ACS Infectious Diseases

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Alternative Pathway to a Glycopeptide-Resistant Cell Wall in the Balhimycin Producer *Amycolatopsis balhimycina*

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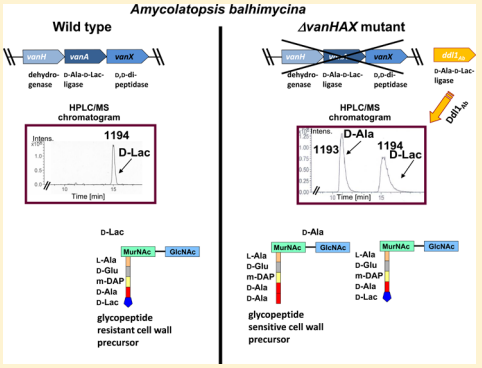
ABSTRACT: Balhimycin, a vancomycin-type glycopeptide, is a lipid II targeting antibiotic produced by *Amycolatopsis balhimycina*. *A. balhimycina* has developed a self-resistance mechanism based on the synergistic action of different enzymes resulting in modified peptidoglycan. The canonical resistance mechanism against glycopeptides is the synthesis of peptidoglycan precursors ending with acyl-D-alanyl-D-lactate (D-Ala-D-Lac) rather than acyl-D-alanyl-D-alanine (D-Ala-D-Ala). This reprogramming is the result of the enzymes VanH, VanA, and VanX. VanH and VanA are required to produce D-Ala-D-Lac; VanX cleaves cytosolic pools of D-Ala-D-Ala, thereby ensuring that peptidoglycan is enriched in D-Ala-D-Lac. In *A. balhimycina*, the Δ*vanHAX*$_{Ab}$ mutant showed a reduced glycopeptide resistance in comparison to the wild type. Nevertheless, Δ*vanHAX*$_{Ab}$ was paradoxically still able to produce D-Ala-D-Lac containing resistant cell wall precursors suggesting the presence of a novel alternative glycopeptide resistance mechanism. In silico analysis, inactivation studies, and biochemical assays led to the characterization of an enzyme, Ddl1$_{Ab}$, as a paraloguous chromosomal D-Ala-D-Lac ligase able to complement the function of VanA$_{Ab}$ in the Δ*vanHAX*$_{Ab}$ mutant. Furthermore, *A. balhimycina* harbors a *vanY*$_{Ab}$ gene encoding a D,D-carboxypeptidase. Transcriptional analysis revealed an upregulated expression of *vanY*$_{Ab}$ in the Δ*vanHAX*$_{Ab}$ mutant. VanY$_{Ab}$ cleaves the endstanding D-Ala from the pentapeptide precursors, reducing the quantity of sensitive cell wall precursors in the absence of VanX$_{Ab}$. These findings represent an unprecedented coordinated layer of resistance mechanisms in a glycopeptide antibiotic producing bacterium.



KEYWORDS: *D-Ala-D-Lac-ligase, vanHAX, vanY, cell wall analysis, glycopeptide producer, lipid II*

Glycopeptide antibiotics such as vancomycin are drugs of choice for treating infections caused by methicillin-resistant *Staphylococcus aureus*, when first-line antimicrobial agents have failed. Glycopeptides inhibit the synthesis of peptidoglycan in Gram-positive bacteria by binding to the acyl-D-alanyl-D-alanine (D-Ala-D-Ala) of the nascent peptidoglycan chains or lipid-linked cell wall subunits and preventing the transpeptidation and transglycosylation reactions.[1,2] Since the introduction of vancomycin in 1953, it took 30 years for the first resistances to appear in the clinic in the form of vancomycin-resistant enterococci (VRE).[3] Vancomycin resistance has since developed into a severe health problem, in part because VRE has become a pervasive nosocomial pathogen.

