In vivo characterization of the activation and interaction of the VanR/VanS two-component regulatory system controlling glycopeptide antibiotic resistance in two related Streptomyces species

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Running Title: Characterization of VanRst/VanSst in *S. toyocaensis*

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ABSTRACT

The VanR/VanS two-component system is responsible for inducing resistance to glycopeptide antibiotics in various bacteria. We have performed a comparative study of the VanR/VanS systems from two streptomyces strains, *Streptomyces coelicolor* and *Streptomyces toyocaensis,* to characterize how the two proteins cooperate for signalling the presence of antibiotics and to define the functional nature of each protein in each strain background. The results indicate that the glycopeptide antibiotic inducer specificity is determined solely by the differences between the amino acid sequences of the VanR/VanS two-component systems present in each strain rather than by any inherent differences in general cell properties, including cell wall structure and biosynthesis. VanRsc functioned with either sensor kinase partner while VanRst only functioned with its cognate partner, VanSst. In contrast to VanRsc which is known to be capable of phosphorylation by acetyl phosphate, VanRst could not be activated in vivo independently of a VanS sensor kinase. A series of amino acid sequence modifications changing residues in the N-terminal receiver (REC) domain of VanRst to the corresponding residues present in VanRsc failed to create a protein capable of being activated by VanSsc and suggests that interaction of the response regulator with its cognate sensor kinase may require a more extended region than the REC domain. A T69S amino acid substitution in the REC domain of VanRst produced a strain exhibiting a weak constitutive resistance indicating that this particular amino acid may play a key role for VanS independent phoshphorylation in the response regulator protein.

INTRODUCTION

Ever since the first clinical isolates of pathogenic strains of vancomycin-resistant Enterococci (VRE) appeared in the late 1980s (1), the spread of vancomycin resistance through bacterial populations has been an acute public health issue, highlighted by the emergence of vancomycin-resistant MRSA (VRSA) in hospitals (2). Vancomycin inhibits cell wall biosynthesis by binding to the D-alanyl-D-alanine (D-Ala-D-Ala) terminus of lipid-attached peptidoglycan (PG) precursors on the outside of the cytoplasmic membrane. This interaction blocks formation of mature PG, principally by denying transpeptidase enzymes access to their substrate and thereby preventing formation of the peptide cross-links between polysaccharide strands that give the cell wall its rigidity (3). However, reprogramming of cell wall biosynthesis such that the 'stem' pentapeptide of PG precursors terminates in D-alanyl-D-lactate (D-Ala-D-Lac), rather than in D-Ala-D-Ala, can escape the action of vancomycin since the binding affinity of the drug for the new precursors is significantly lower than for the original precursors (4-6). Reprogramming can be achieved via expression of dedicated glycopeptide antibiotic resistance gene clusters minimally consisting of a 'core' of five genes – *vanR, vanS, vanH, vanA and vanX*. The polycistronic operon, *vanHAX*, encodes VanH, VanA and VanX enzymes which are required for remodelling cell wall precursors, and transcriptional induction of *vanHAX* is normally regulated by the VanR/VanS two-component system (TCS) which is encoded by the dicistronic operon *vanRS* (7, 8).

TCSs are the most dominant type of signal transduction pathway found in prokaryotes and play an important role in the regulation of metabolism in response to different nutritional or environmental signals. At their simplest, TCS consist of a pair of sensor histidine kinase (SHK) and response regulator (RR) proteins. The SHK responds to a specific inducer signal by modifying the phosphorylation state of the cognate RR. The N-terminus of the SHK can be diverse but usually contains a sensory or 'input'

domain which responds to changes in environmental stimuli. The C-terminus cytoplasmic kinase of the SHK, usually known as a transmitter domain, contains two distinct sub-domains: (i) a well-conserved catalytic and ATP binding domain; and (ii) a less well-conserved dimerization and histidine phosphotransfer domain (9-11). The N-terminal part of the RR plays a role in phosphotransfer and possesses a phosphorylation pocket containing three conserved aspartate residues and one lysine residue. Phosphorylation of a conserved aspartate within the phosphorylation pocket by the SHK, or potentially also by intracellular phosphor donors such as acetyl phosphate, induces a conformational change in the RR thereby activating the C-terminal DNA-binding effector domain (DBED), typically converting the RR into an active transcription activator (12). On exposure to vancomycin, VanS is autophosphorylated using ATP at a conserved C-terminal histidine residue and the phosphoryl group is then transferred to a conserved aspartate in the N-terminus of its cognate RR, VanR (13). Phospho-VanR has an enhanced C-terminal DBED DNA-binding activity and thereby triggers transcription of the *vanHAX* genes and confers resistance to vancomycin. Null mutation of the *vanR* gene consequently always produces a glycopeptide antibiotic sensitive phenotype, and the level of vancomycin resistance correlates with the expression of the *vanHAX* genes.

Resistance to vancomycin and other glycopeptide antibiotics has typically been identified in pathogenic bacteria or in non-pathogenic glycopeptide-producing strains. The resistance gene clusters in glycopeptide-producing bacteria are associated with the glycopeptide biosynthetic gene cluster (14-22). The model actinomycete *Streptomyces coelicolor* is a non-pathogenic, non-glycopeptide producing strain possessing inducible, high level resistance to vancomycin via expression of a cluster of seven resistance genes *vanSRJKHAX* (Fig. 1A). This cluster is organized into four transcription units (*vanRS*, *vanJ*, *vanK* and *vanHAX*) which are all under the control of the VanR/VanS two-component system (defined throughout the following text as VanRsc and VanSsc) (23). The role of all genes in this cluster in conferring vancomycin resistance has been characterized in detail (24-30). On exposure of *S.*

97 *coelicolor* to vancomycin, VanSsc switches from a phosphatase activity to functioning as a kinase and
98 the resulting increase in abundance of phospho-VanRsc activates transcription from the *van* promoters
99 thereby inducing vancomycin resistance (25, 26). In the absence of inducer drug the RR VanRsc is
100 activated by the intracellular small molecule phosphodonor acetyl phosphate, but the phosphatase
101 activity of VanSsc acts to suppress the level of phospho-VanR and prevent expression of the resistance
102 genes (Fig. 1A). A *S. coelicolor vanS*sc deletion mutant consequently constitutively expresses the *van*
103 genes (25). In addition to *S. coelicolor*, glycopeptide resistance has been explored in several other
104 actinomycetes including *Streptomyces toyocaensis*. *S. toyocaensis* produces a "sugarless" glycopeptide
105 A47934 and the resistance genes in this organism are associated with the A47934 biosynthetic cluster
106 (14). From a total of 34 open reading frames identified in the A47934 cluster, 8 genes (*vanS*st, *vanR*st,
107 *vanH*st, *vanA*st, *vanX*st, *murX*, *staP* and *staQ* (the "st" label is used throughout the following text to
108 indicate sequences originating in *S. toyocaensis*)) are predicted to be involved in A47934 resistance
109 (Fig 1B) (14), but molecular genetic studies to understand the biological function of each gene has to
110 date not been attempted.
111 In this work, we use a combinatorial *vanRS* gene swapping approach to investigate the distinctive
112 biological role of the individual VanR and VanS protein components in relation to the differences in
113 glycopeptide inducer specificity present in *S. coelicolor* and *S. toyocaensis*. We discuss the insight the
114 results provide into the mechanisms directing the immediate response to glycopeptide antibiotics
115 involving sensing by VanS and transduction of the signal from VanS to VanR.
116
117 **MATERIALS AND METHODS**
118
119 **Antibiotics, bacterial strains and culture conditions.**

