The VanRS Homologous Two-Component System VnlRS$_{Ab}$ of the Glycopeptide Producer Amycolatopsis balhimycina Activates Transcription of the vanHAX$_{Sc}$ Genes in Streptomyces coelicolor, but not in A. balhimycina

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In enterococci and in Streptomyces coelicolor, a glycopeptide nonproducer, the glycopeptide resistance genes $vanHAX$ are colocalized with $vanRS$. The two-component system (TCS) VanRS activates $vanHAX$ transcription upon sensing the presence of glycopeptides. Amycolatopsis balhimycina, the producer of the vancomycin-like glycopeptide balhimycin, also possesses $vanHAX_{Ab}$ genes. The genes for the VanRS-like TCS VnlRS$_{Ab}$ together with the carboxypeptidase gene $vanY_{Ab}$, are part of the balhimycin biosynthetic gene cluster, which is located 2 Mb separate from the $vanHAX_{Ab}$. The deletion of vnlRS$_{Ab}$ did not affect glycopeptide resistance or balhimycin production. In the A. balhimycina vnlR$_{Ab}$ deletion mutant, the $vanHAX_{Ab}$ genes were expressed at the same level as in the wild type, and peptidoglycan (PG) analyses proved the synthesis of resistant PG precursors. Whereas $vanHAX_{Ab}$ expression in A. balhimycina does not depend on VnlR$_{Ab}$, a VnlR$_{Ab}$-depending regulation of $vanY_{Ab}$ was demonstrated by reverse transcriptase polymerase chain reaction (RT-PCR) and RNA-seq analyses. Although VnlR$_{Ab}$ does not regulate the $vanHAX_{Ab}$ genes in A. balhimycina, its heterologous expression in the glycopeptide-sensitive S. coelicolor $\Delta$vanRS$_{Sc}$ deletion mutant restored glycopeptide resistance. VnlR$_{Ab}$ activates the $vanHAX_{Sc}$ genes even in the absence of VanS. In addition, expression of vnlR$_{Ab}$ increases actinorhodin production and influences morphological differentiation in S. coelicolor.

Introduction

Bacteria need to respond to changes in their environment. Therefore, they require adequate means to gain and process information on the immediate surroundings. Such means are represented by two-component systems (TCSs), which are ubiquitous in all prokaryotes. A typical TCS consists of a sensor histidine kinase (HK) and a response regulator (RR). The HK measures a specific external signal and autophosphorylates at a conserved histidine residue within the cytosol. This phosphoryl group is transferred to the associated RR. The activated RR initiates the cellular response. Most RRs are transcription factors that not only change the gene expression pattern of one or more genes of the cell, but also post-transcriptional and post-translational regulation of RNAs and proteins, respectively, by RRs has been reported. The ability of bacteria to sense the signal enables them to react with an adaptive response.

Of special interest is the glycopeptide-sensing TCS VanRS that controls the expression of glycopeptide resistance genes in gram-positive pathogens, some glycopeptide producers, and other actinomycetes. VanS is a membrane-standing HK. Its C-terminus extends into the cytoplasm and contains the kinase domain and the phosphorylation site. VanS senses the presence of glycopeptides and catalyses adenosine triphosphate-dependent autophosphorylation of a specific histidine residue. Subsequently, VanS transfers the phosphate group to an aspartate residue of VanR, which then activates the transcription of the resistance genes. However, under noninduction conditions, VanS acts as a phosphatase, removing the phosphate group from VanR.

Glycopeptides such as vancomycin, teicoplanin, and telavancin are used for treating infections caused by gram-positive pathogens. They act by binding to the N-acyl-d-alanyl-d-alanine (d-Ala-d-Ala) termini of peptidoglycan (PG) and its precursor lipid II. This binding effectively sequesters the substrate for the transglycosylases and the d,d-transpeptidases,
Two key enzymes of cell wall synthesis, resulting in an inability to grow and subsequently to cell death.

Glycopeptide resistance is mediated by reprogramming cell wall biosynthesis. Ten types of resistances have been characterized so far (VanA-N). In each case, the terminal D-alanine (D-Ala) in the pentapeptide side chain of the PG of gram-positive bacteria is substituted either by a D-lactate (D-Lac) (VanA, B, D, F, and M) or a D-serine (VanC, E, G, L, and VanN). These substitutions result in a 1000-fold or 6-fold decrease in binding affinity of the glycopeptide to its target, respectively. Categorization into the different phenotypes is based on the inducibility, the breadth of resistance to individual compounds, and the level of resistance.

Three of those phenotypes, VanC, VanD, and VanN, are constitutively expressed. All others are inducible to different degrees by different glycopeptides. It was shown that enterococcus and staphylococcus strains expressing glycopeptide resistance genes constitutively are impaired in growth in comparison with strains where the genes are inducible. Apparently, careful control of the expression of these genes is advantageous.

Streptomyces coelicolor A3(2) is neither a pathogen nor a glycopeptide producer, but it is likely to encounter glycopeptides in its natural habitat. Therefore, it benefits from cross-bridge amino acid(s) to the stem pentapeptide of PG precursors.

Materials and Methods

Bacterial strains, plasmids, and primers

The strains and plasmids used for this study are listed in Table 1, the primers used for this study are listed in Table 2. E. coli ET1256729 was used to obtain unmethylated DNA for A. balhimycina transformations.