The glycopeptide resistance mechanism was elucidated in detail in enterococci at the beginning of the 1990s.[4,5] It was first predicted that resistant bacteria, in some manner, produce a thicker call wall, thereby reducing the ability of vancomycin to diffuse into the division septum of the cell required for effective vancomycin treatment.[2] Although this resistance mechanism, called "baiting", was later found in intermediate vancomycin-resistant *S. aureus* strains (VISA),[6] it turned out that the enterococcal resistance was based on reprogramming the cell wall biosynthesis toward the production of an alternative cell wall precursor. The terminal D-alanine (D-Ala) in the pentapeptide side chain of the peptidoglycan was replaced by a D-lactate (D-Lac). This modification reduced the binding affinity of glycopeptides to their target approximately 1000-fold.[4]

In enterococci, the biosynthesis of this alternative cell wall precursor is encoded on transposon Tn1546, an element of conjugative plasmid pIB816.[5] The transposon encodes nine proteins: the transposase Orf1 and resolvase Orf2 for transposition; the two-component regulatory system composed of VanR and VanS; essential resistance determinants VanH, VanA, and VanX; and accessory resistance enzymes VanY and VanZ.[5] VanS is a membrane-spanning histidine kinase that undergoes autophosphorylation in response to glycopeptide antibiotic exposure. VanS subsequently phosphorylates VanR, the cognate response regulator of VanS. The phosphorylated

Received: January 23, 2015
Published: May 5, 2015

DOI: 10.1021/acsinfecdis.5b00011
ACS Infect. Dis. 2015, 1, 243−252

VanR activates the expression of the *vanHAXYZ* genes. VanH is a D-stereospecific lactate dehydrogenase that converts pyruvate to D-Lac.[7] VanA is a D-Ala-D-Ala-ligase family protein that ligates D-Ala and D-Lac to D-Ala-D-Lac-depsipeptide.[8] VanX is a highly selective carboxypeptidase that eliminates the remaining D-Ala-D-Ala-dipeptide that would otherwise be incorporated into the cell wall precursors.[9] In the synthesis of cytosolic and membrane-bound cell wall pentapeptide precursors ending with D-Ala-D-Ala, accessory resistance carboxypeptidase VanY (encoded by *vanY*) cleaves the terminal D-Ala-residue and thereby increases the resistance level. *vanZ*, the second accessory gene within the *van* cluster, confers low-level teicoplanin resistance by an unknown mechanism.[10]

Nine different glycopeptide resistance phenotypes have been described in VRE. Five of those (VanA, B, D, F, and M) follow the model of Tn1546. The other four (VanC, E, G, and N) do not exchange the terminal D-Ala with D-Lac but rather with a D-serine (D-Ser) using a different set of enzymes. However, the basic necessary functions (providing the D-Ser, formation of the D-Ala-D-Ser dipeptide, and elimination of competing sensitive cell wall precursors) are comparable.[11] *vanHAX* homologous genes have also been found in the nonpathogenic bacterial producers of glycopeptides such as *Amycolatopsis orientalis*, *Streptomyces toyocaensis*,[12] *AAmycolatopsis orientalis subsp. lurida*, *Amycolatopsis coloradiensis*,[13,14] *Amycolatopsis japonicum*,[15,16] *Actinoplanes teichomyceticus*[17,18] and WAC1420.[19] It was therefore not only assumed that glycopeptide-resistant enterococci use the same resistance mechanism as producers but also that pathogenic resistance originates from producing actinobacteria. Even the actinomycete *Streptomyces coelicolor* A3(2), which does not produce a glycopeptide, has acquired a *vanHAX*-operon.[20]

In both glycopeptide producers, *A. teichomyceticus* and *S. toyocaensis*, the *vanHAX*-operon is part of their glycopeptide biosynthetic gene clusters. However, *Nonomuraea* ATCC 39727, the producer of A40926 (used for manufacturing the recently approved semisynthetic glycopeptide antibiotic dalbavancin), developed an alternative resistance mechanism employing only a homologue of *vanY* for the synthesis of a resistant cell wall.[21] The resistance mechanism in *Nonomuraea* is based on the synthesis of a peptidoglycan with tetrapeptide side chains that no longer effectively bind the antibiotics. The peptidoglycan is cross-linked by L,D-transpeptidases that use tetrapeptide acyl donors lacking the target for glycopeptides.[22]

Amycolatopsis balhimycina is the producer of the vancomycin-type glycopeptide balhimycin. Because *A. balhimycina* is amenable to genetic manipulation, it was chosen as a model system for analyzing glycopeptide biosynthesis[23,24] and self-resistance.[25] The balhimycin biosynthetic gene cluster contains the $vnlRS_{Ab}$ genes and $vanY_{Ab}$.[26] $vnlRS_{Ab}$ encodes a two-component system with similarity to regulatory resistance genes *vanRS* from *S. coelicolor* A3(2). Additionally, $vanHAX_{Ab}$ is present in the chromosome of *A. balhimycina*, but it is not adjacent to the balhimycin biosynthetic gene cluster. In previous studies we demonstrated that $VanY_{Ab}$ is a D,D-carboxypeptidase with activity toward pentapeptides ending in D-Ala-D-Ala. In contrast to the VanY of VRE, $VanY_{Ab}$ has no carboxyesterase activity.[25]