120 Plasmids and bacterial strains used for this study are listed in Table 1. Except where described here,
121 media and culture conditions were as given previously (31, 32). Vancomycin and teicoplanin solutions
122 were made from commercially available antibiotic powder (Sigma-Aldrich) and used for all bioassays.
123 For bioassays performed using A47934, a neutralized NH_4OH extract solution of A47934 was prepared
124 from the producer strain *S. toyocaensis* as described previously (19). No glycopeptide antibiotics were
125 included in any precultures used in any of the experiments.
126
127 **Construction of vectors**
128 All vectors constructed are listed in Table 1, and primer sequences used in their construction are given
129 in Table S1. To construct pGN073, a 3.5kb DNA fragment containing the *vanRS*sc genes and its native
130 promoter sequence (*p-vanRS*sc) was obtained by PvuII digestion of *S. coelicolor* cosmid H66 then
131 ligated into the EcoRV site of pMS81 (33). A similar 3 kb DNA fragment containing the *vanRS*st genes
132 with its own promoter sequence (*p-vanRS*st) was obtained by SacI digestion of *S. toyocaensis* cosmid
133 pCep5 (14) and blunt-ended by DNA Polymerase I reaction. It was then cloned into pMS81 cut with
134 PvuII to create pHJH2000. pGN067 harboring the *vanR*sc gene with its own promoter sequence (*p-*
135 *vanR*sc) was prepared using the In-Fusion PCR cloning system (Clontech). In order to construct
136 pGN067, a KpnI/NdeI fragment containing the *ermE* promoter sequence in pHJH2001 was replaced by
137 a PCR product amplified using primers pRsc IF PvuII F and pRsc IF R. The *vanR*st gene including its
138 own promoter (*p-vanR*st) was amplified by PCR using primers vanRst I and vanRst II then cloned into
139 the vector pGEM-T easy (Promega) to create pHJH2002. After verification of the sequence, a 1.1 kb
140 DNA fragment of *p-vanR*st was obtained from pHJH2002 by SpeI/PvuII digestion and ligated into the
141 SpeI-PvuII sites of pMS81 to create pHJH2003. The *vanS*sc gene was obtained by PCR amplification
142 using primers vanSFOR and vanSREV, cloned into pGEM-T easy and verified by sequencing prior to
143 restriction digestion. The resulting plasmid pHJH2004 was cut with NdeI to obtain a 1.1 kb DNA

fragment of *vanS*sc which was then ligated into the NdeI site of pIJ10257 (24) to create pDU001. The *vanS*st gene was amplified by PCR using primers vanSst I and vanSst II and the product was cloned into pGEM-T easy to create pHJH2005. After the sequence of *vanS*st in pHJH2005 was confirmed, a 1.1 kb NdeI fragment of *vanS*st was obtained and ligated into the NdeI site of pIJ10257 (24) to create pDU006. To construct pHJH2006, a KpnI/AvrII fragment of *vanS*st obtained from pDU006 was ligated into pIJ6902 (28) cut with KpnI/XbaI.

Site-directed mutagenesis of VanRst

In order to substitute targeted amino acids in the REC domain of the VanRst protein for the corresponding residues present in VanRsc, we introduced site-directed nucleotide changes in the *vanR*st gene to construct recombinant plasmids using the Quick Change Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. pGN078, pGN080, pGN081, pGN082, and pGN083 were created using pJG001 (pIJ10257 plasmid harboring *vanR*st under the control of *ermE*p) as template DNA and the following primer pairs listed in Table S1: vanRst L10P_M12L and vanRst L10P_M12L_anti for pGN078; vanRst L10P_M12L and vanRst L10P_M12L_anti for pGN080; R106Q_I109A_I112L and R106Q_I109A_I112L_anti for pGN081; vanRst_S69T and vanRst _S69T_anti for pGN082; and vanRst_V62I and vanRst_V62I_anti for pGN083. pGN079 was made using pGN078 as template and the primers R106Q_I109A_I112L and R106Q_I109A_I112L_anti (see Table S1). Correct formation of all the mutated *vanR*st variants was confirmed by sequencing. The required strains (as listed in Table 1) were then constructed by conjugal transfer of plasmids from *E. coli* ET12567 (pUZ8002) (31).

Construction of strains

S. toyocaensis Δ*vanRS*st::*apr* (H31) and Δ*vanS*st::*apr* (H33) mutant strains were constructed by replacing the entire coding sequences of the target gene (or genes) with a cassette carrying the apramycin resistance gene (*apr*) and *oriT* of RK2, using the published PCR-targeting method (34). Primer sequences used are listed in Table S1. The cosmid pCep5 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. Using pIJ773 (34) as template, the *apr* disruption cassette were created by PCR using primers vanRst KO I and vanSst KO II for Δ*vanRS*st::*apr*, and vanSst KO I and vanSst KO II for Δ*vanS*st::*apr*. Each resulting cosmid was then introduced into *E. coli* ET12567 carrying pUZ8002 and transferred into *S. toyocaensis* NRRL 15009 by conjugation. Double crossover integrants were isolated as apramycin-resistant, kanamycin-sensitive colonies. Strain H320 was constructed by introducing the *vanS*st gene under the control of a constitutive promoter (*ermEp-vanS*st in pHJH2006 in Table 1) into the Δ*vanRS*st::*apr* (H31) mutant background. An unmarked *S. coelicolor* Δ*vanR*sc (H21) deletion mutant was constructed by introducing cosmid H66Δ*vanR*sc::*apr* into *E. coli* BT340 and excising the *apr-oriT* cassette (which is flanked by FRT sites) via induction of the FLP recominase system (34). The resultant mutant cosmid, H66Δ*vanR*sc, was then introduced into *S. coelicolor* M600 by protoplast transformation and kanamycin-resistant transformants were selected. After growth in the absence of antibiotic selection, colonies that had lost kanamycin resistance and were sensitive to vancomycin were selected and purified then analyzed by PCR to confirm in-frame replacement of the wild type sequence by a non-polar scar sequence. All other *S. coelicolor* and *S. toyocaensis* strains created in this study were constructed by conjugal transfer from *E. coli* strain ET12567 (pUZ8002) carrying the appropriate pMS81 or pIJ10257 plasmid derivative. Exconjugants were selected by hygromycin resistance.

Antibiotic susceptibility tests

All the bioassays, including both paper disc diffusion assays and the determination of minimum inhibitory concentrations (MICs), using *Streptomyces* strains were performed on MMCGT (Minimal Medium plus Casaminoacids Glucose Tiger milk) agar medium (32). For the paper disc diffusion assays, approximately 10^5 spores of each strain were spread on 9 cm diameter round plates and 6 mm paper discs containing 30 μg of glycopeptide antibiotics were then applied to the freshly spread spore lawns (19, 29). Plates were scored after incubation at 30°C for 2-4 days by measuring the diameter of any zone of growth inhibition (ZID) formed such that a ZID of 0 corresponds to complete resistance and scores < 7 indicate very strong resistance (the diameter of the paper discs used is 6 mm). ZID scores defined as "(0)" correspond to strains displaying growth up to the edge of the antibiotic disc but with a zone of detectabtly weaker growth around the disc. MIC values were evaluated using a method previously described (29) involving visual inspection of growth over a range of antibiotic concentrations on minimal agar medium in 96 well plates after 2-4 days of incubation at 30°C. The following concentrations of antibiotics were used in the MIC tests: 0, 0.125, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120 and 140 μg/ml vancomycin; 0, 0.125, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 μg/ml teicoplanin; and 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 50, 60, 70 and 80 μg/ml A47934. Each strain was assigned a single resistance index category for each antibiotic tested based on a combined consideration of the ZID and MIC values.

RNA preparation and quantitative real time PCR (qRT-PCR)

For the analysis of *vanH* transcription in response to glycopeptide antibiotics, 0.5 ml of germinated spores of *S. coelicolor* M600 or *S. toyocaensis* NRRL 15009 were inoculated in 50 ml NMMP liquid medium and grown to mid-log phase. Immediately after 10 ml of the first sample was taken as a non-induced control (0 min), the glycopeptide antibiotics were added to a final concentration of 10 μg/ml and samples taken 30, 60 and 90 min after treatment. Culture samples were immediately centrifuged for

10 s at 4,000 g and the cell pellet resuspended in 10 ml of RNA protect bacteria solution (Qiagen). After 5 min incubation at room temperature, the sample was centrifuged again for 10 s at 4,000 g and the supernatant was discarded. RNA preparation and qRT-PCR were performed according to methods described previously (35). For the qRT-PCR of *S. coelicolor vanH*, we used a pair of previously published primers, qvanH-F and qvanH-R (30) and gene SCO4702 as the internal normalization control (28). Primer pair qvanHst-F and qvanHst-R for pRT-PCR analysis of *S. toyocaensis vanH* transcription were designed using Primer3 (http://frodo.wi.mit.edu/) (see Table S1). Gene WP_037929599 with 94% identity to SCO4702 was used as the internal normalization control. qRT-PCR determinations were performed in triplicate on each RNA sample and average abundances determined.

RESULTS

Viability in an antibiotic bioassay is a good indicator of the ability of the glycopeptide to induce the VanR/VanS system.