![EMBOSS stretcher pairwise sequence alignment of VnlRAb and VanRSc](Image)

**FIG. 1.** (A, B) EMBoss stretcher pairwise sequence alignment of VnlRSc and VanRSSc. (A) EMBoss stretcher pairwise sequence alignment of VnlRSc and VanRSSc. Transmembrane domains are indicated in blue. The extracytosolic domain is highlighted by a red box. (B) EMBoss stretcher pairwise sequence alignment of VnlRSc and VanRSSc. The site of aspartate phosphorylation is indicated by red and that of the proposed autophosphorylation by green arrow. (C) Organization of the resistance genes in *Streptomyces coelicolor* compared with that of *Amycolatopsis balhimycina*. “-” for a mismatch or a gap; “.” for any small positive score; “:” for a similarity, which scores more than 1.0; and “I” for an identity where both sequences have the same residue.
**Table 1. Bacterial Strains Used in This Study**

| Strains | Relevant feature(s) | References |
|---------|---------------------|------------|
| *Streptomyces coelicolor* A3(2) | | |
| M600 | | |
| M600ΔvanRS<sub>sc</sub> (J2301) | SCP1, SCP2 | 30 |
| M600ΔvanRS<sub>sc</sub>[vnlRS<sub>Ab</sub>] | vanRS<sub>sc</sub> deletion mutant | 6 |
| M600ΔvanRS<sub>sc</sub>[vnlRS<sub>Ab</sub>] | ΔvanRS<sub>sc</sub> complemented with pRM4vnlRS<sub>Ab</sub> | This study |
| M600ΔvanRS<sub>sc</sub>[vnlRS<sub>Ab</sub>] | ΔvanRS<sub>sc</sub> complemented with pRM4vnlRS<sub>Ab</sub> | This study |
| M600ΔvanRS<sub>sc</sub>[vnlRS<sub>Ab</sub>D51A] | ΔvanRS<sub>sc</sub> complemented with pRM4vnlRS<sub>Ab</sub>D51A | This study |
| Amycolatopsis balhimycina DSM 5908 | | |
| *A. balhimycina* WT DSM 5908 | Wildtype | 24 |
| *A. balhimycina* ΔvanRS<sub>Ab</sub> | vanRS<sub>Ab</sub> deletion mutant | 27 |
| *A. balhimycina* vnlRS<sub>Ab</sub> | Overexpression of vnlRS<sub>Ab</sub> in *A. balhimycina*, using pRM4vnlRS<sub>Ab</sub> | This study |
| *A. balhimycina* ΔvnlRS<sub>Ab</sub> | ΔvnlRS<sub>Ab</sub> complemented with pRM4vnlRS<sub>Ab</sub> | This study |
| *A. balhimycina* ΔvnlRS<sub>Ab</sub>[vnlRS<sub>Ab</sub>] | vnlRS<sub>Ab</sub> deletion mutant | This study |
| *Escherichia coli* | | |
| XL1-blue | recA1; endA1; gyrA96; tji-1; hsdR17; supE44; relA1; lac [F'proAB, lac<sup>e</sup>ZAM15Tn10(ter<sup>1</sup>)] | 28 |
| ET 12567 pUZ8002 | pUZ8002; <i>kan</i> | 34 |
| ET 12567 | F<sup>*</sup>; dam13::Tn9; dcm-6; hsdM; hsdR; recF143; jj201::Tn10; galK2; galT22; ara14; lacY1; xyl15; leuB6; thi1; tonA31; rpsL136; hisG4; tss78; mili; gltV44 | 29 |
| Plasmids | | |
| pRM4 | pSET152 erm<sup>Ep</sup>*; RBS, F<sub>31</sub> attP-int-derived integration vector | 31 |
| pRM4vnlRS<sub>Ab</sub> | Expression plasmid for vnlRS<sub>Ab</sub> | This study |
| pRM4vnlRS<sub>Ab</sub> | Expression plasmid for vnlRS<sub>Ab</sub> | This study |
| pRM4vnlRS<sub>Ab</sub>D51A | Expression plasmid for vnlRS<sub>Ab</sub>D51A | This study |
| pSP1 | Inactivation vector in *A. balhimycina* | 32 |
| pSPΔvnlRS<sub>Ab</sub> | Erythromycin and ampicillin resistance | |
| pSPΔvnlRS<sub>Ab</sub> | pSP1 carrying a 1579 bp upstream and a 1509 bp downstream fragment of vnlRS<sub>Ab</sub> | This study |

** WT, wild type. **

*A. balhimycina*<sup>24</sup> is the balhimycin-producing wild type (WT) and was used to generate the vnl<sub>S</sub> deletion as well as the vnl<sub>R</sub>-overexpressing strains (this study). Furthermore, Δvnl<sub>S</sub> deletion<sup>27</sup> was used for complementation (this study).

*S. coelicolor* M600<sup>30</sup> were used to generate *S. coelicolor* M600ΔvanRS<sub>sc</sub>. This deletion strain was used to generate complementations with vnlRS<sub>Ab</sub>, vnlRS<sub>Ab</sub>, vnlRS<sub>Ab</sub>, and vnlRS<sub>Ab</sub>D51A (this study).

