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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* Δ*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).[1] 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.[2] 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.[3] 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,[4,5] some glycopeptide producers, and other actinomycetes.[6] VanS is a membrane-standing HK. Its C-terminus extends into the cytoplasm and contains the kinase domain and the phosphorylation site.[7] 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.[8]

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,

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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).[9,10] 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[11] or 6-fold[12] decreased 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.[9]

Three of those phenotypes, VanC,[13] VanD,[14] and VanN,[15,16] 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.[17,18] 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 carrying $vanRS_{Sc}$, $vanHAX_{Sc}$, $vanK_{Sc}$, and $vanJ_{Sc}$ (Fig. 1C). $VanH_{Sc}$ is a D-stereospecific lactate dehydrogenase that converts pyruvate to D-Lac. $VanA_{Sc}$ is a D-Ala-D-Ala-ligase family protein that ligates D-Ala and D-Lac to D-Ala-D-Lac-depsipeptides. $VanX_{Sc}$ is a highly selective carboxypeptidase that cleaves the remaining D-Ala-D-Ala-dipeptide. $VanK_{Sc}$ belongs to the Fem family of enzymes, which add the cross-bridging amino acid(s) to the stem pentapeptide of PG precursors.[19] VanY is a membrane protein conferring resistance to teicoplanin. To identify the precise nature of the ligand signal that activates glycopeptide resistance in *S. coelicolor* A3(2), the VanB-type HK $VanS_{Sc}$, sensing vancomycin, but not teicoplanin, was investigated.[21,22] Investigation on $VanS_{Sc}$ revealed opposed results. On the one hand, it was shown by cross-linking experiments that vancomycin is the direct ligand of the $VanS_{Sc}$.[21] On the other hand, Kwun *et al.*[22] demonstrated that $VanS_{Sc}$ is activated by vancomycin in complex with the D-Ala-D-Ala termini of PG precursors.

The actinomycete *Amycolatopsis balhimycina* produces the glycopeptide balhimycin, a vancomycin-type glycopeptide differing from vancomycin only in the glycosylation pattern.[23,24] The balhimycin biosynthesis gene cluster contains all genes necessary for balhimycin production[25] as well the *vanRS-like* regulatory genes, $vnlRS_{Ab}$ and the accessory resistance gene $vanY_{Ab}$ (Fig. 1C).[26] VanYAb is a carboxypeptidase which cleaves the endstanding D-Ala-D-Ala-dipeptide from the PG precursors.[27] However, the $vanHAX_{Ab}$ genes are encoded more than 2 Mb apart from the balhimycin biosynthesis cluster. Although $VnlR_{Ab}$ does not regulate glycopeptide resistance in *A. balhimycina*,[27] its heterologous expression in the glycopeptide-sensitive *S. coelicolor* strain M600 J2301[6] ($\Delta vanRS_{Sc}$) revealed unexpected effects. $VnlR_{Ab}$ activates the $vanHAX_{Sc}$ genes, increases actinorhodin production, and influences morphological differentiation in *S. coelicolor*.

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.

Escherichia coli XL1-blue[28] was used for cloning purposes, and the methylation-deficient strain *E. coli* ET12567[29] was used to obtain unmethylated DNA for *A. balhimycina* transformations.

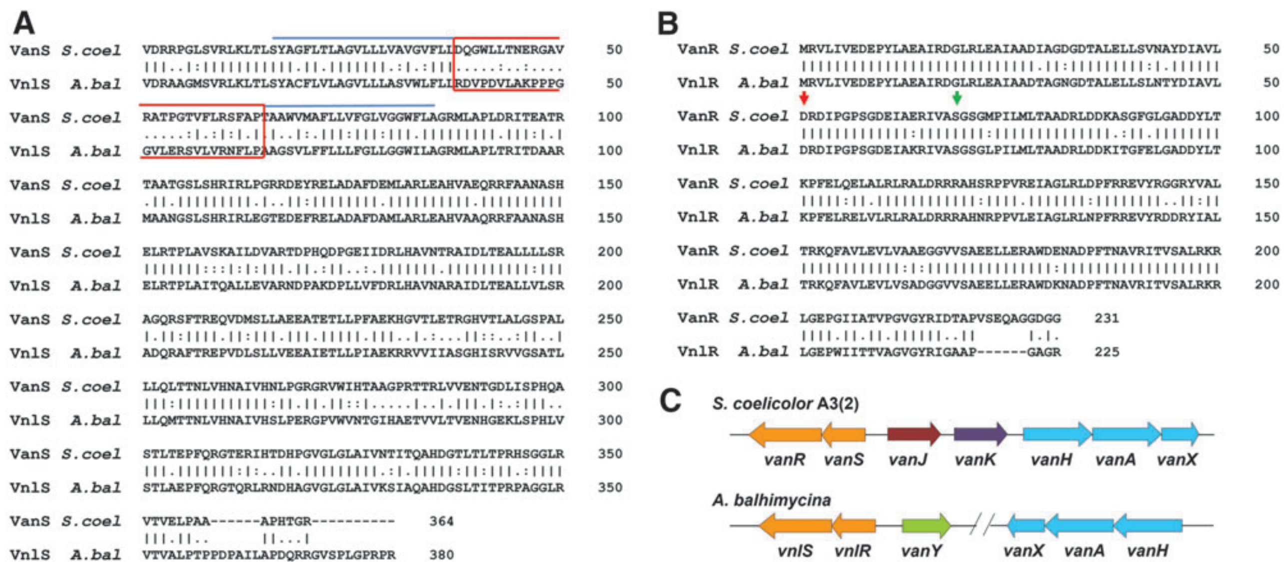

FIG. 1. **(A, B)** EMBOSS stretcher pairwise sequence alignment of $VnlRS_{Ab}$ and $VanRS_{Sc}$. **(A)** EMBOSS stretcher pairwise sequence alignment of $VnlS_{Ab}$ and $VanS_{Sc}$. Transmembrane domains are indicated in *blue*. The extracytosolic domain is highlighted by a *red box*. **(B)** EMBOSS stretcher pairwise sequence alignment of $VnlR_{Ab}$ and $VanR_{Sc}$. 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