The expression of the genes in the vancomycin resistance cluster in a wild type *S. coelicolor* strain M600 is induced by vancomycin and A47934 but not by teicoplanin (23, 25, 29). In a paper disc diffusion bioassay *S. coelicolor* therefore grows to the edge of discs containing vancomycin or A4794, but is markedly inhibited by teicoplanin which in contrast produces a large circular halo of non-growth (Fig. 2A). Consistent with these phenotypes, qRT-PCR analysis indicates that transcription of the *vanH* gene (a reporter for expression of the *vanHAX* operon) is highly induced by both vancomycin and A47934 in *S. coelicolor*, but not by teicoplanin which totally failed to induce transcription even after exposure for 90 min (Fig. 2A). Maximal induction of *vanH* by A47934 is approximately half that observed for the response to vancomycin and the MIC observed is also markedly lower. In contrast to *S. coelicolor*, *S. toyocaensis* (NRRL 15009), the producer of the glycopeptide antibiotic A47934, is

resistant to A47934 but susceptible to both vancomycin and teicoplanin (Fig. 2B). Quantification of *vanH* transcription in *S. toyocaensis* shows strong inducible expression in response to A47934 but not to vancomycin or teicoplanin (Fig. 2B). A low level of *vanH* transcription was detectable in response to teicoplanin and this corresponds with a slight reduction in the diameter of the teicoplanin inhibitory halo compared to vancomycin in the plate bioassay and an increase in the MIC value (Fig. 2B). Thus the viability of the cells in the presence of glycopeptides, as determined by disc diffusion bioassay and measurement of the MIC, is a good indication of whether the particular antibiotic is a strong, weak, poor or non-inducer of the resistance controlled by VanR/VanS. Assignment of a resistance index based on a combined consideration of the zone of inhibition diameter (ZID) and MIC bioassay values is used in the subsequent sections as a measure of the activation of a resistance system by a particular antibiotic in a particular strain.

Investigating glycopeptide-induced signal transduction through the *S. coelicolor* and *S. toyocaensis* VanR/VanS two-component systems.

The amino acid sequences of the VanS and VanR proteins present in *S. coelicolor* and *S. toyocaensis* are highly conserved (90% identity for VanR and 67% identity for VanS) but the mechanism of VanR/VanS signal transduction in each strain is different (see Fig. 2). To investigate glycopeptide-induced signal transduction through the VanR/VanS systems we constructed *S. coelicolor* and *S. toyocaensis* strains carrying all possible pair-wise combinations of VanR and VanS from each strain, and determined their antibiotic resistance indexes using the panel of three glycopeptide antibiotics A47934, teicoplanin and vancomycin (Fig. 3; see also Fig. S1 and S2). Since this approach focusses on the resistance phenotypes that are the functional end product of VanR/VanS activation, the readout reports on the combined efficiency of the following processes: detection of the glycopeptide by the VanS sensor; transduction of the activation signal from VanS to VanR by phosphorylation; induction of

expression from the *van* gene promoters; and translation of the *van* gene transcripts to produce functional enzymes. The level of phospho-VanR can be influenced by both phosphorylation and dephosphorylation. Strains using *S. coelicolor* as the host were constructed based on unmarked in-frame deletion mutants in which the coding sequence for the *vanR* (this study), *vanS* (25) or *vanRS* (25) genes had been removed. Genes were added back into these mutants in single copy to produce the required combinations by using vectors that integrate stably into the chromosome at the ϕBT1 attachment site (33). A similar strategy was used for the *S. toyocayensis* strains except that these were based on deletion mutants in which the coding sequence of *vanS* or *vanRS* had been replaced by an apramycin resistance cassette, and genes were added in single copy at the ϕBT1 and ϕC31 integration sites to produce all the combinations. All strains constructed grew as vigorously in the absence of antibiotics as their parental strains with the exception of the *S. coelicolor* Δ*vanS*sc and *S. toyocaensis* Δv*anS*st strains expressing *vanR*sc (H2473, H2297 and H2311) which exhibited reduced growth. These three strains however show a higher than expected resistance in the glycopeptide antibiotic tests (see below) and the growth defects are therefore not significant in this context.

i) Glycopeptide antibiotic inducer specificity depends on the identity of the VanR/VanS two-component system rather than the host background.

Introduction of the *S. toyocaensis vanRS* genes (*vanRS*st) into the *S. coelicolor* Δ*vanRS*sc null mutant (producing strain H2060) switched the natural inducer specificity of *S. coelicolor* to that of *S. toyocaensis* i.e. it was resistant to A47934, but sensitive to vancomycin or teicoplanin (Fig 3A; see also Fig. S1e). Similarly, introduction of the *S. coelicolor vanRS* genes (*vanRS*sc) into the *S. toyocaensis* Δ*vanRS*st null mutant (producing strain H2360) also switched the inducer specificity so that the resulting *S. toyocaensis* strain was, like wild type *S. coelicolor*, resistant to vancomycin and A47934 but sensitive to teicoplanin (Fig 3B; see also Fig. S1l). Both results indicate that all the heterologously expressed proteins are functional in their respective strains.

ii) The non-cognate pairing of VanRst/VanSsc cannot induce glycopeptide resistance in either *S. coelicolor* or *S. toyocaensis* but the reciprocal non-cognate pairing VanRsc/VanSst is functional.

The non-cognate combination of VanRst/VanSsc failed to induce resistance to any of the glycopeptide antbiotics regardless of the host background (Fig. 3; see also Fig. S1f and S1n). In contrast, the reciprocal non-cognate combination of VanRsc/VanSst was productive. Strains H36 (*S. coelicolor* background) and H2309 (*S. toyocaensis* background) are both resistant to A47934 but susceptible to vancomycin and teicoplanin (Fig. 3; see also Fig. S1g and S1m). The inducer specificity towards the glycopeptide antibiotics is therefore again determined according to the origin of the VanS sensor (VanSst) and not the host background. Interestingly however, both H36 and H2309 also exhibit significantly increased resistance toward teicoplanin (see Fig. S1g and S1m). These results are consistent with cross-talk between VanRsc and VanSst in responding to the presence of glycopeptide antibiotics, and interestingly also suggest that VanRsc may in some conditions act as a better RR partner for VanSst than its natural cognate partner, VanRst.

iii) VanRst is apparently not activatable by the intracellular phosphate donor acetyl phosphate.

S. coelicolor Δ*vanS*sc (H2473) exhibits significantly increased resistance to all three glycopeptide antibiotics relative to the Δ*vanRS*sc (Fig 3A; see also Fig. S1d). This was shown to be associated with a constitutive but moderate expression of the *van* resistance reporter gene *vanH* even in the absence of any glycopeptide (Fig. S3). In contrast, *S. toyocaensis* Δ*vanS*st (H2118) is susceptible to A47934 and does not exhibit constitutive resistance to any of the antibiotics tested (Fig. 3B; see also Fig. S1k). The wild type resistance phenotype was fully restored in this strain by addition of a single integrated copy of *vanS*st excluding the possibility of any polar effect of *vanS*st deletion (data not shown). *S. coelicolor* VanS (VanSsc) is known to negatively regulate VanRsc function in the absence of inducer drug such that a *S. coelicolor vanS* null mutant (Δ*vanS*sc) exhibits constitutive resistance via a phosphorylation of VanRsc that is dependent upon acetyl phosphate production (25). The absence of a similar constitutive

glycopeptide resistance phenotype in *S. toyocaensis* Δ*vanS*st implies that VanRst cannot be phosphorylated in a VanSst independent manner. Introduction of *vanR*sc into *S. toyocaensis* Δ*vanRS*st (producing strain H2311) produces a constitutive resistance phenotype (Fig. 3B; see also Fig. S2f) consistent with acetyl phosphate dependent phosphorylation of VanRsc taking place in this strain background as in the native host *S. coelicolor*. In contrast, the introduction of *vanR*st into the *S. coelicolor* Δ*vanRS*sc (producing strain H2062) or *S. toyocaensis* Δ*vanRS*st (producing strain H2476) backgrounds yielded strains which are susceptible to all three glycopeptide antibiotics (Fig. 3; see also Fig. S2c and S2e respectively) suggesting that phosphorylation of VanRst using acetylphosphate as the substrate is inefficient. *S. coelicolor* strain H2297 (see Fig. S2b) carrying a ϕBT1 integrated copy of *vanR*sc exhibited the same phenotype as strain H2473 (see Fig. S1d) where *vanR*sc is present at its native location and served as a control for the genetic location of the *vanR* expression constructs.

Site directed mutagenesis of the N-terminal REC domain of VanRst to more resemble the VanRsc REC domain fails to produce a VanSsc activatable protein but creates a variant subject to VanS-independent activation.