The overexpression plasmids pRM4vnlRS<sub>Ab</sub>, pRM4vnlRS<sub>Ab</sub>, pRM4vnlRS<sub>Ab</sub>, and pRM4vnlRS<sub>Ab</sub>D51A are derived from pRM4<sup>31</sup>, a pSET152-derived nonreplicative, F<sub>31</sub> integrative vector with an integrated constitutive erm<sup>Ep</sup>* promoter, an artificial ribosomal binding site, and an apramycin resistance cassette.

The deletion vector pSPΔvnlRS<sub>Ab</sub> is derived from pSP1<sup>32</sup> in which flanking regions of vnlRS<sub>Ab</sub> were cloned.

**Media and culture conditions**

*A. balhimycina* grown in 100 ml TSB medium (Difco) for 48 hr and 2 ml of this preculture were used to inoculate the main cultures either in 100 ml R5<sup>30</sup> or in TSB medium. R5 medium was used to stimulate balhimycin production, while TSB medium was used when balhimycin production should be prevented. After 48 hr of cultivation, the mycelium was used to isolate PG precursors, to extract DNA, or to perform resistance assays against different glycopeptides. To isolate RNA, the cells were grown 15/39/63 hr. Balhimycin production assays were performed after 5 days of growth.

*S. coelicolor* M145 and M600 were grown on Cullum-agar plates for sporulation. Isolated spores were used to inoculate 10 ml R5 medium as preculture for cell wall precursor extraction or DNA extraction. For RNA isolation, 2 ml of a 48-hr-old TSB preculture was used to inoculate 100 ml of HA medium. The cells were harvested after 69 hr.

To compare the growth of the different *S. coelicolor* strains, 10 μl spores (∼1.5 × 10<sup>17</sup>) of each strain were streaked on a YM plate. The plate was incubated for 7 days.

*A. balhimycina* and *S. coelicolor* were grown at 30°C, and liquid cultures were shaken at 180 rpm.

*A. balhimycina* and *S. coelicolor* strains were cultivated in 100 ml of R5 medium in an orbital shaker (220 rpm) in 500-ml baffled Erlemeyer flasks at 27°C.

Liquid/solid media were supplemented with 100 μg/ml apramycin to select for strains carrying integrated antibiotic resistance genes.
E. coli was grown in Luria-Bertani broth (Roth) at 37°C using 100 mg/ml apramycin or 150 mg/ml ampicillin for selection of plasmid-containing colonies. Liquid cultures were shaken at 180 rpm.

**Plasmid construction**

For the heterologous expression in *S. coelicolor DvanRSSc*, the entire coding regions of the *vnlRAb* (Table 2 primer 1 + 3), *vnlRSAb* (Table 2 primer 1 + 2), and *vnlSAb* (primer 4 + 2) were amplified using Kapa-Hifi proofreading polymerase and the corresponding primers in brackets.

The *vnlRSAb* (758 bp), *vnlRSAb* (1908 bp), and *vnlSAb* (1198 bp) polymerase chain reaction (PCR) products were integrated into pRM431 through the primer-attached restriction sites (NdeI-EcoRI) downstream of the *ermEp* promoter.

Site-directed mutagenesis by overlap extension was performed for the exchange of aspartate at position 51 to an alanine with the primers 5 + 6 (Table 2). The 758 bp PCR product *vnlRSAbD51A* was integrated into pRM4 through the primer-attached restriction sites (NdeI-EcoRI) downstream of the *ermEp* promoter.

For the in-frame deletion of *vnlSAb* (1125 bp), a 1579 bp upstream fragment (Table 2 primer 7 + 8) and a 1198 bp downstream fragment (Table 2 primer 9 + 10) of *vnlSAb* were amplified from *A. balhimycina* genomic DNA using Kapa-Hifi proofreading polymerase and the corresponding primers in brackets. The plasmid pSPDvnlS was constructed by integration of the fragments in pSP130 through the primer-attached restriction sites at the 5′ and 3′ ends (EcoRI/XbaI and XbaI/SphI).

**DNA transfer**

Transformation of *E. coli* XL1-blue was performed as described previously. Plasmids pRM4vnRSAb, pRM4vnRSAb, pRM4vnSAb, and pRM4vnSAb,D51A were transferred into *S. coelicolor* through intergeneric conjugation. Plasmid integration was confirmed by colony PCR using the primer pair 12 + 13 (Table 2) or primer 11 (Table 2) in combination with a reverse primer of corresponding gene.

pRM4vnRSAb and pRM4vnRSAb,D51A were transferred into *A. balhimycina* through the direct transformation method using unmethylated plasmid DNA isolated from *E. coli* ET12567. Integration of plasmid was verified by PCR using primer pair 11 + 3 (Table 2).
For deletion of \( vnlS_{A.~balhimycina} \), the \( A.~balhimycina \) WT was transformed with pSPA\( vnlS_{A.~balhimycina} \) by direct transformation. The integration of the plasmid into the chromosome through homologous recombination was confirmed by PCR screening for the erythromycin resistance cassette, using primers \( \text{ery for and ery rev} \) (Table 2). To obtain deletion mutants, a second homologous recombination event was provoked by stressing plasmid-carrying colonies as described by Puk et al.\(^{38} \) Colonies were examined for sensitivity to erythromycin, and the deletions were verified by PCR analysis, using primers \( 16+17 \) (Table 2).

