w/v) Difco Casaminoacids, 0.75% (v/v) Tiger Milk (Kieser et al., 2000), 0.5% (w/v) glucose. Except where described below, media and culture conditions were as given previously (Kieser et al., 2000; Hong et al., 2002).

Protoplast transformation and conjugal plasmid transfer from *E. coli* to *Streptomyces* spp.

To bypass the methyl-specific restriction system of *S. coelicolor*, cosmids and plasmids were passed through the *dam dcm hsdS* *E. coli* strain ET12567 prior to protoplast transformation or conjugation. *E. coli* ET12567 carrying the non-transmissible, oriT-mobilizing ‘driver’ plasmid pUZ8002 was used for conjugation. *Streptomyces* protoplast transformation and conjugation from *E. coli* to *Streptomyces* were carried out as described by Kieser et al. (2000).

Isolating suppressors of femX

J3130 was streaked on MS agar containing 10 µg ml⁻¹ vancomycin and 23 single colonies were restreaked on MS agar without vancomycin. A single femX suppressor was then isolated from each of these plates to ensure the mutants were not clonal. Genomic DNA was isolated from each suppressor strain as described (Kieser et al., 2000) and the vanRS genes, including 100 bp flanking DNA on either side, were amplified by polymerase chain reaction (PCR) using the primers vanRFLANKI plus vanRFLANKII, and vanSFLANKI plus vanSFLANKII (Table 2) and sequenced using the same primers.

**Construction of insertion and deletion mutants**

ΔvanS::apr (J3199), ΔvanRS::apr (J2175) and Δ(pta ackA)::apr (J3202) insertion mutants were constructed by replacing most or all of the entire coding sequence with a cassette carrying the apramycin resistance gene (apr) and oriT of RK2, using the PCR-targeting method of Gust et al. (2003). The appropriate cosmid (H66 or IF6) was introduced into *E. coli* BW25113 carrying pIJ790, and the target gene was disrupted by electroporation of the cells with the PCR-amplified apr-oriT cassette, generated using primers carrying the appropriate gene-specific extensions (Table 2). The resulting cosmids [H66ΔvanS::apr, H66ΔvanRS::apr and IF6Δ(pta ackA)::apr] were introduced into *E. coli* ET12567 carrying pUZ8002 and transferred into *S. coelicolor* M600 by conjugation. Apramycin-resistant (AprR), kanamycin-sensitive (KanS) exconjugants were identified and purified, and the disruptions were confirmed by PCR and Southern analysis.

Unmarked ΔvanS (J3200) and ΔvanRS (J3201) deletion mutants were also constructed; cosmids H66ΔvanS::apr and H66ΔvanRS::apr were individually introduced into *E. coli* BT340 and deletion of the apr-oriT cassette (which is flanked by FRT sites) was obtained after induction of the FLP recombinase (Gust et al., 2003). The mutant cosmids thus obtained, H66ΔvanS and H66ΔvanRS, were introduced into J3199 and J2175, respectively, by protoplast transformation, and KanR transformants were selected. After growth in the absence of antibiotic selection, colonies that had lost both apramycin and kanamycin resistance were selected and purified (J3200 and J3201; Table 2) and analysed by PCR to confirm replacement of apr-oriT cassette by the non-polar ‘scar’ sequence (Gust et al., 2003). J3203 [Δ(pta ackA)::apr]...
\( \Delta \text{vanS} \) was constructed by introducing the \( \Delta (pta \ ackA)::apr \) allele into the unmarked \( \Delta \text{vanS} \) mutant, J3200, using the method described above.

Construction of a D51A allele of vanR and complementation of a vanR null mutant

A D51A allele of vanR was created using a pair of complementary primers containing the point mutation (D51A1 and D51A2; Table 2) and two flanking primers from the 5′-and 3′ regions of vanR (vanRFLANKI and vanRFLANKII; Table 2). Briefly, two PCR reactions were set up using vanRFLANKI plus D51A2 and vanRFLANKII plus D51A1. The two PCR products were gel purified, mixed, and used as template for a third PCR reaction containing only the flanking primers, and the resulting full-length PCR product was gel purified. The wild-type vanR allele was amplified from M600 genomic DNA using only vanRFLANKI and vanRFLANKII. The PCR products were cloned into SmaI-cut pUC19, sequenced and subcloned into HindIII-KpnI-cut pMS82 (Gregory et al., 2003) to create pIJ6959 (D51A) and pIJ6961 (wild-type), which were