The specificity of SHK/RR interactions is believed to be determined by coevolving residues located in the N-terminal dimerization and histidine phosphotransfer (DHp) domain of the SHK and in the receiver (REC) domain of the RR (36, 37). Mutation of these coevolving residues, either in the SHK or the RR, can allow the specificity of the cognate pairs to be rewired, as previously shown with two closely related SHK-RR systems in *Escherichia coli*, EnvZ-OmpR and RstB-RstA (37, 38). In this work the VanRst/VanSsc RR/SHK pairing failed to induce glycopeptide resistance in either the *S. coelicolor* or *S. toyocaensis* strain background suggesting an imperfect interaction between the VanRst and VanSsc proteins. VanRsc and VanRst differ in their REC domain by only eight amino acids residues however, so we attempted to improve this interaction by gradually altering the amino acids in

VanRst to correspond to those found in VanRsc using site-directed mutagenesis (Fig. 4). The activity of the mutagenized *vanR*st variants was tested by single copy integration into the *S. coelicolor* Δ*vanR*sc and *S. toyocaensis* Δ*vanR*st strain backgrounds, assessing the phenotype of the resulting strains toward A47934, teicoplanin and vancomycin. None of the mutations in VanRst tested restored resistance to wild-type levels when paired with VanSsc in *S. coelicolor* (Fig. 5A; see also Fig. S4), but all were functional in restoring A47934 resistance when used with their natural VanSst partner in *S. toyocaensis* (Fig. 5B; see also Fig. S4). One variant VanRst(T69S) did however partially complement the *S. coelicolor* Δ*vanR*sc phenotype showing an increased resistance to glycopeptide antibiotics (strain H2328 in Fig. 5A; see also Fig. S4d). Strikingly this variant also shows activity in the *S. coelciolor* Δ*vanRS*sc and *S. toyocaensis* Δ*vanRS*st mutant strains which lack any VanS kinase (strains H2329 and H2338 in Fig. 5; see also Fig. S5d and S5k), indicating that it can be activated independently of VanS, presumably via acetyl phosphate. None of the other variants tested were active in these Δ*vanRS* backgrounds (Fig. 5; see also Fig. S5). Interestingly, a *S. toyocaensis* strain harboring VanRst(L10P+M12L+R106Q+I109A+I112L)/VanSst (H2506) exhibited significantly increased resistance to teicoplanin but not to vancomycin (Fig. 5B; see also Fig. S4n). This phenotype is similar to that of *S. toyocaensis* strain H2309 (Δ*vanR*st harboring a plasmid expressing *vanRsc*) (see Fig. 3B and Fig. S1m) implying that the five amino acids changes in VanRst may be sufficient to reproduce the interaction behavior of VanRsc with VanSst.

DISCUSSION

In many bacterial glycopeptide antibiotic resistance systems the important role of recognizing the presence of the antibiotic and transducing an appropriate signal to the chromosome is performed by a VanR/VanS two-component system, but relatively little is known about how the two proteins involved

359 cooperate to achieve this. In this work we have exploited the differing responses to glycopeptide
360 antibiotics exhibited by the VanR/VanS sensory systems in two streptomyces strains, *Streptomyces*
361 *coelicolor* and *Streptomyces toyocaensis*, to explore this area and to define the functioning of each
362 protein in each strain background. The A47934 resistance gene cluster in *S. toyocaensis* was first
363 identified and reported in 2002 (14), but the molecular mechanism of A47934 resistance as well as the
364 functioning of its key regulatory two-component system, VanRst/VanSst, has remained poorly defined.
365 This study is the first investigation into this system and compares its activity with the analogous and
366 well characterized two-component system VanRsc/VanSsc from *S. coelicolor* (25, 26).
367 Notably, exchanging just the complete VanR/VanS two-component system between the two
368 *Streptomyces* strains was sufficient to switch the glycopeptide resistance profiles. The resistance
369 profiles are therefore determined by the differences between the amino acid sequences of the
370 VanR/VanS proteins present rather than by inherent differences in cell wall structure or biosynthesis
371 between the strains. The activity of extracellular D,D-carboxypeptidases has been shown to alter the
372 efficacy of glycopeptide antibiotics in actinomycete strains expressing the VanHAX enzymes by
373 exchanging the D-Lac reprogrammed into cytoplasmic peptidoglycan precursors for D-Ala or Gly in
374 the periplasm (39), but differences in the control by VanR/VanS is clearly the dominant factor between
375 *S. coelicolor* and *S. toyocaensis*. The incomplete restoration of resistance to vancomycin in *S.*
376 *toyocaensis* H2360 (Δ*vanRS*st + *vanRS*sc) compared to wild-type *S. coelicolor* could however suggest
377 some contribution from differences in carboxypeptidase activity between strains but this could also be
378 due to a possible increase in expression of *staP* from the A47934 cluster which is known to have
379 deleterious effects on growth (29). The ability to predictably alter the glycopeptide resistance profiles
380 by swapping the VanR/VanS systems strongly supports the proposal that VanS responds directly to an
381 antibiotic ligand such that VanSst is able to sense and interact productively with A47923 but not with

382 teicoplanin or vancomycin, whereas VanSsc interacts productively with vancomycin or A47934 but not
383 with teicoplanin.
384 Gene swaps producing hybrid combinations of either VanRsc/VanSst or VanRst/VanSsc as the sole
385 sensory system for glycopeptides produced results which indicate differences in the activities of the
386 two RR regulator proteins. VanRsc was able to transduce the signal detected by VanSst in both the *S.*
387 *coelicolor* and *S. toyocaensis* backgrounds (producing strains H36 and H2309) while VanRst was
388 unable to accomplish this in either background when similarly paired with VanSsc (producing strains
389 H2123 and H3129) (Fig. 3; see also Fig. S1). In addition to being unable to interact productively with
390 VanSsc, VanRst was found to be different to VanRsc in being resistant to VanS independent activation.
391 In *S. coelicolor* VanRsc can be activated in the absence of VanSsc by phosphorylation that is dependent
392 upon the *ackA* (SCO5424) and *pta* (SCO5425) genes required for acetyl phosphate production (25) (see
393 H2473 or H2297 in Fig. 3A; see also Fig. S1d and S2b). The *S. toyocaensis* genome sequence (40) also
394 contains orthologous *ackA* and *pta* sequences (85% and 87% of nucleotide sequence identity to the *S.*
395 *coelicolor* sequences, respectively), and VanRsc can also be activated independently of VanS in this
396 strain background (H2311 in Fig. 3B; see also Fig. S2f). The absence of VanS independent activation
397 of VanRst in *S. toyocaensis* is therefore consistent with acetyl phosphate being a poor net
398 phosphodonor for VanRst. Response regulators can exhibit wide differences in the half-life of their
399 phsophorylated forms and it is possible that the half-life of phospho-VanRst is short compared to
400 phospho-VanRsc. The antibiotic resistance phenotypes of the strains in which VanSst is partnered with
401 VanRsc also indicates that, in common with VanSsc, VanSst must possess phosphatase activity in
402 addition to its kinase function (H36 and H2309 in Fig. 3; see also Fig. S1g and S1m). If this was not the
403 case then these strains would exhibit the same constitutive resistance to all three antibiotics shown by
404 strains H2297 and H2311. Although VanSst in *S. toyocaensis* has retained the phosphatase activity seen
405 in the *S. coelicolor* system, it is evidently not required for keeping expression of the resistance genes to

406 a minimum in the absence of glycopeptide inducers since VanRst is not phosporylated independently of
407 VanS.
408 It is noteworthy that VanRsc and VanRst behave so differently when their amino acid sequences,
409 particularly in their REC domains, are highly similar (90% identity; see Fig.4). Although not
410 exhaustive, the results of site-directed mutagenesis targeting the N-terminal REC domain of VanRst
411 suggests additional sequences outside this region may be important for correct interaction with the
412 VanS kinases, and goes against the general belief that this domain of RRs is the only region responsible
413 for the interaction with a cognate SHK partner. Barbieri et al. (2010) previously demonstrated that the
414 extent of the interdomain interface made by the REC domain with the DBED in several RRs from the
415 OmpR/PhoB family influenced the rate of phosphotransfer from acetyl phosphate in vitro (41). Proteins
416 with substantial interdomain interfaces phosphorylate poorly possibly because the interdomain
417 interactions stabilize an inactive conformation of the RR which is not catalytically competent to accept
418 phosphate from acetyl phosphate (41). Although the authors have shown that the interdomain
419 interaction did not influence the rate of phosphotransfer from the cognate SHK, this might not be
420 necessarily true for a non-cognate SHK. Our in vivo experiments show that in contrast to VanRsc,
421 VanRst does not efficiently undergo phosphorylation using acetyl phosphate. While this could be due
422 to a very short half-life of the phosphorylated form, it is also consistent with unphosphorylated VanRst
423 occupying a conformation where the REC and DBED domains are in tighter contact than in VanRsc
424 thus preventing phosphoacceptance from acetyl phosphate. Such structural differences between VanRsc
425 and VanRst could also be responsible for different promiscuities with respect to phosphorylation by
426 cognate or non-cognate SHKs. We hypothesize that VanRst requires very precise interaction with the
427 SHK in order to release the inactive conformation putatively stabilized by substantial interdomain
428 contact between the REC and DBED domains. This requirement would restrict the specificity of
429 VanRst to only its cognate SHK, VanSst. In contrast, VanRsc evidently occupies a conformation more

amenable to phosphorylation using acetyl phosphate making it generally more accessible also for activation by a non-cognate SHK. Interestingly, the VanRst mutant variant VanRst(T69S) is more readily activatable in the absence of VanSst (or VanSsc) than the wild-type sequence and strains expressing this variant in these genetic backgrounds exhibit a similar constitutive but low-level glycopeptide resistance (see H2329 and H2338 in Fig. 5; see also Fig. S5d and S5k).