### Sequence alignment

The amino acid (AA) sequences of \( VnLR_{A.~balhimycina} \), \( VnR_{S.~coelicolor} \), and \( VnS_{S.~coelicolor} \) are available under accession number Y16952 (named \( VnRS \)), (SCO3590), and (SCO3589), respectively. Alignment of the AA sequences was performed by EMBL stretcher\(^{39} \); (www.ebi.ac.uk/Tools/psa/emboss_stretcher/).

### Resistance test, reverse transcriptase polymerase chain reaction analyses, PG precursor, and cell wall analysis

Resistance test, reverse transcriptase polymerase chain reaction (RT-PCR) analyses, extraction of PG precursors, PG isolation, and the high-performance liquid chromatography–mass spectrometry (HPLC-MS) analyses were performed as described.\(^{27,40} \)

### Balhimycin concentration

The balhimycin concentration in 1 ml culture was quantified using HPLC with a photodiode array detector (HPLC-DAD) as described.\(^{40} \) The balhimycin concentration was calculated to 100 \( \mu \text{g/ml} \) total DNA.

### Inference of biomass concentration from DNA quantification

For the quantification of total DNA in 1 ml culture, an acid extraction of DNA coupled with a colorimetric method\(^{41} \) was performed by measuring the absorbance at 600 nm. To analyze the amount of DNA, a standard curve with salmon sperm DNA was generated.

### Results

**A. balhimycina includes a VanRS homologous TCS (VnLR_{A.~balhimycina}) encoded in the balhimycin biosynthetic gene cluster**

In most of the antibiotic-producing bacteria, the antibiotic biosynthetic gene clusters include resistance genes. One exception is the balhimycin producer \( A.~balhimycina \). In this study, the glycopeptide resistance genes \( vanHAX_{A.~balhimycina} \) are located 2 Mb apart from the balhimycin biosynthetic gene cluster. In addition, the resistance is characterized by another unusual feature: the counterpart of the well-known TCS \( VanRS \), which is known to regulate \( vanHAX \) expression in pathogens and in \( S.~coelicolor \) is encoded by genes \( (vnlRS_{A.~balhimycina}) \), which are part of the biosynthetic gene cluster and are therefore not colocated with the \( vanHAX_{A.~balhimycina} \) genes.

\( VanR_{S.~coelicolor} \) was reported to sense glycopeptides and to activate the expression of the \( vanHAX_{S.~coelicolor} \) genes.\(^{19} \) To elucidate the differences of the two actinomycete TCSSs, we compared the AA sequence of \( VnLR_{A.~balhimycina} \) with the sequence of \( VanR_{S.~coelicolor} \). Sequence alignment using EMBL stretcher\(^{39} \) revealed 82% sequence similarity between \( VnLR_{A.~balhimycina} \) and \( VanR_{S.~coelicolor} \) (Sco3590) and 73% between \( VnRS_{A.~balhimycina} \) and \( VanSS_{S.~coelicolor} \) (SCO3589) (Fig. 1A, B). Based on the high similarity, a corresponding function of both RRs could be proposed.

In \( S.~coelicolor \), \( VanSS_{S.~coelicolor} \) phosphorylates \( VanR_{S.~coelicolor} \) at the aspartate at AA position 51. Replacement of this residue with an alanine completely destroyed the activity of \( VanR_{S.~coelicolor} \).\(^{6} \) It has been shown that in \( S.~coelicolor \), in the absence of vancomycin, acetylphosphate phosphorylates \( VanR_{S.~coelicolor} \), whereas \( VanS_{S.~coelicolor} \) acts as a phosphatase to decrease the level of \( VanR_{S.~coelicolor} \),\(^{*} \) P. On exposure to vancomycin, \( VanS_{S.~coelicolor} \) activity switches from a phosphatase to a kinase and vancomycin resistance is induced.\(^{6} \) Furthermore, Novotna et al.\(^{42} \) specified a serine residue at AA position 69 important for autophosphorylation through acetyl phosphate.

Sequence comparison revealed that \( VnLR_{A.~balhimycina} \) contains both, a conserved aspartate at AA position 51 (D51) and a serine at AA position 69, the position that probably becomes phosphorylated.\(^{27} \) This raises the interesting question on the function of this TCS in \( A.~balhimycina \).

Since overexpression of RRs of two-component signal transduction systems often modulates multidrug resistance,\(^{43,44} \) we overexpressed \( VnLR_{A.~balhimycina} \) to analyze the effects on resistance and antibiotic production. \( VnLR_{A.~balhimycina} \) was cloned under the control of the constitutive promoter \( ermE^{-}p \) into the integrative plasmid \( pRM4 \) (pRM4\( vnlR_{A.~balhimycina} \)). \( pRM4\( vnlR_{A.~balhimycina} \) was transferred into \( A.~balhimycina \) WT and into the \( A.~balhimycina \) \( \Delta vnlR_{A.~balhimycina} \) mutant,\(^{27} \) resulting in the recombinant strains \( A.~balhimycina \) \( \Delta vnlR_{A.~balhimycina} \) \( [vnlR_{A.~balhimycina}] \) and \( A.~balhimycina \) \( \Delta vnlR_{A.~balhimycina} \) \( \Delta vnlR_{A.~balhimycina} \) \( [vnlR_{A.~balhimycina}] \), respectively. The phenotypes of the recombinant strains overexpressing \( VnLR_{A.~balhimycina} \) and (as a control) that of the deletion mutant \( A.~balhimycina \) \( \Delta vnlR_{A.~balhimycina} \) \( \Delta vnlR_{A.~balhimycina} \), were compared with the WT phenotype (Fig. 2). All strains produced balhimycin at the same level (Fig. 2A). No differences in resistance against balhimycin were observed. Using a method optimized for actinomycetes,\(^{27} \) muropeptides from all \( A.~balhimycina \) strains cultivated under balhimycin production conditions were isolated. HPLC/MS chromatograms showed the similar muropeptide composition pattern for all strains (Fig. 2B).