	Relevant feature(s)	*References*
Strains		
Streptomyces coelicolor A3(2)		
M600	SCP1$^-$ SCP2$^-$	30
M600 Δ*vanRS$_{Sc}$* (J2301)	*vanRS$_{Sc}$* deletion mutant	6
M600 Δ*vanRS$_{Sc}$* [*vnlRS$_{Ab}$*]	Δ*vanRS$_{Sc}$* complemented with pRM4*vnlRS$_{Ab}$*	This study
M600 Δ*vanRS$_{Sc}$* [*vnlR$_{Ab}$*]	Δ*vanRS$_{Sc}$* complemented with pRM4*vnlR$_{Ab}$*	This study
M600 Δ*vanRS$_{Sc}$* [*vnlS$_{Ab}$*]	Δ*vanRS$_{Sc}$* complemented with pRM4*vnlS$_{Ab}$*	This study
M600 Δ*vanRS$_{Sc}$* [*vnlR$_{Ab}$*D51A]	Δ*vanRS$_{Sc}$* complemented with pRM4*vnlR$_{Ab}$*D51A	This study
Amycolatopsis balhimycina DSM 5908		
A. balhimycina WT DSM 5908	Wildtype	24
A. balhimycina Δ*vnlR$_{Ab}$*	*vnlR$_{Ab}$* deletion mutant	27
A. balhimycina [*vnlR$_{Ab}$*]	Overexpression of *vnlR$_{Ab}$* in *A. balhimycina*, using pRM4*vnlR$_{Ab}$*	This study
A. balhimycina Δ*vnlR$_{Ab}$* [*vnlR$_{Ab}$*]	Δ*vnlR$_{Ab}$* complemented with pRM4vnlR$_{Ab}$	This study
A. balhimycina Δ*vnlS$_{Ab}$*	*vnlS$_{Ab}$* deletion mutant	This study
Escherichia coli		
XL1-blue	*recA1; endA1; gyrA96; tji-1; hsdR17; supE44; relA1; lac* [F'*proAB, lac*^{q}ZΔM15Tn*10*(*tet^r*)]	28
ET 12567 pUZ8002	pUZ8002; *kan^r*	34
ET 12567	F$^-$; *dam13::*Tn*9; dcm-6; hsdM; hsdR; recF143; zjj201::*Tn*10*; *galK2; galT22; ara14; lacY1; xyl15; leuB6; thi1; tonA31; rpsL136; hisG4; tsx78; mtli; glnV44*	29
Plasmids		
pRM4	pSET152 *ermE*p*, RBS, Φ31 *attP-int*-derived integration vector	31
pRM4vnlRS$_{Ab}$	Expression plasmid for *vnlRS$_{Ab}$*	This study
pRM4vnlR$_{Ab}$	Expression plasmid for *vnlR$_{Ab}$*	This study
pRM4vnlS$_{Ab}$	Expression plasmid for *vnlS$_{Ab}$*	This study
pRM4vnlR$_{Ab}$D51A	Expression plasmid for *vnlR$_{Ab}$*, Exchange of Asp at position 51 with Ala	This study
pSP1	Inactivation vector in *A. balhimycina* Erythromycin and ampicillin resistance	32
pSPΔvnlS$_{Ab}$	pSP1 carrying a 1579 bp upstream and a 1509 bp downstream fragment of *vnlS$_{Ab}$*	This study

WT, wild type.

A. balhimycina[24] is the balhimycin-producing wild type (WT) and was used to generate the *vnlS* deletion as well as the *vnlR*-overexpressing strains (this study). Furthermore, Δ*vnlR* deletion[27] was used for complementation (this study).

S. coelicolor M600[30] were used to generate *S. coelicolor* M600 Δ*vanRS*.[6] This deletion strain was used to generate complementations with *vnlRS$_{Ab}$*, *vnlR$_{Ab}$*, *vnlS$_{Ab}$*, and *vnlR$_{Ab}$*-D51A (this study).

The overexpression plasmids pRM4vnlRS$_{Ab}$, pRM4vnlR$_{Ab}$, pRM4vnlS$_{Ab}$, and pRM4vnlR$_{Ab}$D51A are derived from pRM4[31], a pSET152-derived nonreplicative, ΦC31 integration vector with an integrated constitutive *ermEp** promoter, an artificial ribosomal binding site, and an apramycin resistance cassette.

The deletion vector pSPΔvnlS$_{Ab}$ is derived from pSP1[32] in which flanking regions of *vnlS$_{Ab}$* 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[30] 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 ($\sim 1.5 \times 10^7$) 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 Erlenmeyer flasks at 27°C.

Liquid/solid media were supplemented with 100 μg/ml apramycin to select for strains carrying integrated antibiotic resistance genes.

Table 2. Primers Used in This Study

	Primer	Sequence	Relevant feature(s)	References
1	vnlRS-compl.1	CATCGG**C**ATATGCGCGTGCTGATCGTCGAG	Cloning of *vnlRS$_{Ab}$*	This study
2	vnlRS-compl. 2	GAATT**C**CTGCGCGACTCCAGCGTTT CTCAGCGGAAG		
3	vnlR-over	GAATT**C**AGGCGTAGCTGAGG	Cloning of *vnlR$_{Ab}$*	This study
4	vnlS-cloning	ATCATATGAGCGTCCGCCTCAAAC	Cloning of *vnlS$_{Ab}$*	This study
5	vnlRD51A1	GAATATCCCGGgCGAGGACGG	Recombinant primers for aa exchange	This study
6	vnlRD51A2	CCGTCCTCGcCCGGGATATT		
7	vnlSdelFrg1for	TTAGAATTCGATTGTCCGCGAGAAATG	Cloning of *vnlS$_{Ab}$* upstream region	This study
8	vnlSdelFrg1rev	TAATCTAGACCCGGCCGCTCTGTC		
9	vnlSdelFrg2for	TTATCTAGACCGGCCGGGTCACCTC	Cloning of *vnlS$_{Ab}$* downstream region	This study
10	vnlSdelFrg2rev	ATTGCATGCGGGCGCAAGTGAGT TTCGGTCATCG		
11	pSETerme rev	ATGCTAGTCGCGGTTGA	Integration of Plasmid and Insert	This study
12	attBli-fwd	TTCTGGAAATCCTCGAAGGC	Integration of plasmid through ΦC31	
13	attPint-rev	TGTGCATGCGCCCACGAATG		
14	ery for	AAGGGAGAAAGGCGGACAGG	Proof of erythromycin resistance cassette	This study
15	ery rev	GTCGCTTCTGCGCAAGTACC		
16	*vnlS*proof for	TGCTCGAAGTCCTCGTTTCC	Integration and deletion verification	This study
17	*vnlS*proof rev	GCAAGTACGTGAGCGATCAG		
18	vanS$_{Sc}$_RT_1	CTCCAACTGACCACGAACCT	RT-PCR analysis in *S. coelicolor* M600	This study
19	vanS$_{Sc}$_RT_2	GGTCGGTGTGTATGCGTTC		
20	vanR$_{Sc}$_RT_1	TGCTGAGTGTCAACGCCTAC		
21	vanR$_{Sc}$_RT_2	CGAACTGCTTCCTGGTCAAC		
22	vanA$_{Sc}$_RT_1	ACCGTGACAGGAGACGAGAC		
23	vanA$_{Sc}$_RT_2	CTGGTGGATCCGGAAGAAT		
24	hrdBSc_RT_1	TGACCAGATTCCGGCCACTC		This study
25	hrdBSc_RT_2	CTTCGCTGCGACGCTCTTTC		
26	sigB for	CGTAGGTCGAGAACTTGAAC	RT-PCR analysis in *A balhimycina* DSM5908	[41]
27	sigB rev	GTGTCTACCTCAACGGTATC		
28	vanH1	GGGACAAGCCCATCAAGAAC		[27]
29	vanA2	GAGCGGACTTGACGGAGATG		
30	*vanY*_RT_fwd	TCGGCACGAGGATTG		
31	*vanY*_RT_rev	TTCACGCACAGTTCG		