The investigation of vancomycin resistance in bacteria has been the subject of extensive research and considerable effort has been put into studying the VanR/VanS TCS in particular, mostly using pathogenic VRE strains as a model system. A detailed topological understanding of how the phosphorelay system in the VanR/VanS complex operates has however remained elusive due to a lack of structural data. Understanding the interplay between the VanS effector ligand on the one hand, and downstream activation of VanR by VanS on the other, will be important for the development of future strategies for circumventing inducible glycopeptide antibiotic resistance in clinical infections. Furthermore, TCSs are one of the most abundant and ubiquitous adaptive signal transduction pathways present in bacteria, making them attractive targets for the modulation of bacterial function by novel bioactive molecules, particularly in pathogenic strains.

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REFERENCES

1. **Leclercq R, Derlot E, Duval J, Courvalin P.** 1988. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. N. Engl. J. Med. **319:**157-161.

2. **Pearson H**. 2002 'Superbug' hurdles key drug barrier. Nature **418:**469-470.

3. **Barna JCJ, Williams DH.** 1984. The structure and mode of action of glycopeptide antibiotics of the vancomycin group. Annu. Rev. Microbiol. **38:**339-357.

4. **Arthur M, Molinas C, Reynolds P, Courvalin P.** 1993. Characterization of Tn1546, a Tn3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J. Bacteriol. **175:**117-127.

5. **Arthur M, Depardieu F, Reynolds P, Courvalin P.** 1996. Quantitative analysis of the metabolism of soluble cytoplasmic peptidoglycan precursors of glycopeptide-resistant enterococci. Mol. Microbiol. **21:**33-44.

6. **Arthur M, Reynolds P, Courvalin P.** 1996. Glycopeptide resistance in enterococci. Trends Microbiol. **4:**401-407.

7. **Arthur M, Molinas C, Courvalin P.** 1992. The VanS-VanR two-component regulatory system controls synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J. Bacteriol. **174:**2582-2591.

8. **Arthur M, Departdieu F, Courvalin P.** 1999. Regulated interactions between partner and non-partner sensors and response regulators that control glycopeptide resistance gene expression in enterococci. Microbiol. **145:**1849-1858.

9. **Kofoid EC, Parkinson JS.** 1988. Transmitter and receiver modules in bacterial signalling proteins. Proc. Natl. Acad. Sci. U. S. A. **85:**4981-4985.

10. **Parkinson JS, Kofoid EC.** 1992. Communication modules in bacterial signaling proteins. Annu. Rev. Genet. **26:**71-112.

11. **Gao R, Stock AM.** 2009. Biological insights from structures of two-component proteins. Annu. Rev. Microbiol. **63:**133-154.

12. **Lukat GS, McCleary WR, Stock AM, Stock JB. 1992.** Phosphorylation of bacterial response regulator proteins by low molecular weight phospho-donors. Proc. Natl. Acad. Sci. U. S. A. **89:**718-722.

13. **Wright, GD, Holman TR, Walsh CT.** 1993. Purification and characterization of VanR and the cytosolic domain of VanS: a two-component regulatory system required for vancomycin resistance in *Enterococcus faecium* BM4147. Biochemistry **32**:5057–5063.

14. **Pootoolal J, Thomas MG, Marshall CG, Neu JM, Hubbard BK, Walsh CT, Wright GD.** 2002. Assembling the glycopeptide antibiotic scaffold: The biosynthesis of A47934 from *Streptomyces toyocaensis* NRRL15009. Proc. Natl. Acad. Sci. U.S.A. **99:**8962-8967.

15. **Sosio M, Stinchi S, Beltrametti F, Lazzarini A, Donadio S.** 2003. The gene cluster for the biosynthesis of the glycopeptide antibiotic A40926 by *nonomuraea* species. Chem. Biol. **10:**541-549.

16. **Li TL, Huang F, Haydock SF, Mironenko T, Leadlay PF, Spencer JB.** 2004. Biosynthetic gene cluster of the glycopeptide antibiotic teicoplanin: characterization of two glycosyltransferases and the key acyltransferase. Chem. Biol. **11:**107-119.

17. **Shawky RM, Puk O, Wietzorrek A, Pelzer S, Takano E, Wohlleben W, Stegmann E.** 2007. The border sequence of the balhimycin biosynthesis gene cluster from *Amycolatopsis balhimycina* contains *bbr*, encoding a StrR-like pathway-specific regulator. J. Mol. Microbiol. Biotechnol. **13:**76-88.

18. **Jeong H, Sim YM, Kim HJ, Lee DW, Lim SK, Lee SJ.** 2013. Genome sequence of the vancomycin-producing *Amycolatopsis orientalis* subsp. *orientalis* strain KCTC 9412T. Genome Announc. **1:**e00408-00413.

19. **Truman AW, Kwun MJ, Cheng J, Yang SH, Shu J-W, Hong H-J.** 2014. Antibiotic resistance mechanisms inform discovery: Identification and characterization of a novel *Amycolatopsis* strain producing Ristocetin. Antimicrob. Agents Chemother. **58:**5687-5695.

20. **Kwun MJ, Hong H-J.** 2014. Genome sequence of the A47934 glycopeptide antibiotic producer strain, *Streptomyces toyocaensis* NRRL 15009. Genome Announc. **2:**e00749-14.

21. **Kwun MJ, Hong H-J.** 2014. Draft genome sequence of *Amycolatopsis lurida* NRRL 2430, producer of the glycopeptide family antibiotic ristocetin. Genome Announc. **2:**e01050-14.

22. **Kwun MJ, Cheng J, Yang SH, Lee DR, Shu J-W, Hong H-J.** 2014. Draft genome sequence of ristocetin-producing strain *Amycolatopsis* sp. Strain MJM2582 isolated in South Korea. Genome Announc. **2:**e01091-14.

23. **Hong H-J, Hutchings MI, Neu JM, Wright GD, Paget MS, Buttner MJ.** 2004. Characterisation of an inducible vancomycin resistance system in *Streptomyces coelicolor* reveals a novel gene (*vanK*) required for drug resistance. Mol. Microbiol. **52:**1107-1121.

24. **Hong H-J, Hutchings MI, Hill L, Buttner MJ.** 2005. The role of the novel Fem protein VanK in vancomycin resistance in *Streptomyces coelicolor*. J. Biol. Chem. **280:**13055-13061.

25. **Hutchings MI, Hong H-J, Buttner MJ.** 2006. The vancomycin resistance VanRS signal transduction system of *Streptomyces coelicolor*. Mol. Microbiol. **59:**923-935.

26. **Hong H-J, Hutchings MI, Buttner MJ.** 2008 Vancomycin resistance VanRS two-component systems. Adv. Exp. Med. Biol. **631:**200-213.

27. **Koteva K, Hong H-J, Wang XD, Nazi I, Hughes D, Naldrett MJ, Buttner MJ, Wright GD.** 2010. A vancomycin photoprobe identifies the histidine kinase VanSsc as a vancomycin receptor. Nat. Chem. Biol. **6:**327-329.

28. **Hesketh A, Hill C, Mokhtar J, Novotna G, Tran N, Bibb M, Hong H-J.** 2011. Genome-wide dynamics of a bacterial response to antibiotics that target the cell envelope. BMC genomics **12:**226.

29. **Novotna G, Hill C, Vicent K, Liu C, Hong H-J.** 2012. A novel membrane protein, VanJ, conferring resistance to teicoplanin. Antimicrob. Agents Chemother. **56:**1784-1796.

30. **Kwun MJ, Novotna G, Hesketh AR, Hill L, Hong H-J**. 2013. In vivo studies suggest that induction of VanS-dependent vancomycin resistance requires binding of the drug to D-Ala-D-Ala termini in the peptidoglycan cell wall. Antimicrob. Agents Chemother. **57:**4470-4480.

31. **Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA.** 2000. Practical *Streptomyces* Genetics, The John Innes Foundation, Norwich, UK.

32. **Hong H-J, Paget MSB, Buttner MJ.** 2002. A signal transduction system in *Streptomyces coelicolor* that activates the expression of a putative cell wall glycan operon in response to vancomycin and other cell wall-specific antibiotics. Mol. Microbiol. **44:**1199-1211.