In addition to muropeptides, the PG precursors were analyzed. For this purpose, we cultivated the strains under balhimycin production conditions and conditions under which balhimycin production is disabled. Under production as well as under nonproduction conditions, \( A.~balhimycina \) WT, \( A.~balhimycina \) \( \Delta vnlR_{A.~balhimycina} \) \( \Delta vnlR_{A.~balhimycina} \), and \( A.~balhimycina \) \( \Delta vnlR_{A.~balhimycina} \) \( \Delta vnlR_{A.~balhimycina} \) \( \Delta vnlR_{A.~balhimycina} \), produced resistant PG precursors ending with \( \text{d-Ala-d-Lac} \) (Fig. 2C). Only \( A.~balhimycina \) WT and \( A.~balhimycina \) \( \Delta vnlR_{A.~balhimycina} \) \( \Delta vnlR_{A.~balhimycina} \) produced resistant PG precursors.
produced traces of precursors ending with d-Ala-d-Lac under nonproduction conditions (Fig. 2C). These results suggest that VnlRAb does not regulate the synthesis of resistance PG in *A. balhimycina*.

RT-PCR analyses revealed that a *vanHAX*Ab transcript was detectable in *A. balhimycina* ΔvnlRAb, confirming that the expression of *vanHAX*Ab is independent of vnlRAb (Fig. 3).

In *A. balhimycina*, sensing of glycopeptides through VnlSAb is not required for expressing the resistance genes.

In enterococci and in *S. coelicolor*, the RR VanRsSc becomes phosphorylated by the HK VanSSc. To analyze whether and how VnlRAb interacts with VnlSAb, we constructed an in-frame ΔvnlSAb mutant of *A. balhimycina* using the inactivation plasmid pSPΔvnlSAb. This plasmid containing a 1509 bp downstream fragment and a 1579 bp upstream fragment of vnlSAb was introduced into *A. balhimycina*.

**FIG. 2.** Analysis of balhimycin production, muropeptide composition, and PG precursors in *A. balhimycina* WT (1), *A. balhimycina* ΔvnlRAb (2), *A. balhimycina* [vnlRAb] (3), and *A. balhimycina* ΔvnlRAb [vnlRAb] (4). (A) Production of balhimycin measured by HPLC (*n* = 5). (B) HPLC/MS chromatogram of the muropeptides (positive mode). The first bracket embraces the peaks representing muropeptide monomers, the second the muropeptide dimers. (C) Extracted ion chromatograms of the negative mode from the PG precursors isolated from cells grown in R5 (balhimycin production) and in TSB (no balhimycin production). d-Lac, Pentapeptide precursors ending on d-Ala-d-Lac 1194 m/z at retention time ~18 min. d-Ala, Pentapeptide precursors ending on d-Ala-d-Ala 1193 m/z at retention time ~12 min. HPLC, high-performance liquid chromatography; MS, mass spectrometry; PG, peptidoglycan; WT, wild type.

**FIG. 3.** RT-PCR analyses of *vanHAX*Ab and *vanY*Ab in *A. balhimycina* WT, *A. balhimycina* ΔvnlRAb, and in *A. balhimycina* WT overexpressing vnlRAb (WT [vnlRAb]). RNA was isolated at different time points (15/39/63 hr) from the three strains cultivated in balhimycin production medium R5. sigB: transcription of the housekeeping gene sigB. *vanHAX*Ab and *vanY*Ab: transcription of *vanHAX*Ab and *vanY*Ab. For PCR, genomic DNA (gDNA) was used as positive control.
through direct transformation. Successive homologous recombination resulted in the deletion of vnlSAb. A. balhimycina ΔvnlSAb showed neither a defect in balhimycin production nor resistance toward glycopeptides. In addition, no changes in the PG precursor and in the nascent PG composition in comparison with A. balhimycina WT were observed (data not shown). These results suggested that sensing the presence of glycopeptides does not correlate with balhimycin production and glycopeptide resistance. Apparently, the expression of the vanHAXAb genes occurs independently of VnlSAb.

VnlRAb is able to activate vanHAXSc transcription in S. coelicolor

In silico analyses revealed similar characteristics of VnlRSAb compared with VanRSSc. However, as shown above, the VnlRSAb system in A. balhimycina, in contrast to VanRSSc in S. coelicolor, does not regulate the vanHAXAb. To clarify the contradictory findings, the genes encoding the TCS VnlRSAb as well as VnlRAb and VnlSAb individually were transferred into the S. coelicolor mutant strain, in which the vanRSSc genes were deleted, to elucidate the ability of VnlRAb to activate the vanHAXSc genes in the S. coelicolor mutant. vnlRSAb, vnlRAb, and vnlSAb were introduced into S. coelicolor ΔvanRSSc under the control of the constitutive promoter ermEp* using the integrative plasmid pRM4vnlRAb. The growth of the recombinant strains was tested on glycopeptide-containing plates.