RT-PCR, reverse transcriptase polymerase chain reaction.

E. coli was grown in Luria-Bertani broth (Roth) at 37°C using 100 µg/µl apramycin or 150 µg/µl ampicillin for selection of plasmid-containing colonies. Liquid cultures were shaken at 180 rpm.

Plasmid construction

For the heterologous expression in *S. coelicolor* Δ*vanRS$_{Sc}$*, the entire coding regions of the *vnlR$_{Ab}$* (Table 2 primer 1 + 3), *vnlRS$_{Ab}$* (Table 2 primer 1 + 2), and *vnlS$_{Ab}$* (primer 4 + 2) were amplified using Kapa-Hifi proofreading polymerase and the corresponding primers in brackets.

The *vnlR$_{Ab}$* (758 bp), *vnlRS$_{Ab}$* (1908 bp), and *vnlS$_{Ab}$* (1198 bp) polymerase chain reaction (PCR) products were integrated into pRM4[31] through the primer-attached restriction sites (Nde-EcoRI) downstream of the *ermEp** promoter.

Site-directed mutagenesis by overlap extension[33] 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 *vnlR$_{Ab}$*D51A was integrated into pRM4 through the primer-attached restriction sites (Nde-EcoRI) downstream of the *ermEp** promoter.

For the in-frame deletion of *vnlS$_{Ab}$* (1125 bp), a 1579 bp upstream fragment (Table 2 primer 7 + 8) and a 1509 bp downstream fragment (Table 2 primer 9 + 10) of *vnlS$_{Ab}$* were amplified from *A. balhimycina* genomic DNA using Kapa-Hifi proofreading polymerase and the corresponding primers in brackets. The plasmid pSPΔvnlS was constructed by integration of the fragments in pSP1[30] through the primer-attached restriction sites at the 5′ and 3′ ends (EcoRI/XbaI and XbaI/SphI) (pSPΔvnlS).

DNA transfer

Transformation of *E. coli* XL1-blue[28] and ET12567 (pUZ8002)[34] was performed as described previously.[35,36]

Plasmids pRM4vnlRS$_{Ab}$, pRM4vnlR$_{Ab}$, pRM4vnlS$_{Ab}$, and pRM4vnlR$_{Ab}$D51A were transferred into *S. coelicolor* through intergeneric conjugation.[30] 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.

pRM4vnlR$_{Ab}$ and pRM4vnlR$_{Ab}$D51A were transferred into *A. balhimycina* through the direct transformation method[32,37] 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$_{Ab}$*, *A. balhimycina* WT was transformed with pSPΔvnlS$_{Ab}$ 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 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 VnlRS$_{Ab}$, VanR$_{Sc}$, and VanS$_{Sc}$ are available under accession number Y16952 (named VanRS), (SCO3590), and (SCO3589), respectively.

Alignment of the AA sequences was performed by EMBOSS 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 μg/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 (VnlRS$_{Ab}$) 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$_{Ab}$* 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$_{Ab}$*), which are part of the biosynthetic gene cluster and are therefore not colocated with the *vanHAX$_{Ab}$* genes.

VanRS$_{Sc}$ of *S. coelicolor* was reported to sense glycopeptides and to activate the expression of the *vanHAX$_{Sc}$* genes.[19] To elucidate the differences of the two actinomycete TCSs, we compared the AA sequence of VnlRS$_{Ab}$ with the sequence of VanRS$_{Sc}$. Sequence alignment using EMBOSS stretcher[39] revealed 82% sequence similarity between VnlR$_{Ab}$ and VanR$_{Sc}$ (Sco3590) and 73% between VnlS$_{Ab}$ and VanS$_{Sc}$ (Sco3589) (Fig. 1A, B). Based on the high similarity, a corresponding function of both RRs could be proposed.

In *S. coelicolor*, VanS$_{Sc}$ phosphorylates VanR$_{Sc}$ at the aspartate at AA position 51. Replacement of this residue with an alanine completely destroyed the activity of VanR$_{Sc}$.[6] It has been shown that in *S. coelicolor*, in the absence of vancomycin, acetylphosphate phosphorylates VanR$_{Sc}$, whereas VanS$_{Sc}$ acts as a phosphatase to decrease the level of VanR$_{Sc}$ ~ P. On exposure to vancomycin, VanS 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$_{Ab}$ contains both, a conserved aspartate at AA position 51 (D51) and a serine at AA position 69, the position that probably becomes autophosphorylated through acetyl phosphate (Fig. 1B), indicating an analogous phosphorylation pattern of VnlR$_{Ab}$ compared with VanR$_{Sc}$.

The RR VnlR$_{Ab}$ does not control expression of the vanHAX$_{Ab}$ *genes in* A. balhimycina

In *A. balhimycina*, *vanHAX$_{Ab}$* expression does not depend on VnlRS$_{Ab}$. The deletion of *vnlR$_{Ab}$* had no effect on glycopeptide resistance and did not result in any obvious phenotype.[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$_{Ab}$* in *A. balhimycina* to analyze the effects on resistance and antibiotic production. *vnlR$_{Ab}$* was cloned under the control of the constitutive promoter *ermE*p* into the integrative plasmid pRM4 (pRM4vnlR$_{Ab}$). pRM4vnlR$_{Ab}$ was transferred into *A. balhimycina* WT and into the *A. balhimycina* Δ*vnlR$_{Ab}$* mutant,[27] resulting in the recombinant strains *A. balhimycina* [*vnlR$_{Ab}$*] and *A. balhimycina* Δ*vnlR$_{Ab}$* [*vnlR$_{Ab}$*], respectively. The phenotypes of the recombinant strains overexpressing VnlR$_{Ab}$ and (as a control) that of the deletion mutant *A. balhimycina* Δ*vnlR$_{Ab}$* 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* [*vnlR$_{Ab}$*], *A. balhimycina* Δ*vnlR$_{Ab}$*, and *A. balhimycina* Δ*vnlR$_{Ab}$* [*vnlR$_{Ab}$*] produced resistant PG precursors ending with D-Ala-D-Lac (Fig. 2C). Only *A. balhimycina* WT and *A. balhimycina* Δ*vnlR$_{Ab}$* [*vnlR$_{Ab}$*]