33. **Gregory MA, Till R, Smith MCM.** 2003. Integration site for *Streptomyces* phage ϕBT1 and development of site-specific integration vectors. J. Bacteriol. **185:**5320-5323.

34. **Gust B, Challis GL, Fowler K, Kieser T, Chater KF.** 2003. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc. Natl. Acad. Sci. U. S. A. **18:**1541-1546.

35. **Kwun MJ, Hong H-J**. 2014. The activity of glycopeptide antibiotics against resistant bacteria correlates with their ability to induce the resistance system. Antimicrob. Agents Chemother. **58:**6306-6310.

36. **Casino P, Rubio V, Marina A.** 2009. Structural insight into partner specificity and phosphoryl transfer in two-component signal transduction. Cell **139:**325-336.

37. **Capra EJ, Perchuk BS, Lubin EA, Ashenberg O, Skerker JM, Laub MT.** 2010. Systematic dissection and trajectory-scanning mutagenesis of the molecular interface that ensures specificity of two-component signaling pathways. PLoS Genet. **6:**e1001220.

546 38. **Skerker JM, Perchuk BS, Siryaporn A, Lubin EA, Ashenberg O, Goulian M, Laub MT.**
547 2008. Rewiring the specificity of two-component signal transduction systems. Cell **133:**1043-1054.
548 39. **Hugonnet JE, Haddache N, Veckerlé C, Dubost L, Marie A, Shikura N, Mainardi JL, Rice**
549 **LB, Arthur M.** 2014. Peptidoglycan cross-linking in glycopeptide resistant Actinomycetales.
550 Antimicrob. Agents Chemother. **58**:1749-1756.
551 40. **Kwun MJ, Hong H-J.** 2014. Genome Sequence of *Streptomyces toyocaensis* NRRL 15009,
552 Producer of the Glycopeptide Antibiotic A47934. Genome Announc. **2:**4 e00749-14.
553 41. **Barbieri CM, Mack TR, Robinson VL, Miller MT, Stock AM.** 2010. Regulation of response
554 regulator autophosphorylation through interdomain contacts. J. Biol. Chem. **285:**32325-32335.
555
556 **FIGURE LEGENDS**
557
558 **FIG 1** (**A**) A model illustrating the function and regulation of vancomycin resistance via the
559 VanRsc/VanSsc TCS in *S. ceolicolor*. In the absence of inducer drug, the small molecule phosphate
560 donor acetyl phosphate phosphorylates and activates VanR for inducing vancomycin resistance but this
561 is effectively supressed by the phophatase activity of VanS. In the presence of inducer drug, VanS
562 interacts with inducer drug and switches its activity from phosphatase to kinase, leading to a strong
563 phosphorylation of VanR. The active phospho-VanR then binds to the four promoters of the *van* gene
564 cluster and induces transcription of the *van* genes to render the *S. coelicolor* cells resistant to
565 vancomycin. (**B**) Genetic organization of the A47934 resistance in *S. toyocaensis*.
566
567 **FIG 2** The bioassay activity of glycopeptide antibiotics against resistant strains correlates with the
568 ability of the antibiotics to induce the VanR/VanS system. Bioassay analyzing the activity of three
569 selected glycopeptide antibiotics A47934 (A), teicoplanin (T) and vancomycin (V) and their MIC

against *S. coelicolor* M600 (**A**) and *S. toyocaensis* NRRL15009 (**B**). Paper discs containing 30 µg of each antibiotic were placed on freshly spread lawns of each strain, and plates were incubated for 2 days at 30°C. A clear and strong dark colored halo around the paper discs indicate the zone of inhibition. The corresponding antibiotic zone of inhibition diameter (ZID; mm) and MIC (µg/ml) values are presented below the bioassay plates, together with quantification of the response in *vanH* transcription in each strain to each antibiotic as determined by qRT-PCR. Cells were grown to mid-exponential phase in NMMP liquid medium and exposed to 10 µg/ml of each antibiotic. Total RNAs were extracted from samples taken immediately before the addition of antibiotic (0) and at subsequent 30 min intervals up to 90 min (30, 60, 90). The x axis indicates time (min) after addition of the antibiotic, and the y axis shows the fold change in normalized *vanH* transcript abundance calculated relative to the abundance at time 0 which was defined as 1.

FIG 3 Antibiotic resistance indexes summarizing the activity of different combinations of VanRS proteins in providing resistance against treatment with A47934 (A), teicoplanin (T) and vancomycin (V) in the *S. coelicolor* (**A**) and *S. toyocaensis* (**B**) genetic backgrounds. The status and origin of each component in the VanR/VanS system in each strain tested is illustrated in the first column of each table alongside the strain numbers (VanR/VanS proteins shaded dark grey originate from *S. coelicolor* and in contrast those shown in white originate from *S. toyocaensis*). Resistance indexes were determined from the ZID (in mm) and MIC (in µg/ml) values as described in the Materials and Methods, and the keys to these categories are presented beneath each table. The genotype of all strains is described in detail in Table 1, and the data from which the indexes are derived are presented in Figs. S1 and S2.

FIG 4 Amino acid sequence alignment of VanRsc and VanRst. The black bar beneath the sequences indicates the conserved N-terminal REC domain. The eight amino acid residues which differ in the

REC domain between VanRsc and VanRst are highlighted. The VanRst variants constructed by site-directed mutagenesis to be more similar to VanRsc are summarized on the right hand side.

FIG 5 Antibiotic resistance indexes summarizing the activity of the VanRst variants described in Fig. 4 in providing resistance against treatment with A47934 (A), teicoplanin (T) and vancomycin (V) in the *S. coelicolor* (**A**) and *S. toyocaensis* (**B**) genetic backgrounds. The status and origin of each component in the VanR/VanS system in each strain tested is illustrated in the first column of each table alongside the strain numbers (VanR/VanS proteins shaded dark grey originate from *S. coelicolor* and in contrast those shown in white originate from *S. toyocaensis*). Resistance indexes were determined from the ZID (in mm) and MIC (in μg/ml) values as described in the Materials and Methods, and the keys to these categories are presented beneath each table. The genotype of all strains is described in detail in Table 1, and the data from which the indexes are derived are presented in Figs. S4 and S5.

Table 1. Plasmids and bacterial strains used in this study.