Introduction of vnlRSAb and of vnlRAb alone into S. coelicolor M600 ΔvanRSSc resulted in balhimycin-resistant strains (Fig. 4). In contrast, expression of vnlSAb alone did not change the glycopeptide-sensitive phenotype of the S. coelicolor ΔvanRSSc mutant. These results indicated that VnlRAb from A. balhimycina is able to activate the transcription of vanHAXSc in S. coelicolor M600 also in the absence of VnlSAb. Since in S. coelicolor M600 VanRSSc ~ P can be generated in a VanSAb-independent manner using acetylphosphate, we suggest a similar activation of VnlRAb in the absence of VnlSAb or VanSSc.

The activation of the vanHAXSc genes in the complemented S. coelicolor M600 ΔvanRSSc mutant with vnlRSAb and vnlRAb was further analyzed by RT-PCR. For this purpose, RNA was isolated from 25-hr-old liquid cultures grown without addition of any glycopeptide. A vanHAXSc transcript was detected when S. coelicolor M600 ΔvanRSSc was complemented with vnlRSAb or with vnlRAb alone. However, in S. coelicolor M600 and in the S. coelicolor M600 ΔvanRSSc mutant, transcription of the vanHAXSc failed (Fig. 5), confirming the functionality of VnlRAb as transcriptional activator in S. coelicolor.

To investigate whether transcription of the vanHAXSc genes indeed resulted in the formation of glycopeptide-resistant PG precursors, which caused the resistant phenotype, we used HPLC/MS to analyze the PG precursor composition of S. coelicolor M600 ΔvanRSSc and of S. coelicolor M600 ΔvanRSSc complemented either with vnlRSAb or vnlRAb. Complementing S. coelicolor M600 ΔvanRSSc with vnlRSAb or with vnlRAb restored the synthesis of resistant PG precursors. In the presence of balhimycin exclusively, PG precursors ending with α-Ala-d-Lac were synthesized (Fig. 6C, D). The PG precursor composition of the glycopeptidase-sensitive S. coelicolor M600 ΔvanRSSc mutant was analyzed.

**FIG. 4.** Growth and resistance of the S. coelicolor M600 ΔvanRSSc complemented with different combinations of vnlRSAb. (A) Growth on YM agar containing no antibiotic. (B) Growth on YM agar containing apramycin (100 mg/ml) (Apra 100) to prove plasmid integration. (C) Growth on YM agar containing balhimycin (10 mg/ml) (Bal 10). (D) Growth on YM agar containing teicoplanin (10 mg/ml) (Teico 10). (E) Growth on YM agar containing no antibiotic. M600, S. coelicolor M600.

**FIG. 5.** RT-PCR analyses of S. coelicolor M600 and different S. coelicolor M600 mutants. RNA was isolated after 25 hr of cultivation in the absence of any glycopeptide. hrdB: transcription of the housekeeping gene hrdBSc, vanSSc, vanRSc, and vanHAXSc; transcription of vanSSc, vanRSc, and vanHAXSc. For PCR, genomic DNA (gDNA) was used as positive control.
FIG. 6. Extracted ion chromatograms (negative mode) of the PG precursors isolated from cells grown in R5 medium without antibiotic or with 25 mg/ml balhimycin (bal25). M600 ∆vanRS<sub>Sc</sub>, S. coelicolor M600 ∆vanRS<sub>Sc</sub>; d-Lac, pentapeptide precursors ending on d-Ala-d-Lac 1194 m/z at retention time ~18 min; d-Ala, penta-peptide precursors ending on d-Ala-d-Ala 1193 m/z at retention time ~14 min.

after growing the strain in the absence of balhimycin. In this mutant, only sensitive cell wall precursors ending on d-Ala-d-Ala (1193 m/z) eluting at a retention time of 10–11 min were detected (Fig. 6A).

The phosphorylation site D51 is essential for the function of VnlRAb

To define the phosphorylation site of VnlRAb, D51, which was identified as a likely phosphorylation site by sequence composition (Fig. 1), was replaced by an alanine by exchanging nucleotide A to C at position 161 of vnlRAb using the recombinant PCR method. The exchange was verified by sequence analysis. The mutated gene was cloned into the integrative vector pRM4 under the control of the erm<sup>E</sup> promoter and introduced into S. coelicolor M600 ∆vanRS<sub>Sc</sub>. The resulting recombinant strain was not able to grow in the presence of the tested glycopeptides (Fig. 4). Therefore, we propose D51 as the VnlRAb phosphorylation site.