FIG. 2. Analysis of balhimycin production, muropeptide composition, and PG precursors in *A. balhimycina* WT (1), *A. balhimycina* $\Delta vnlR_{Ab}$ (2), *A. balhimycina* [$vnlR_{Ab}$] (3), and *A. balhimycina* $\Delta vnlR_{Ab}$ [$vnlR_{Ab}$] (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.

produced traces of precursors ending with D-Ala-D-Ala under nonproduction conditions (Fig. 2C). These results suggest that $VnlR_{Ab}$ 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* $\Delta vnlR_{Ab}$, confirming that the expression of $vanHAX_{Ab}$ is independent of $vnlR_{Ab}$ (Fig. 3).

In A. balhimycina*, sensing of glycopeptides through $VnlS_{Ab}$ is not required for expressing the resistance genes*

In enterococci and in *S. coelicolor*, the RR $VanR_{Sc}$ becomes phosphorylated by the HK $VanS_{Sc}$. To analyze whether and how $VnlR_{Ab}$ interacts with $VnlS_{Ab}$, we constructed an in-frame $\Delta vnlS_{Ab}$ mutant of *A. balhimycina* using the inactivation plasmid pSPΔvnlS$_{Ab}$. This plasmid containing a 1509 bp downstream fragment and a 1579 bp upstream fragment of $vnlS_{Ab}$ was introduced into *A. balhimycina*

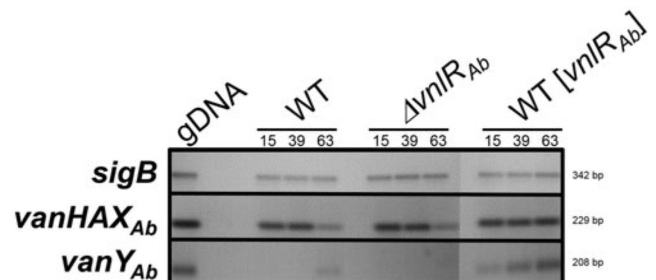

FIG. 3. RT-PCR analyses of $vanHAX_{Ab}$ and $vanY_{Ab}$ in *A. balhimycina* WT, *A. balhimycina* $\Delta vnlR_{Ab}$, and in *A. balhimycina* WT overexpressing $vnlR_{Ab}$ (WT [$vnlR_{Ab}$]). RNA was isolated at different time points (15/39/63 hr) from the three strains cultivated in balhimycin production 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 $vnlS_{Ab}$. *A. balhimycina* $\Delta vnlS_{Ab}$ 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 $vanHAX_{Ab}$ genes occurs independently of $VnlS_{Ab}$.

$VnlR_{Ab}$ is able to activate $vanHAX_{Sc}$ transcription in S. coelicolor

In silico analyses revealed similar characteristics of $VnlRS_{Ab}$ compared with $VanRS_{Sc}$. However, as shown above, the $VnlRS_{Ab}$ system in *A. balhimycina*, in contrast to $VanRS_{Sc}$ in *S. coelicolor*, does not regulate the $vanHAX_{Ab}$. To clarify the contradictory findings, the genes encoding the TCS $VnlRS_{Ab}$ as well as $VnlR_{Ab}$ and $VnlS_{Ab}$ individually were transferred into the *S. coelicolor* mutant strain, in which the $vanRS_{Sc}$ genes were deleted,[6] to elucidate the ability of $VnlR_{Ab}$ to activate the $vanHAX_{Sc}$ genes in the *S. coelicolor* mutant. $vnlRS_{Ab}$, $vnlR_{Ab}$, *and* $vnlS_{Ab}$ were introduced into *S. coelicolor* $\Delta vanRS_{Sc}$ under the control of the constitutive promoter *ermE*p* using the integrative plasmid pRM4vnlR_{Ab}. The growth of the recombinant strains was tested on glycopeptide-containing plates.

Introduction of $vnlRS_{Ab}$ and of $vnlR_{Ab}$ alone into *S. coelicolor* M600 $\Delta vanRS_{Sc}$ resulted in balhimycin-resistant strains (Fig. 4). In contrast, expression of $vnlS_{Ab}$ alone did not change the glycopeptide-sensitive phenotype of the *S. coelicolor* $\Delta vanRS_{Sc}$ mutant. These results indicated that $VnlR_{Ab}$ from *A. balhimycina* is able to activate the transcription of $vanHAX_{Sc}$ in *S. coelicolor* M600 also in the absence of $VnlS_{Ab}$. Since in *S. coelicolor* M600 $VanR_{Sc}\sim P$ can be generated in a $VanS_{Sc}$-independent manner using acetylphosphate,[6] we suggest a similar activation of $VnlR_{Ab}$ in the absence of $VnlS_{Ab}$ or $VanS_{Sc}$.

The activation of the $vanHAX_{Sc}$ genes in the complemented *S. coelicolor* M600 $\Delta vanRS_{Sc}$ mutant with $vnlRS_{Ab}$ and $vnlR_{Ab}$ 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 $vanHAX_{Sc}$ transcript was detected when *S. coelicolor* M600 $\Delta vanRS_{Sc}$ was complemented with $vnlRS_{Ab}$ or with $vnlR_{Ab}$ alone. However, in *S. coelicolor* M600 and in the *S. coelicolor* M600 $\Delta vanRS_{Sc}$ mutant, transcription of the $vanHAX_{Sc}$ failed (Fig. 5), confirming the functionality of $VnlR_{Ab}$ as transcriptional activator in *S. coelicolor*.