| Table 2. Bacterial strains, plasmids and primers used in this study. |
|-----------------------------|-----------------------------|
| **Strains** | **Relevant genotype/comments** | **Source/reference** |
| S. coelicolor A3(2) | SCP1+ SCP2- | Kieser et al. (2000) |
| M600 | &Delta;femX::apr SCP1+ SCP2- | Hong et al. (2005) |
| J3130 | &Delta;vanS::apr SCP1+ SCP2- | This study |
| J3199 | &Delta;vanS SCP1+ SCP2- | This study |
| J3200 | &Delta;vanRS::apr SCP1+ SCP2- | This study |
| J3201 | &Delta;vanRS SCP1+ SCP2- | This study |
| J3202 | &Delta;(pta ackA)::apr SCP1+ SCP2- | This study |
| J3203 | &Delta;(pta ackA)::apr &Delta;vanS SCP1+ SCP2- | This study |
| E. coli | | |
| ET12567 (pUZ8002) | ET12567 containing helper plasmid pUZ8002 | Paget et al. (1999) |
| **Plasmids** | | |
| pU773 | pBluescript KS (+) containing the apramycin resistance gene apr and the oriT of plasmid RP4, flanked by FRT sites | Gust et al. (2003) |
| pMS82 | φBT1 attP-int derived integration vector for the conjugal transfer of DNA from E. coli to Streptomyces spp. (HygR) | Gregory et al. (2003) |
| pRT801 | φBT1 attP-int derived integration vector for conjugal transfer of DNA from E. coli to Streptomyces spp. (AprR) | Gregory et al. (2003) |
| pIJ6961 | pMS82 carrying vanR | This study |
| pIJ6959 | pMS82 carrying vanR D51A | This study |
| pIJ10277 | pRT801 carrying vanRSst | This study |
| **Primers** | | |
| vanH S1 FOR | TTCGACCTCTATATAGAAGCGAGCT | |
| vanH S1 REV | TGAGATGCTGCCGTACGCAGCGG | |
| vanRFOR | CATATGGATAGGCGCCGGGCA | |
| vanRREV | GGATCCCTATCCACCGTCGCGC | |
| vanSFOR | CATATGGGACGCATGCTCGC | |
| vanSFOR | GGATCCTCACCTGCGCCTGTT | |
| vanSFLANKI | GTGCAGCATCATCACGCAGGAG | |
| vanSFLANKII | TCCGTGCCTCGTACCGCACT | |
| D51A1 | GGCGTCCTCGGGCGGGACATC | |
| D51A2 | GATGTCGCCGCGGAGAGGAG | |
| vanSFLANKI | GGCCTGGCGCTCCGACC | |
| vanSFLANKII | TCACCTGGCGTGGTGGGCAAG | |
| VanS KO I | GGCGCGGTGAGAGCGAGCATCAGCCGGCAGTCTCTCCT | |
| VanS KO II | GTCAGAAGCTACGGCTAGCGAGGCGGGCCTGCTGCCAGGACC | |
| vanR KO I | TGGCATGATGGCAATGCGTACCTGGGACACTC | |
| pta KO I | AGGATTCTGGCTACTACCCACTCTACGAAAGGAAGAGACGCG | |
| ackA KO II | GCCACCGAGGTGTCGCCAGCCACGACGTCCGCTTACC | |

\( \Delta \text{vanS} \) was constructed by introducing the \( \Delta (pta \ ackA)::apr \) allele into the unmarked \( \Delta \text{vanS} \) mutant, J3200, using the method described above.

Construction of a D51A allele of vanR and complementation of a vanR null mutant

A D51A allele of vanR was created using a pair of complementary primers containing the point mutation (D51A1 and D51A2; Table 2) and two flanking primers from the 5′-and 3′ regions of vanR (vanRFLANKI and vanRFLANKII; Table 2). Briefly, two PCR reactions were set up using vanRFLANKI plus D51A2 and vanRFLANKII plus D51A1. The two PCR products were gel purified, mixed, and used as template for a third PCR reaction containing only the flanking primers, and the resulting full-length PCR product was gel purified. The wild-type vanR allele was amplified from M600 genomic DNA using only vanRFLANKI and vanRFLANKII. The PCR products were cloned into Smal-cut pUC19, sequenced and subcloned into HindIII-KpnI-cut pMS82 (Gregory et al., 2003) to create pIJ6959 (D51A) and pIJ6961 (wild-type), which were

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introduced into the vanRS mutant J2175 by conjugal transfer from *E. coli* strain ET12567 (pUZ8002), selecting exconjugants with hygromycin.