Plasmid/Strains	Description/Genotype	Reference

Plasmids		
pMS81	ϕBT1 *attP-int* derived integration vector for the conjugal transfer of DNA from *E. coli* to *Streptomyces* spp. (Hyg^R)	(35)
pIJ10257	pMS81 carrying *ermEp* with ribosome binding site and multicloning sites (Hyg^R)	(26)
pIJ6902	integrative (∅C31 *attP-int*) and conjugative (*oriT* RK2), *tipAp* expression vector (Apr^R)($Thio^R$)	(30)
pHJH2000	pMS81 carryig *vanRS*st including *van* promoter sequence	This study
pHJH2002	pGEM-T easy carrying *vanR*st including *van* promoter sequence	This study
pHJH2003	pMS81 carryig carrying *vanR*st including *van* promoter sequence	This study
pHJH2005	pGEM-T easy carrying *vanS*st	This study
pDU006	pIJ10257 carryig *vanS*st under *ermEp*	This study
pHJH2006	pIJ6902 carryig *vanS*st under *ermEp*	This study
pJG001	pIJ10257 carrying *vanR*st under *ermEp*	This study
pGN078	pIJ10257 carrying *vanR*st (L10P, M12L) under *ermE*p	This study
pGN083	pIJ10257 carrying *vanR*st (V62I) under *ermEp*	This study
pGN082	pIJ10257 carrying *vanR*st (T69S) under *ermEp*	This study
pGN080	pIJ10257 carrying *vanR*st (Q92G) under *ermEp*	This study
pGN081	pIJ10257 carrying *vanR*st (R106Q, I109A, I112L) under *ermEp*	This study
pGN079	pIJ10257 carrying *vanR*st (L10P, M12L, R106Q, I109A, I112L) under *ermEp*	This study
pGN073	pMS81 carrying *vanRS*sc including *van* promoter sequence	This study
pHJH2001	pIJ10257 carrying *vanR*sc under *ermEp*	This study
pGN067	pMS81 carrying *vanR*sc including *van* promoter sequence	This study
pHJH2004	pGEM-T easy carrying *vanS*sc	This study
pDU001	pIJ10257 carrying *vanS*sc under *ermEp*	This study
Strains		
M600	*S. coelicolor* $SCP1^-$ $SCP2^-$	(33)
H27	*S. coelicolor* M600 + pMS81	(31)
J3201	*S. coelicolor* Δ*vanRS*sc $SCP1^-$ $SCP2^-$	(27)
H21	*S. coelicolor* Δ*vanR*sc $SCP1^-$ $SCP2^-$	This study
J3200	*S. coelicolor* Δ*vanS*sc $SCP1^-$ $SCP2^-$	(27)
H28	*S. coelicolor* J3201 + pMS81	This study
H2474	*S. coelicolor* H21 + pMS81	This study
H2473	*S. coelicolor* J3200 + pMS81	This study
H2060	*S. coelicolor* J3201 + pHJH2000	This study
H2123	*S. coelicolor* H21 + pHJH2003	This study
H36	*S. coelicolor* J3200 + pDU006	This study
H2297	*S. coelicolor* J3201 + pGM067	This study
H2062	*S. coelicolor* J3201 + pHJH2003	This study
H2255	*S. coelicolor* H21 + pGN078	This study
H2259	*S. coelicolor* H21 + pGN083	This study
H2328	*S. coelicolor* H21 + pGN082	This study
H2263	*S. coelicolor* H21 + pGN080	This study
H2500	*S. coelicolor* H21 + pGN081	This study
H2504	*S. coelicolor* H21 + pGN079	This study
H2257	*S. coelicolor* J3201 + pGN078	This study
H2261	*S. coelicolor* J3201 + pGN083	This study
H2329	*S. coelicolor* J3201 + pGN082	This study
H2265	*S. coelicolor* J3201 + pGN080	This study
H2501	*S. coelicolor* J3201 + pGN081	This study
H2505	*S. coelicolor* J3201 + pGN079	This study
NRRL15009	*S. toyocaensis* A47934 producer, wild type	(16)
H2251	*S. toyocaensis* NRRL15009 + pMS81	This study
H31	*S. toyocaensis* Δ*vanRS*st*::apr*	This study
H320	*S. toyocaensis* Δ*vanRS*st*::apr* + pHJH2006	This study
H33	*S. toyocaensis* Δ*vanS*st*::apr*	This study
H229	*S. toyocaensis* H31 + pMS81	This study
H2205	*S. toyocaensis* H320 + pMS81	This study
H2118	*S. toyocaensis* H33 + pMS81	This study
H2360	*S. toyocaensis* H31 + pGN073	This study
H2309	*S. toyocaensis* H320 + pGN067	This study
H312	*S. toyocaensis* H33 + pDU001	This study
H2476	*S. toyocaensis* H31 + pHJH2003	This study
H2311	*S. toyocaensis* H31 + pGN067	This study
H2233	*S. toyocaensis* H320 + pGN078	This study
H2239	*S. toyocaensis* H320 + pGN083	This study
H2336	*S. toyocaensis* H320 + pGN082	This study
H2245	*S. toyocaensis* H320 + pGN080	This study
H2502	*S. toyocaensis* H320 + pGN081	This study
H2506	*S. toyocaensis* H320 + pGN079	This study
H2231	*S. toyocaensis* H31 + pGN078	This study
H2237	*S. toyocaensis* H31 + pGN083	This study
H2338	*S. toyocaensis* H31 + pGN082	This study
H2243	*S. toyocaensis* H31 + pGN080	This study
H2503	*S. toyocaensis* H31 + pGN081	This study
H2507	*S. toyocaensis* H31 + pGN079	This study
ET12567 pUZ8002	*E. coli* ET12567 containing helper plasmid pUZ8002	(33)
BW25113 pIJ790	*E. coli* BW25113 containing helper plasmid pIJ790	(36)
BT340	*E. coli* DH5α containing pCP20 (temperature-sensitive FLP recombination plasmid)	(36)

629

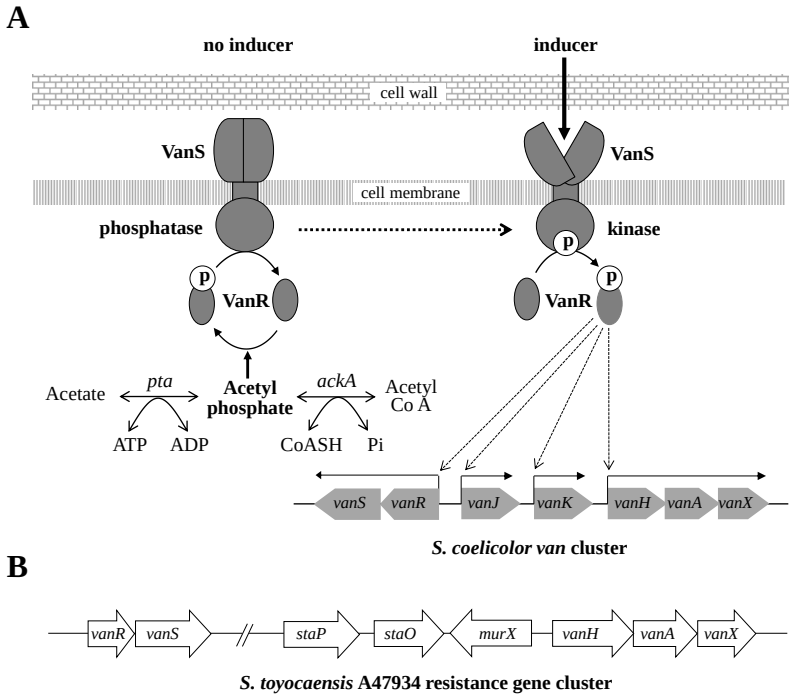


FIG 1 (**A**) A model illustrating the function and regulation of vancomycin resistance via the VanRsc/VanSsc TCS in *S. ceolicolor*. In the absence of inducer drug, the small molecule phosphate donor acetyl phosphate phosphorylates and activates VanR for inducing vancomycin resistance but this is effectively supressed by the phophatase activity of VanS. In the presence of inducer drug, VanS interacts with inducer drug and switches its activity from phosphatase to kinase, leading to a strong phosphorylation of VanR. The active phospho-VanR then binds to the four promoters of the *van* gene cluster and induces transcription of the *van* genes to render the *S. coelicolor* cells resistant to vancomycin. (**B**) Genetic organization of the A47934 resistance in *S. toyocaensis*.

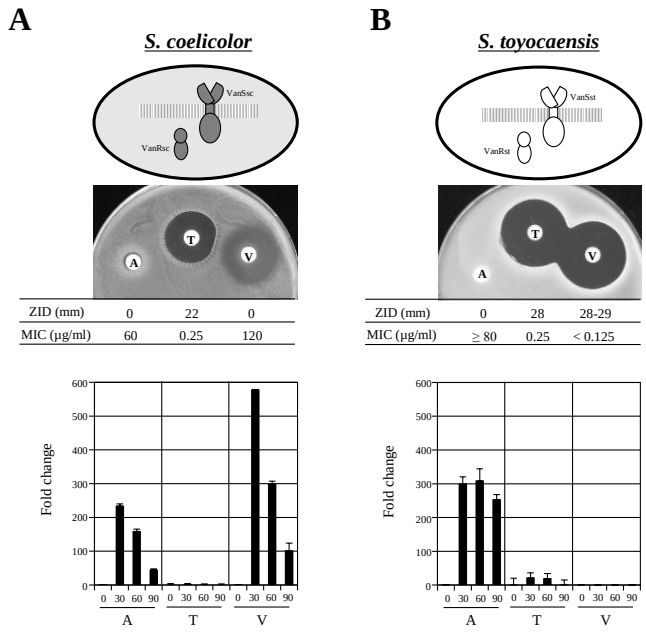
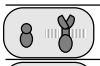
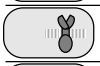
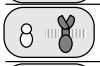


FIG 2 The bioassay activity of glycopeptide antibiotics against resistant strains correlates with the ability of the antibiotics to induce the VanR/VanS system. Bioassay analyzing the activity of three selected glycopeptide antibiotics A47934 (A), teicoplanin (T) and vancomycin (V) and their MIC against *S. coelicolor* M600 (**A**) and *S. toyocaensis* NRRL15009 (**B**). Paper discs containing 30 µg of each antibiotic were placed on freshly spread lawns of each strain, and plates were incubated for 2 days at 30˚C. A clear and strong dark colored halo around the paper discs indicate the zone of inhibition. The corresponding antibiotic zone of inhibition diameter (ZID; mm) and MIC (µg/ml) values are presented below the bioassay plates, together with quantification of the response in *vanH* transcription in each strain to each antibiotic as determined by qRT-PCR. Cells were grown to mid-exponential phase in NMMP liquid medium and exposed to 10 µg/ml of each antibiotic. Total RNAs were extracted from samples taken immediately before the addition of antibiotic (0) and at subsequent 30 min intervals up to 90 min (30, 60, 90). The x axis indicates time (min) after addition of the antibiotic, and the y axis shows the fold change in normalized *vanH* transcript abundance calculated relative to the abundance at time 0 which was defined as 1.