To investigate whether transcription of the $vanHAX_{Sc}$ 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 $\Delta vanRS_{Sc}$ and of *S. coelicolor* M600 $\Delta vanRS_{Sc}$ complemented either with $vnlRS_{Ab}$ or $vnlR_{Ab}$. Complementing *S. coelicolor* M600 $\Delta vanRS_{Sc}$ with $vnlRS_{Ab}$ or with $vnlR_{Ab}$ restored the synthesis of resistant PG precursors. In the presence of balhimycin exclusively, PG precursors ending with D-Ala-D-Lac were synthesized (Fig. 6C, D). The PG precursor composition of the glycopeptide-sensitive *S. coelicolor* M600 $\Delta vanRS_{Sc}$ mutant was analyzed

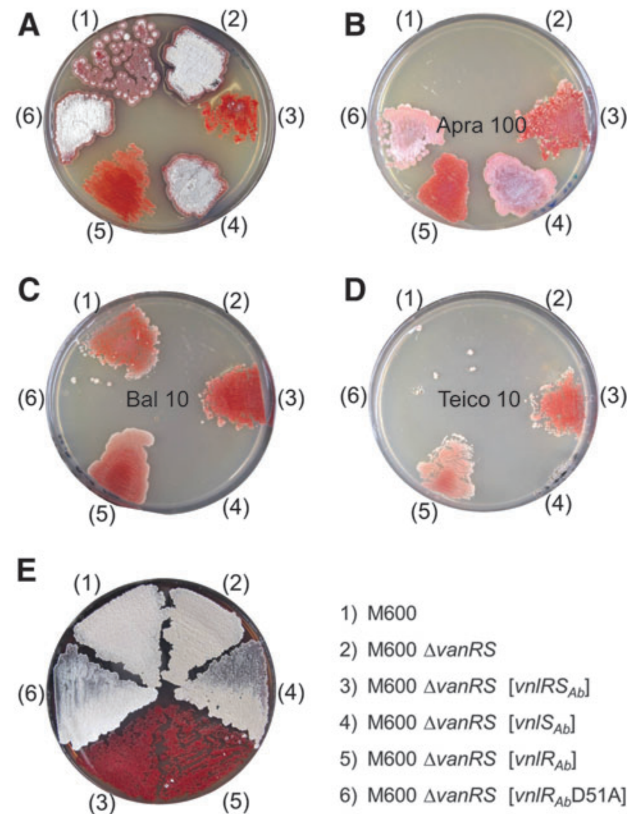

FIG. 4. Growth and resistance of the *S. coelicolor* M600 $\Delta vanRS_{Sc}$ complemented with different combinations of $vnlRS_{Ab}$. **(A)** Growth on YM agar containing no antibiotic. **(B)** Growth on YM agar containing apramycin (100 mg/ml) (Apra 100) to prove plasmid integration. **(C)** Growth 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.

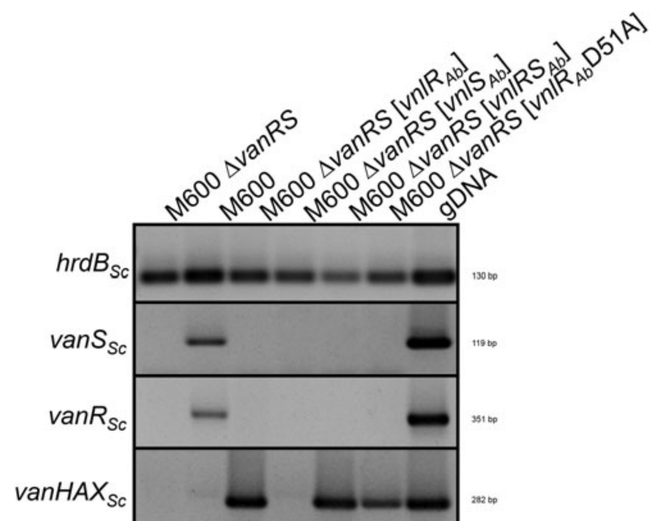

FIG. 5. RT-PCR analyses of *S. coelicolor* M600 and different *S. coelicolor* M600 mutants. RNA was isolated after 25 hr of cultivation in the absence of any glycopeptide. *hrdB*: transcription of the housekeeping gene $hrdB_{Sc}$. $vanS_{Sc}$, $vanR_{Sc}$, and $vanHAX_{Sc}$: transcription of $vanS_{Sc}$, $vanR_{Sc}$, and $vanHAX_{Sc}$. For PCR, genomic DNA (gDNA) was used as positive control.

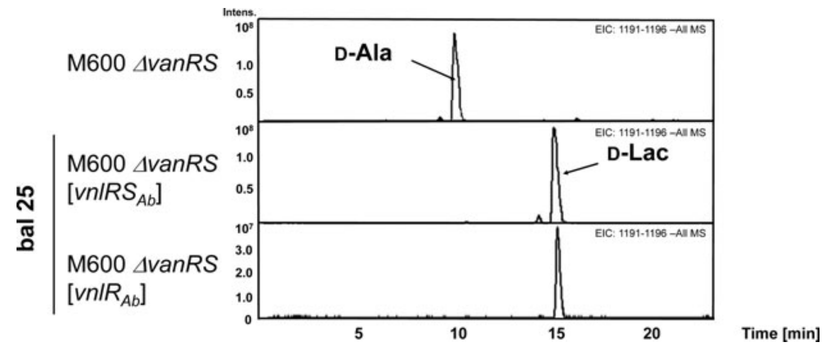

FIG. 6. Extracted ion chromatograms (negative mode) of the PG precursors isolated from cells grown in R5 medium without antibiotic or with 25 mg/ml balhimycin (bal25). M600 Δ$vanRS_{Sc}$: *S. coelicolor* M600 Δ$vanRS_{Sc}$; D-Lac, pentapeptide precursors ending on D-Ala-D-Lac 1194 m/z at retention time ~18 min; D-Ala, pentapeptide precursors ending on D-Ala-D-Ala 1193 *m/z* at retention time ~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 $VnlR_{Ab}$

To define the phosphorylation site of $VnlR_{Ab}$, 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 $vnlR_{Ab}$ 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 *ermE*p* promoter and introduced into *S. coelicolor* M600 Δ$vanRS_{Sc}$. The resulting recombinant strain was not able to grow in the presence of the tested glycopeptides (Fig. 4). Therefore, we propose D51 as the $VnlR_{Ab}$ phosphorylation site.

$VnlR_{Ab}$ *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,[42] 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* Δ$vanRS_{Sc}$ [$vnlRS_{Ab}$], *S. coelicolor* Δ$vanRS_{Sc}$ [$vnlR_{Ab}$], *S. coelicolor* Δ$vanRS_{Sc}$ [$vnlS_{Ab}$], and *S. coelicolor* Δ$vanRS_{Sc}$ [$vnlRS_{Ab}$ D51A] were grown on teicoplanin-containing plates. Surprisingly, the recombinant strains (*S. coelicolor* Δ$vanRS_{Sc}$ [$vnlRS_{Ab}$], *S. coelicolor* Δ$vanRS_{Sc}$ [$vnlR_{Ab}$]) were able to grow also on teicoplanin-containing plates, whereas growth of *S. coelicolor* M600 WT was inhibited (Fig. 4). These results indicated that $VnlR_{Ab}$ is able to induce teicoplanin resistance in *S. coelicolor* M600 by probably activating further genes required for teicoplanin resistance.