**Overexpression of VanS and VanR**

Full-length vanR was amplified using primers vanRFOR and vanRREV, and the cytosolic domain of VanS (c-VanS, corresponding to codons 85–364) was amplified using primers vanSFOR and vanSREV. The forward primers contained Ndel sites in frame with the translation start codon. The PCR products were cloned into Smal-cut pUC19, sequenced and subcloned into Ndel-HindIII-cut pET15b (Stratagene). The His-tagged proteins were overexpressed in BL21λDE3 (2 l) by induction with 0.1 mM IPTG at OD600 = 0.6 at 37°C for 3 h.

**Purification of VanR**

Cells were resuspended in 4 ml of binding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM imidazole), passed twice through a French Press, and this was repeated three times. The concentrated protein was centrifuged for 10 min at 10 000 r.p.m. in an Eppendorf 5415 centrifuge for 10 min. The concentrated protein was filtered through a 0.2-µm filter, and c-VanS was purified by nickel affinity chromatography as described for VanR. Fractions containing VanR were stored at 4°C in elution buffer.

**Purification of c-VanS**

Cells were resuspended in 35 ml of 20 mM Tris-HCl (pH 8.0), passed twice through a French Press, and the lysate was clarified by centrifugation. The supernatant and pellet both contained VanR, but only the soluble protein was further purified. The supernatant was filtered through a 0.2-µm filter (Millipore) and applied to a 5 ml HiTrap Chelating Ni²⁺ column. The column was washed with binding buffer until the UV trace was stable, and VanR was eluted with a linear 5–500 mM imidazole gradient (30 ml). Fractions containing VanR were stored at 4°C in elution buffer.

**In vitro phosphorylation assays**

Immediately before use, purified VanR protein (0.5 ml) was applied to a Millipore Ultrafree-0.5 centrifugal filter device and centrifuged for 10 min at 10 000 r.p.m. in an Appendorf 5415 D microcentrifuge for 10 min. The concentrated protein was diluted back to 0.6 ml with 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, and this was repeated three times. For the autophosphorylation reaction, VanS (40 pmol) was incubated with 10 µCi [γ³²P] ATP at room temperature for 1 h in 50 mM HEPES, pH 7.2, 5 mM MgCl₂ (50 µl total volume) and samples were taken at 0.5, 1, 2, 5, 15, 30 and 60 min and quenched immediately by addition of an equal volume of SDS-PAGE loading dye. For the phosphotransfer reaction, 40 pmol VanS was incubated for 60 min with 10 µCi [γ³²P] ATP and then mixed with VanR protein (20 pmol) at room temperature (50 µl total volume). Samples were taken at 1, 5, 15 and 30 min and quenched by addition of SDS-PAGE loading dye. [³²P] acetyl phosphate was prepared as described previously (Molle and Buttner, 2000) and incubated with VanR (20 pmol) at room temperature for 15 min (5 µl total volume). Reactions were quenched by addition of an equal volume of SDS-PAGE loading dye. All samples were loaded onto 15% SDS-polyacrylamide gels and separated at 200 V for 60 min at room temperature. Gels were dried and exposed to X-ray film overnight.

**Complementation of the S. coelicolor vanRS null mutant with S. toyocaensis vanRS (vanRSst)**

A 3 kb SacI fragment carrying the *S. toyocaensis vanRS* operon was made blunt-ended using DNA PolI and was cloned into the conjugative vector pRT801 cut with PvuII. The resulting plasmid, pIJ10277, was introduced into the *S. coelicolor vanRS* null mutant J3201 by conjugal transfer from *E. coli* strain ET12567/pUZ8002, and exconjugants were selected with apramycin.

**RNA preparation and S1 nuclease protection assays**

RNA was prepared as described previously (Hong et al., 2002; 2004). Germinated spores of *S. coelicolor* strains were inoculated into NMMP (Kieser et al., 2000) and grown to an OD₆₀₀ of 0.3–0.6 at 30°C. Immediately after the first 10 ml sample was taken, inducer (10 µg ml⁻¹ vancomycin or 10 µg ml⁻¹ A47934) was added to the remaining 40 ml of culture broth and 10 ml samples were taken at subsequent 30 min intervals up to 90 min. The vanH probe was a 270 bp 5’ end-labelled fragment generated by PCR using primers vanH S1 FOR and vanH S1 REV (Table 2) and *S. coelicolor* M600 chromosomal DNA as a template. For all assays, 30 µg RNA and 25 pmol labelled probe were dissolved in 20 µl of NaTCA buffer and hybridized at 45°C overnight after denaturation at 65°C for 15 min.

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