VnlRAb expands the glycopeptide resistance in S. coelicolor

The glycopeptide resistance mechanism in S. coelicolor belongs to the VanB type of resistance, meaning that glycopeptide resistance can only be induced by vancomycin or vancomycin-type glycopeptides, whereas teicoplanin (a type IV glycopeptide) fails to activate resistance, resulting in a teicoplanin-sensitive phenotype of S. coelicolor. In contrast, A. balhimycina is resistant against vancomycin- as well as teicoplanin-type glycopeptides. To analyze whether the RR is responsible for determination of the glycopeptide resistance type, the recombinant strains S. coelicolor ∆vanRS<sub>Sc</sub> [vnlRS<sub>Ab</sub>], S. coelicolor ∆vanRS<sub>Sc</sub> [vnlRA<sub>Ab</sub>], S. coelicolor ∆vanRS<sub>Sc</sub> [vnlS<sub>Ab</sub>], and S. coelicolor ∆vanRS<sub>Sc</sub> [vnlRAb D51A] were grown on teicoplanin-containing plates. Surprisingly, the recombinant strains (S. coelicolor ∆vanRS<sub>Sc</sub> [vnlRS<sub>Ab</sub>], S. coelicolor ∆vanRS<sub>Sc</sub> [vnlRA<sub>Ab</sub>]) were able to grow also on teicoplanin-containing plates, whereas growth of S. coelicolor M600 WT was inhibited (Fig. 4). These results indicated that VnlRAb is able to induce teicoplanin resistance in S. coelicolor M600 by probably activating further genes required for teicoplanin resistance.

VnlRAb influences antibiotic production in S. coelicolor

To analyze if the heterologous expression of VnlRAb, in addition to the activation of the vanHAX<sub>Sc</sub> genes, causes further (morphological) changes in S. coelicolor M600, the growth and production of actinorhodin were investigated without the addition of any antibiotic. Similar titers of spores (1.5 × 10<sup>7</sup>) of S. coelicolor M600, S. coelicolor ∆vanRS<sub>Sc</sub>, S. coelicolor ∆vanRS<sub>Sc</sub> [vnlRS<sub>Ab</sub>], S. coelicolor ∆vanRS<sub>Sc</sub> [vnlRA<sub>Ab</sub>], S. coelicolor ∆vanRS<sub>Sc</sub> [vnlS<sub>Ab</sub>], and S. coelicolor ∆vanRS<sub>Sc</sub> [vnlRA<sub>Ab</sub> D51A] were plated on YM medium. Surprisingly, the heterologous expression of vnlRS<sub>Ab</sub> or vnlRA<sub>Ab</sub> alone in S. coelicolor M600 ∆vanRS<sub>Sc</sub> caused retardation in growth and increased actinorhodin production (Fig. 4E).

These results suggested that VnlRAb is not only able to activate the vanHAX<sub>Sc</sub> genes in S. coelicolor M600 and to change its glycopeptide resistance type but it also has effects on other genes in S. coelicolor M600.

VnlRAb is responsible for the activation of vanY<sub>Ab</sub>

Heterologous expression of VnlRAb in S. coelicolor confirmed that it can take over the VanRS<sub>c</sub> function to induce the expression of the vanHAX<sub>Ab</sub> genes and, in addition, can apparently induce the expression of further genes. In contrast, it is not involved in regulation of the vanHAX<sub>Sc</sub> genes in A. balhimycina. Since regulatory genes are often colocalized with its target genes, we speculated that VnlRAb might control vanY<sub>Ab</sub>, which is located directly adjacent to vnlRAb and which encodes a carboxypeptidase. Previous studies showed that VanY<sub>Ab</sub> cleaves the d-Ala-d-Ala dipeptide from the PG precursors, but it is not able to cleave the d-Ala-d-Lac depsipeptide. To investigate, whether VnlRAb regulates the expression of vanY<sub>Ab</sub>, transcriptional analyses were performed. RT-PCR analyses revealed that vanY<sub>Ab</sub> was only transcribed when vanY<sub>Ab</sub> was expressed under the control of the strong promoter erm<sup>Ep</sup> (Fig. 2 (A. balhimycina [vnlRAb]). In A. balhimycina WT, transcription was detectable on a low level only after 63 hr of cultivation and in the A. balhimycina ∆vnlRAb mutant, vanY<sub>Ab</sub> transcription was not induced at all (Fig. 2). This result was confirmed by RNA-seq analyses where we compared the transcription level of vanY<sub>Ab</sub> in the A. balhimycina WT and A. balhimycina ∆vnlRAb (data not shown). The transcription of vanY<sub>Ab</sub> was 25-fold decreased in A. balhimycina ∆vnlRAb compared with A. balhimycina WT. We therefore concluded that the RR VnlRAb in A. balhimycina is involved in controlling the expression of resistance mediated by VanY<sub>Ab</sub>.

Discussion

Glycopeptide resistance in pathogens and in S. coelicolor is mediated by the action of VanHAX. The expression of the vanHAX genes is regulated by the TCS VanRS, the genes of which are colocalized with vanHAX. In the presence of glycopeptides, VanS becomes autophosphorylated
and phosphorylates VanR, which subsequently activates transcription of vanHAX. VanH, VanA, and VanH program the biosynthesis of the PG precursors, resulting in lipid II with an N-terminal d-Ala-d-Lac depsipeptide instead of the normally occurring d-Ala-d-Ala termini, the target of the glycopeptides. A. balhimicina produces the vancomycin-like glycopeptide balhimycin and has to protect itself from the action of the glycopeptide.45 The genome of A. balhimicina includes vanHAXAb genes and vanRS-like genes (vanRSAb). However, in contrast to other glycopeptide-resistant bacteria, the vanHAXAb genes in A. balhimicina are located 2 Mb apart from the vanRSAb genes, which are part of the balhimycin biosynthetic gene cluster.40

RT-PCR experiments revealed that VnlRAb is not involved in the activation of the vanHAXAb genes in A. balhimicina. Subsequent PG analyses confirmed that a vnlRAb deletion mutant cannot synthesize resistant muropeptides. Since vnlRAb is colocalized with the balhimycin biosynthetic genes, an alternative role of VnlRAb as regulator of balhimycin synthesis was assumed, but the deletion of the vnlRAb did not affect balhimycin production. Hence, VnlRAb is not the central regulator activating the vanHAXAb resistance genes or the balhimycin biosynthetic genes.