$VnlR_{Ab}$ *influences antibiotic production in* S. coelicolor

To analyze if the heterologous expression of $VnlR_{Ab}$, in addition to the activation of the $vanHAX_{Sc}$ 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 (1.5×10^7) of *S. coelicolor* M600, *S. coelicolor* Δ$vanRS_{Sc}$, *S. coelicolor* Δ$vanRS_{Sc}$ [$vnlRS_{Ab}$], *S. coelicolor* Δ$vanRS_{Sc}$ [$vnlR_{Ab}$], *S. coelicolor* Δ$vanRS_{Sc}$ [$vnlS_{Ab}$], and *S. coelicolor* Δ$vanRS_{Sc}$ [$vnlRS_{Ab}$ D51A] were plated on YM medium. Surprisingly, the heterologous expression of $vnlRS_{Ab}$ or $vnlR_{Ab}$ alone in *S. coelicolor* M600 Δ$vanRS_{Sc}$ caused retardation in growth and increased actinorhodin production (Fig. 4E).

These results suggested that $VnlR_{Ab}$ is not only able to activate the $vanHAX_{Sc}$ genes in *S. coelicolor* M600 and to change its glycopeptide resistance type but it also has effects on other genes in *S. coelicolor* M600.

$VnlR_{Ab}$ *is responsible for the activation of* $vanY_{Ab}$

Heterologous expression of $VnlR_{Ab}$ in *S. coelicolor* confirmed that it can take over the $VanR_{Sc}$ function to induce the expression of the $vanHAX_{Ab}$ genes and, in addition, can apparently induce the expression of further genes. In contrast, it is not involved in regulation of the $vanHAX_{Ab}$ genes in *A. balhimycina*. Since regulatory genes are often colocalized with its target genes, we speculated that $VnlRS_{Ab}$ might control $vanY_{Ab}$, which is located directly adjacent to $vnlR_{Ab}$ and which encodes a carboxypeptidase. Previous studies showed that $VanY_{Ab}$ cleaves the D-Ala-D-Ala dipeptide from the PG precursors, but it is not able to cleave the D-Ala-D-Lac depsipeptide.[27] To investigate, whether $VnlR_{Ab}$ regulates the expression of $vanY_{Ab}$, transcriptional analyses were performed. RT-PCR analyses revealed that $vanY_{Ab}$ was only transcribed when $vnlR_{Ab}$ was expressed under the control of the strong promoter *ermE*p* (Fig. 2 (*A. balhimycina* [$vnlR_{Ab}$]). In *A. balhimycina* WT, transcription was detectable on a low level only after 63 hr of cultivation and in the *A. balhimycina* Δ$vnlR_{Ab}$ mutant, $vanY_{Ab}$ transcription was not induced at all (Fig. 2). This result was confirmed by RNA-seq analyses where we compared the transcription level of $vanY_{Ab}$ in the *A. balhimycina* WT and *A. balhimycina* Δ$vnlR_{Ab}$ (data not shown). The transcription of $vanY_{Ab}$ was 25-fold decreased in *A. balhimycina* Δ$vnlR_{Ab}$ compared with *A. balhimycina* WT. We therefore concluded that the RR $VnlR_{Ab}$ in *A. balhimycina* is involved in controlling the expression of resistance mediated by $VanY_{Ab}$.

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 reprogram 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. balhimycina* produces the vancomycin-like glycopeptide balhimycin and has to protect itself from the action of the glycopeptide.[45] The genome of *A. balhimycina* includes *vanHAX$_{Ab}$* genes and *vanRS*-like genes (*vnlRS$_{Ab}$*). However, in contrast to other glycopeptide-resistant bacteria, the *vanHAX$_{Ab}$* genes in *A. balhimycina* are located 2 Mb apart from the *vnlRS$_{Ab}$* genes, which are part of the balhimycin biosynthetic gene cluster.[40]

RT-PCR experiments revealed that VnlR$_{Ab}$ is not involved in the activation of the *vanHAX*$_{Ab}$ genes in *A. balhimycina*. Subsequent PG analyses confirmed that a *vnlR$_{Ab}$* deletion mutant cannot synthesize resistant muropeptides. Since *vnlR$_{Ab}$* is colocalized with the balhimycin biosynthetic genes, an alternative role of VnlR$_{Ab}$ as regulator of balhimycin synthesis was assumed, but the deletion of the *vnlR$_{Ab}$* did not affect balhimycin production. Hence, VnlR$_{Ab}$ is not the central regulator activating the *vanHAX*$_{Ab}$ resistance genes or the balhimycin biosynthetic genes.

To further investigate the potential target gene(s) of VnlR$_{Ab}$, we analyzed the transcription of *vanY$_{Ab}$*, which encodes a carboxypeptidase and which is located adjacent to the *vnlRS$_{Ab}$* genes in the balhimycin biosynthetic gene cluster.[25] RT-PCR and RNA-seq analyses revealed that *vanY$_{Ab}$* expression was 25-fold decreased in *A. balhimycina* ΔvnlR$_{Ab}$ compared with *A. balhimycina* WT.

VanY$_{Ab}$ 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] VanY$_{Ab}$ has no D,D-carboxyesterase activity. The tetrapeptides are the substrates for the L,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. balhimycina* 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. balhimycina*.[45] We therefore speculate that by activating the expression of *vanY$_{Ab}$*, VnlR$_{Ab}$ is involved in regulating an alternative, VanHAX$_{Ab}$-independent glycopeptide resistance mechanism in *A. balhimycina*. This fact is further confirmed by RT-PCR analysis, where it was shown that VanY$_{Ab}$ is expressed in *A. balhimycina* Δ*vanHAX$_{Ab}$*.[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 (VanY$_n$) for the synthesis of a resistant PG precursor.[48] As described for *A. balhimycina*, VanY$_n$ cleaves the C-terminal D-Ala from the pentapeptide as well as from the D-Ala-D-Ala dipeptide. The tetrapeptides are subsequently cross-linked by Ldt, resulting in glycopeptide-resistant cell wall.[47] The surprising features of VnlR$_{Ab}$ are that although it does not regulate the transcription of the *vanHAX$_{Ab}$* genes in *A. balhimycina*, it is able to activate *vanHAX$_{Sc}$* transcription in *S. coelicolor*, and that it activates teicoplanin resistance in *S. coelicolor*.