A

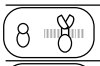
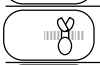
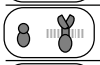
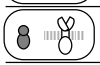
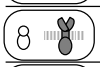
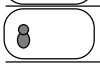
Resulting constructs in *S. coelicolor*			A47934	Teicoplain	Vancomycin
	VanRsc/VanSsc	(H27)	+++	-	+++++
	- -	(H28)	-	-	-
	- VanSsc	(H2474)	-	-	-
	VanRsc -	(H2473) or (H2297)	++	+	++
	VanRst/VanSst	(H2060)	++++	-	-
	VanRst/VanSsc	(H2123)	-	-	-
	VanRsc/VanSst	(H36)	+++++	+	-
	VanRst -	(H2062)	-	-	-

A47934
- : ZID > 14, MIC < 5
+ : ZID 7-14, MIC 4-30
++ : ZID (0), MIC 30-40
+++ : ZID < 7, MIC 40-60
++++: ZID < 7, MIC 60-70
+++++ : ZID < 7, MIC > 70

Teicoplanin
- : ZID > 20, MIC < 1
+ : ZID < 20, MIC 1-5

Vancomycin
- : ZID > 20, MIC < 1
+ : ZID 7-20, MIC 1-30
++ : ZID (0), MIC 30-60
+++ : ZID < 7, MIC 30-60
++++: ZID < 7, MIC 60-80
+++++ : ZID < 7, MIC > 80

B

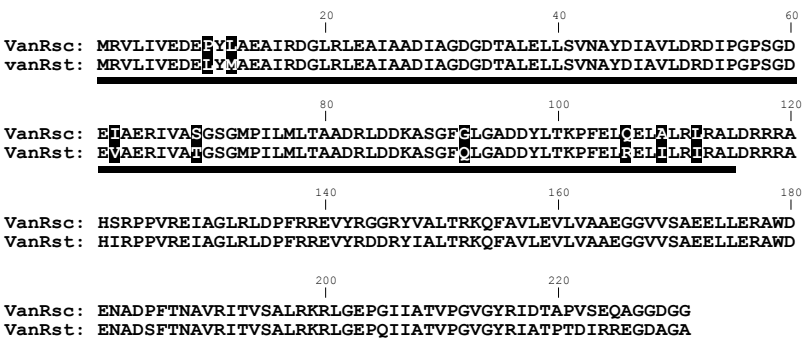
Resulting constructs in *S. toyocaensis*			A47934	Teicoplain	Vancomycin
	VanRst/VanSst	(H2251)	+++++	-	-
	- -	(H229)	-	-	-
	- VanSst	(H2205)	-	-	-
	VanRst -	(H2118) or (H2476)	-	-	-
	VanRsc/VanSsc	(H2360)	++++	-	+
	VanRsc/VanSst	(H2309)	+++++	+	-
	VanRst/VanSsc	(H312)	-	-	-
	VanRsc -	(H2311)	++	+	+

A47934
- : ZID > 19, MIC < 5
+ : ZID 7-19, MIC 4-40
++ : ZID (0), MIC 40-60
+++ : ZID < 7, MIC 40-60
++++: ZID < 7, MIC 60-80
+++++ : ZID < 7, MIC > 80

Teicoplanin
- : ZID > 28, MIC < 1
+ : ZID < 28, MIC 1-5

Vancomycin
- : ZID > 28, MIC < 1
+ : ZID < 28, MIC 1-5

FIG 3 Antibiotic resistance indexes summarizing the activity of different combinations of VanRS proteins in providing resistance against treatment with A47934 (A), teicoplanin (T) and vancomycin (V) in the *S. coelicolor* (**A**) and *S. toyocaensis* (**B**) genetic backgrounds. The status and origin of each component in the VanR/VanS system in each strain tested is illustrated in the first column of each table alongside the strain numbers (VanR/VanS proteins shaded dark grey originate from *S. coelicolor* and in contrast those shown in white originate from *S. toyocaensis*). Resistance indexes were determined from the ZID (in mm) and MIC (in µg/ml) values as described in the Materials and Methods, and the keys to these categories are presented beneath each table. The genotype of all strains is described in detail in Table 1, and the data from which the indexes are derived are presented in Figs. S1 and S2.



Mutations in N-terminus REC domain of VanRst

VanRst(1) = VanRst(L10P+M12L)

VanRst(2) = VanRst(V62I)

VanRst(3) = VanRst(T69S)

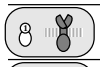
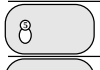
VanRst(4) = VanRst(Q92G)

VanRst(5) = VanRst(R106Q+I109A+I112L)

VanRst(6) = VanRst(L10P+ M12L+R106Q+I109A+I112L)

FIG 4 Amino acid sequence alignment of VanRsc and VanRst. The black bar beneath the sequences indicates the conserved N-terminal REC domain. The eight amino acid residues which differ in the REC domain between VanRsc and VanRst are highlighted. The VanRst variants constructed by site-directed mutagenesis to be more similar to VanRsc are summarized on the right hand side.

A

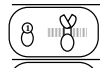
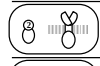
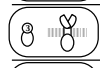
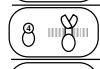
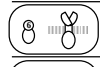
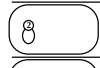
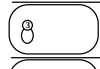
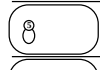
Resulting constructs in *S. coelicolor*	A47934	Teicoplain	Vancomycin
 VanRst(1)/VanSsc (H2255)	-	-	-
 VanRst(2)/VanSsc (H2259)	-	-	-
 VanRst(3)/VanSsc (H2328)	+	+	-
 VanRst(4)/VanSsc (H2265)	-	-	-
 VanRst(5)/VanSsc (H2500)	-	-	-
 VanRst(6)/VanSsc (H2504)	-	-	-
 VanRst(1) - (H2257)	-	-	-
 VanRst(2) - (H2261)	-	-	-
 VanRst(3) - (H2329)	+	+	-
 VanRst(4) - (H2265)	-	-	-
 VanRst(5) - (H2501)	-	-	-
 VanRst(6) - (H2505)	-	-	-

A47934
- : ZID > 14, MIC < 5
+ : ZID 7-14, MIC 4-30
++ : ZID (0), MIC 30-40
+++ : ZID < 7, MIC 40-60
++++: ZID < 7, MIC 60-70
+++++ : ZID < 7, MIC > 70

Teicoplanin
- : ZID > 20, MIC < 1
+ : ZID < 20, MIC 1-5

Vancomycin
- : ZID > 20, MIC < 1
+ : ZID 7-20, MIC 1-30
++ : ZID (0), MIC 30-60
+++ : ZID < 7, MIC 30-60
++++: ZID < 7, MIC 60-80
+++++ : ZID < 7, MIC > 80

B

Resulting constructs in *S. toyocaensis*	A47934	Teicoplain	Vancomycin
 VanRst(1)/VanSst (H2233)	+++++	-	-
 VanRst(2)/VanSst (H2239)	+++++	-	-
 VanRst(3)/VanSst (H2336)	+++++	-	-
 VanRst(4)/VanSst (H2245)	+++++	-	-
 VanRst(5)/VanSst (H2502)	+++++	-	-
 VanRst(6)/VanSst (H2506)	+++++	+	-
 VanRst(1) - (H2231)	-	-	-
 VanRst(2) - (H2237)	-	-	-
 VanRst(3) - (H2338)	+	+	-
 VanRst(4) - (H2243)	-	-	-
 VanRst(5) - (H2503)	-	-	-
 VanRst(6) - (H2507)	-	-	-

A47934
- : ZID > 19, MIC < 5
+ : ZID 7-19, MIC 4-40
++ : ZID (0), MIC 40-60
+++ : ZID < 7, MIC 40-60
++++: ZID < 7, MIC 60-80
+++++ : ZID < 7, MIC > 80

Teicoplanin
- : ZID > 28, MIC < 1
+ : ZID < 28, MIC 1-5

Vancomycin
- : ZID > 28, MIC < 1
+ : ZID < 28, MIC 1-5

FIG 5 Antibiotic resistance indexes summarizing the activity of the VanRst variants described in Fig. 4 in providing resistance against treatment with A47934 (A), teicoplanin (T) and vancomycin (V) in the *S. coelicolor* (**A**) and *S. toyocaensis* (**B**) genetic backgrounds. The status and origin of each component in the VanR/VanS system in each strain tested is illustrated in the first column of each table alongside the strain numbers (VanR/VanS proteins shaded dark grey originate from *S. coelicolor* and in contrast those shown in white originate from *S. toyocaensis*). Resistance indexes were determined from the ZID (in mm) and MIC (in µg/ml) values as described in the Materials and Methods, and the keys to these categories are presented beneath each table. The genotype of all strains is described in detail in Table 1, and the data from which the indexes are derived are presented in Figs. S4 and S5.