To further investigate the potential target gene(s) of VnlRAb, we analyzed the transcription of vanYAb, which encodes a carboxypeptidase and is located adjacent to the vnlRSAb genes in the balhimycin biosynthetic gene cluster.25 RT-PCR and RNA-seq analyses revealed that vanYAb expression was 25-fold decreased in A. balhimicina ΔvnlRAb compared with A. balhimicina WT.

VanYAb is a d, d-carboxypeptidase, which cleaves the endstanding d-Ala from lipid II, resulting in the formation of tetrapeptides.27 In contrast to other described carboxypeptidases,46 VanYAb has no d, d-carboxypeptidase activity. The tetrapeptides are the substrates for the d, d-transpeptidase (Ldt), which subsequently cross-links the tetrapeptide acyl donors at the third AA. This results in PG with 3–3 cross-linked tetra- and tripeptides, which are devoid of the d-Ala-d-Ala-ending peptides, and which can therefore not serve as target of glycopeptides anymore.47 Investigations of the PG of A. balhimicina revealed the presence of 3–3 cross-linked tetra- and tripeptides.40,45 Furthermore, we could identify at least three ldt genes in the genome of A. balhimicina.45 We therefore speculate that by activating the expression of vanYAb, VnlRAb is involved in regulating an alternative, VanHAXAb-independent glycopeptide resistance mechanism in A. balhimicina. This fact is further confirmed by RT-PCR analysis, where it was shown that VanYAb is expressed in A. balhimicina ΔvanHAXAb.40

This observation is in accordance with the findings in Nonomuraea ATCC 39727, the producer of the dalbavancin precursor A40926. Nonomuraea ATCC 39727 does not encode VanHAX homologs, but possesses a VanY homolog (VanYn) for the synthesis of a resistant PG precursor.48 As described for A. balhimicina, VanYn cleaves the C-terminal d-Ala from the pentapeptide as well as from the d-Ala-d-Ala dipptide. The tetrapeptides are subsequently cross-linked by Ldt, resulting in glycopeptide-resistant cell wall.47 The surprising features of VnlRAb are that although it does not regulate the transcription of the vanHAXAb genes in A. balhimicina, it is able to activate vanHAXSc transcription in S. coelicolor, and that it activates teicoplanin resistance in S. coelicolor.

Activation of the vanHAXSc transcription can be explained by the binding of VnlRa in the promoter region of vanHAXSc. Sequence comparison of the promoter regions of vanHAXSc and vanHAXAb not only revealed conserved motifs but also some differences (data not shown). Although many attempts have been made to analyze putative promoter sequences in gel mobility assays, no shifts could be observed. This is probably due to the fact that after purification, the protein lost its functionality (data not shown). Therefore, determination of the exact binding motive of VnlRAb still requires alternative approaches.

VnlRAb was not only able to restore vancomycin resistance in an S. coelicolor ΔvanRSAb mutant after heterologous expression but it even conferred teicoplanin resistance to this mutant, although S. coelicolor WT is sensitive toward teicoplanin. Recent comparative study of the VanR-VanS systems from two Streptomyces strains, S. coelicolor and Streptomyces toyoacensis (the producer of the sugarless glycopeptide A47934), indicated that the glycopeptide antibiotic inducer specificity is determined solely by the differences between the AA sequences of the VanR-VanS systems present in each strain rather than by any inherent differences in general cell properties, including cell wall structure and biosynthesis.42 On the one hand, the results obtained in this work support this finding; since vnlRa is under the control of the ermEp promoter, VnlRAb is constitutively expressed and activates the transcription of the vanHAXJKSc genes in S. coelicolor independent of the presence of any glycopeptide. The activation of vanHAXJKSc resulted in the synthesis of PG with pentapeptides ending in d-Ala-d-Lac depsipeptide, which are resistant against vancomycin and teicoplanin. On the other hand, the second explanation contradicts the work of Novotna et al.42; it is likely that VnlRAb activates the transcription of additional unknown genes, which mediate teicoplanin resistance.

The diverse functionality of VnlRAb in the glycopeptide producer A. balhimicina and in the nonproducer S. coelicolor provides the starting point of evolutionary analyses of glycopeptide resistance. In pathogenic bacteria and in S. coelicolor of glycopeptides, resistance is strictly regulated and is induced by the presence of glycopeptides. In contrast, glycopeptide producers overcome this regulation not only by the constitutive expression of the vanHAX genes but also by the development of a vanHAX-independent resistance mechanism. However, the ability of VnlRAb to activate transcription of vanHAX in S. coelicolor is an indication of a common origin of the three glycopeptide resistance mechanisms, the inducible one, the constitutively expressed, and the vanHAX-independent mechanism. Whether and how the complex resistance mechanism has evolved in the glycopeptide producers and whether and how it was transferred into resistance pathogens have to be subjects of future investigation.

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