Activation of the *vanHAX$_{Sc}$* transcription can be explained by the binding of VnlR$_{Ab}$ at the promoter region of *vanHAX$_{Sc}$*. Sequence comparison of the promoter regions of *vanHAX$_{Sc}$* and *vanHAX$_{Ab}$* not only revealed conserved motives 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 VnlR$_{Ab}$ still requires alternative approaches.

VnlR$_{Ab}$ was not only able to restore vancomycin resistance in an *S. coelicolor* Δ*vanRS$_{Ab}$* 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 toyocaensis* (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 TCS 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 *vnlR$_{Ab}$* is under the control of the *ermEp** promoter, VnlR$_{Ab}$ is constitutively expressed and activates the transcription of the *vanHAXJK$_{Sc}$* genes in *S. coelicolor* independent from the presence of any glycopeptide. The activation of *vanHAXJK$_{Sc}$* resulted in the synthesis of PG with pentapeptides ending on 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 VnlR$_{Ab}$ activates the transcription of additional unknown genes, which mediate teicoplanin resistance.

The diverse functionality of VnlR$_{Ab}$ in the glycopeptide producer *A. balhimycina* 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 VnlR$_{Ab}$ 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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Disclosure Statement

The authors disclose that there are no commercial associations that might create a conflict of interest in connection with submitted manuscripts.

References

1. Laub, M.T., and M. Goulian. 2007. Specificity in two-component signal transduction pathways. Annu. Rev. Genet. 41:121–145.
2. Perry, J., K. Koteva, and G. Wright. 2011. Receptor domains of two-component signal transduction systems. Mol. Biosyst. 7:1388–1398.
3. Galperin, M.Y. 2010. Diversity of structure and function of response regulator output domains. Curr. Opin. Microbiol. 13:150–159.
4. Arthur, M., C. Molinas, and P. Courvalin. 1992. The VanS-VanR two-component regulatory system controls synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J. Bacteriol. 174:2582–2591.
5. Gardete, S., and A. Tomasz. 2014. Mechanisms of vancomycin resistance in *Staphylococcus aureus*. J. Clin. Invest. 124:2836–2840.
6. Hutchings, M.I., H.J. Hong, and M.J. Buttner. 2006. The vancomycin resistance VanRS two-component signal transduction system of *Streptomyces coelicolor*. Mol. Microbiol. 59:923–935.
7. Cheung, J., and W.A. Hendrickson. 2010. Sensor domains of two-component regulatory systems. Curr. Opin. Microbiol. 13:116–123.
8. Gold, H.S. 2001. Vancomycin-resistant enterococci: mechanisms and clinical observations. Clin. Infect. Dis. 33:210–219.
9. Pootoolal, J., J. Neu, and G.D. Wright. 2002. Glycopeptide antibiotic resistance. Annu. Rev. Pharmacol. Toxicol. 42:381–408.
10. Xu, X., D. Lin, G. Yan, X. Ye, S. Wu, Y. Guo, D. Zhu, F. Hu, Y. Zhang, F. Wang, G.A. Jacoby, and M. Wang. 2010. *vanM*, a new glycopeptide resistance gene cluster found in *Enterococcus faecium*. Antimicrob. Agents Chemother. 54:4643–4647.
11. Bugg, T.D., G.D. Wright, S. Dutka-Malen, M. Arthur, P. Courvalin, and C.T Walsh. 1991. Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. Biochemistry. 30:10408–10415.
12. Billot-Klein, D., D. Blanot, L. Gutmann, and J. van Heijenoort. 1994. Association constants for the binding of vancomycin and teicoplanin to N-acetyl-D-alanyl-D-alanine and N-acetyl-D-alanyl-D-serine. Biochem. J. 304 (Pt 3):1021–1022.
13. Reynolds, P.E., H.A. Snaith, A.J. Maguire, S. Dutka-Malen, and P. Courvalin. 1994. Analysis of peptidoglycan precursors in vancomycin-resistant *Enterococcus gallinarum* BM4174. Biochem. J. 301 (Pt 1):5–8.
14. Perichon, B., P. Reynolds, and P. Courvalin. 1997. VanD-type glycopeptide-resistant *Enterococcus faecium* BM4339. Antimicrob. Agents Chemother. 41:2016–2018.
15. Lebreton, F., F. Depardieu, N. Bourdon, M. Fines-Guyon, P. Berger, S. Camiade, R. Leclercq, P. Courvalin, and V. Cattoir. 2011. D-Ala-D-Ser VanN-type transferable vancomycin resistance in *Enterococcus faecium*. Antimicrob. Agents Chemother. 55:4606–4612.
16. Nomura, T., K. Tanimoto, K. Shibayama, Y. Arakawa, S. Fujimoto, Y. Ike, and H. Tomita. 2012. Identification of VanN-type vancomycin resistance in an *Enterococcus faecium* isolate from chicken meat in Japan. Antimicrob. Agents Chemother. 56:6389–6392.
17. Foucault, M.L., P. Courvalin, and C. Grillot-Courvalin. 2009. Fitness cost of VanA-type vancomycin resistance in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. 53:2354–2359.
18. Foucault, M.L., F. Depardieu, P. Courvalin, and C. Grillot-Courvalin. 2010. Inducible expression eliminates the fitness cost of vancomycin resistance in enterococci. Proc. Natl. Acad. Sci. U S A 107:16964–16969.
19. Hong, H.J., M.I. Hutchings, J.M. Neu, G.D. Wright, M.S. Paget, and M.J. Buttner. 2004. Characterization of an inducible vancomycin resistance system in *Streptomyces coelicolor* reveals a novel gene (*vanK*) required for drug resistance. Mol. Microbiol. 52:1107–1121.
20. Novotna, G., C. Hill, K. Vincent, C. Liu, H.J. Hong. 2012. A novel membrane protein, VanJ, conferring resistance to teicoplanin. Antimicrob. Agents Chemother. 56:1784–1796.
21. Koteva, K., H.J. Hong, X.D. Wang, I. Nazi, D. Hughes, M.J Naldrett, M.J. Buttner, and G.D. Wright. 2010. A vancomycin photoprobe identifies the histidine kinase VanSsc as a vancomycin receptor. Nat. Chem. Biol. 6:327–329.
22. Kwun, M.J., G. Novotna, A.R. Hesketh, L. Hill, and H.-J. Hong. 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.
23. Chatterjee, S., E.K.S. Vijayakumar, S.R. Nadkarni, M.V. Patel, J. Blumbach, B.N. Ganguli, H.W. Fehlhaber, H. Kogler, and L. Vertesy. 1994. Balhimycin, a New Glycopeptide Antibiotic with an unusual hydrated 3-amino-4-oxoaldopyranose sugar moiety. J. Org. Chem. 59:3480–3484.
24. Nadkarni, S.R., M.V. Patel, S. Chatterjee, E.K. Vijayakumar, K.R. Desikan, J. Blumbach, B.N. Ganguli, and M. Limbert. 1994. Balhimycin, a new glycopeptide antibiotic produced by *Amycolatopsis sp*. Y-86,21022. Taxonomy, production, isolation and biological activity. J. Antibiot. (Tokyo). 47:334–341.
25. Pelzer, S., R. Sussmuth, D. Heckmann, J. Recktenwald, P. Huber, G. Jung, and W. Wohlleben. 1999. Identification and analysis of the balhimycin biosynthetic gene cluster and its use for manipulating glycopeptide biosynthesis in *Amycolatopsis mediterranei* DSM5908. Antimicrob. Agents Chemother. 43:1565–1573.
26. Shawky, R.M., O. Puk, A. Wietuorrek, S. Pelzer, E. Takano, W. Wohlleben, E. Stegmann. 2007. The border sequence of the balhimycin biosynthetic gene cluster from Amycolatopsis balhimicina contains bbr, encoding a StrR-like pathway-specific regulator. J. Mol. Microbiol. Biotechnol. 13:76–88.
27. Schäberle, T.F., W. Vollmer, H.J. Frasch, S. Huttel, A. Kulik, M. Rottgen, A.K. von Thaler, W. Wohlleben, and E. Stegmann. 2011. Self-resistance and cell wall composition in the glycopeptide producer *Amycolatopsis balhimycina.* Antimicrob. Agents Chemother. 55:4283–4289.

28. Bullock, W.O., J.M. Fernandez, and J.M. Short. 1987. Xl1-Blue—a high-efficiency plasmid transforming Reca *Escherichia-Coli* strain with beta-galactosidase selection. Biotechniques. 5:376–378.
29. MacNeil, D.J., K.M. Gewain, C.L. Ruby, G. Dezeny, P.H. Gibbons, and T. MacNeil. 1992. Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. Gene. 111:61–68.
30. Kieser, T., M.J. Bibb, M.J. Buttner, K.F. Chater, and D.A. Hopwood. 2000. Practical Streptomyces Genetics: John Innes Foundation, Norwich Research Park, Colney, Norwich, United Kingdom.
31. Menges, R., G. Muth, W. Wohlleben, and E. Stegmann. 2007. The ABC transporter Tba of *Amycolatopsis balhimycina* is required for efficient export of the glycopeptide antibiotic balhimycin. Appl. Microbiol. Biotechnol. 77: 125–134.
32. Pelzer, S., W. Reichert, M. Huppert, D. Heckmann, and W. Wohlleben. 1997. Cloning and analysis of a peptide synthetase gene of the balhimycin producer *Amycolatopsis mediterranei* DSM5908 and development of a gene disruption/replacement system. J. Biotechnol. 56:115–128.
33. Ho, S.N., H.D. Hunt, R.M. Horton, J.K. Pullen, and L.R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene. 77: 51–59.
34. Flett, F., V. Mersinias, and C.P. Smith. 1997. High efficiency intergeneric conjugal transfer of plasmid DNA from *Escherichia coli* to methyl DNA-restricting streptomycetes. FEMS Microbiol. Lett. 155:223–229.
35. Cohen, S.N., A.C. Chang, and L. Hsu. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA. 69:2110–2114.
36. Morrison, D.A. 1979. Transformation and preservation of competent bacterial cells by freezing. Methods Enzymol. 68:326–331.
37. Madon, J., and R. Hutter. 1991. Transformation system for *Amycolatopsis* (*Nocardia*) mediterranei: direct transformation of mycelium with plasmid DNA. J. Bacteriol. 173: 6325–6331.
38. Puk, O., D. Bischoff, C. Kittel, S. Pelzer, S. Weist, E. Stegmann, R.D. Sussmuth, and W. Wohlleben. 2004. Biosynthesis of chloro-beta-hydroxytyrosine, a nonproteinogenic amino acid of the peptidic backbone of glycopeptide antibiotics. J. Bacteriol. 186:6093–6100.
39. Rice, P., I. Longden, and A. Bleasby. 2000. EMBOSS: the European Molecular Biology Open Software Suite. Trends Genet. TIG. 16:276–277.
40. Frasch, H.J., L. Kalan, R. Kilian, T. Martin, G. Wright, and E. Stegmann. 2015. Alternative pathway to a glycopeptide-resistant cell wall in the balhimycin producer *Amycolatopsis balhimycina*. ACS Infect. Dis. 1:243–252.
41. Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62:315–323.
42. Novotna, G.B., M.J. Kwun, and H.J. Hong. 2015. 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. Antimicrob. Agents Chemother. 60:1627–1637.
43. Nishino, K., and A. Yamaguchi. 2001. Overexpression of the response regulator EvgA of the two-component signal transduction system modulates multidrug resistance conferred by multidrug resistance transporters. J. Bacteriol. 183:1455–1458.
44. Lin, M.F., Y.Y. Lin, H.W. Yeh, and C.Y. Lan. 2014. Role of the BaeSR two-component system in the regulation of *Acinetobacter baumannii adeAB* genes and its correlation with tigecycline susceptibility. BMC Microbiol. 14:119.
45. Stegmann, E., H.J. Frasch, R. Kilian, and R. Pozzi. 2015. Self-resistance mechanisms of actinomycetes producing lipid II-targeting antibiotics. Int. J. Med. Microbiol. 305: 190–195.
46. Wright, G.D., C. Molinas, M. Arthur, P. Courvalin, and C.T. Walsh. 1992. Characterization of vanY, a DD-carboxypeptidase from vancomycin-resistant *Enterococcus faecium* BM4147. Antimicrob. Agents Chemother. 36: 1514–1518.
47. Hugonnet, J.E., N. Haddache, C. Veckerle, L. Dubost, A. Marie, N. Shikura, J.L. Mainardi, L.B. Rice, and M. Arthur. 2014. Peptidoglycan cross-linking in glycopeptide-resistant *Actinomycetales*. Antimicrob. Agents Chemother. 58:1749–1756.
48. Marcone, G.L., F. Beltrametti, E. Binda, L. Carrano, L. Foulston, A. Hesketh, M. Bibb, and F. Marinelli. 2010. Novel mechanism of glycopeptide resistance in the A40926 producer *Nonomuraea* sp. ATCC 39727. Antimicrob. Agents Chemother. 54:2465–2472.

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