In this work we show that the *A. balhimycina* $\Delta vanHAX_{Ab}$ mutant still produces balhimycin and synthesizes resistant cell wall precursors. Genetic and biochemical analyses revealed the presence of a second chromosomal D-Ala-D-Lac-ligase (Ddl1), which complements the function of $VanA_{Ab}$ in the $\Delta vanHAX_{Ab}$ mutant. Therefore, we propose a $vanHAX_{Ab}$-independent, alternative glycopeptide resistance mechanism in *A. balhimycina* that also results in the synthesis of a drug-resistant cell wall.

RESULTS AND DISCUSSION

Roles of $vanHAX_{Ab}$ and $vanY_{Ab}$ in *A. balhimycina*. Nearly all antibiotic biosynthetic gene clusters contain genes encoding resistance against the respective antibiotic.[19] The expression of these resistance genes is often coregulated with that of the biosynthesis genes. However, in the balhimycin biosynthetic gene cluster only the accessory resistance gene $vanY_{Ab}$ was identified. The essential $vanHAX_{Ab}$ genes are located more than 2 Mb apart from the balhimycin gene cluster and are expressed constitutively.[25]

To investigate the involvement of $vanHAX_{Ab}$ in self-resistance, we constructed a $\Delta vanHAX_{Ab}$ in-frame deletion mutant (Methods section). The absence of $vanHAX_{Ab}$ was confirmed by PCR analyses and Southern hybridization (data not shown). The glycopeptide resistance of the *A. balhimycina* $\Delta vanHAX_{Ab}$ was reduced (minimal inhibition concentration (MIC) of 0.25 mg/mL balhimycin) in comparison to the wild type (MIC >5 mg/mL balhimycin), demonstrating that the $vanHAX_{Ab}$ genes are functional and are resistance determinants in *A. balhimycina* (Figure 1A).

It was recently shown that $VanY_{Ab}$ increases balhimycin resistance after heterologous expression in *S. coelicolor* A3(2), which naturally harbors *vanHAX* but not *vanY* genes.[25] To characterize its function in *A. balhimycina*, an in-frame $\Delta vanY_{Ab}$ mutant was constructed (Methods section). The deletion of $vanY_{Ab}$ was confirmed by PCR analyses (data not shown). The $\Delta vanY_{Ab}$ mutant was able to grow in the presence of up to 250−500 μg/mL balhimycin and produced balhimycin at a level comparable to that of the wild type (100 mg/L), indicating that $vanY_{Ab}$ is not required for the expression of glycopeptide resistance in the presence of the constitutively expressed $vanHAX_{Ab}$ genes. RT-PCR analyses revealed that a $vanY_{Ab}$ transcript was detectable in the $\Delta vanHAX_{Ab}$ mutant as early as 15 h following inoculation but in the wild type only after 63 h of growth (Figure 2). To further analyze the role of $vanY_{Ab}$, we constructed a $\Delta vanHAX_{Ab}/vanY_{Ab}$ double mutant. The deletion of both genes was confirmed by PCR and Southern blot analyses (data not shown). This double mutant showed the same phenotype as $\Delta vanHAX_{Ab}$ (reduced MIC values). It seems that $VanY_{Ab}$ is not absolutely required for the resistance observed in the $\Delta vanHAX_{Ab}$ mutant.

In contrast to the results obtained in the balhimycin producer, *Nonomuraea* ATCC 39727 does not contain *vanHAX* genes; therefore, the *Nonomuraea* $vanY_n$ mutant strain showed reduced levels of glycopeptide resistance without affecting A40926 (a teicoplanin-type glycopeptide) production.[21]

To investigate whether antibiotic production depends on self-resistance, we analyzed balhimycin production in the $\Delta vanHAX_{Ab}$. The bioassay against *Bacillus subtilis* (Figure 1B) and HPLC-UV/vis analyses revealed that the $\Delta vanHAX_{Ab}$ mutant is able to produce balhimycin, albeit at a level of ca. 40% compared to that of the wild type.

Cell Wall Analysis of the $\Delta vanHAX_{Ab}$ Mutant. To analyze whether in the *A. balhimycina* $\Delta vanHAX_{Ab}$ mutant the balhimycin resistance is the result of the synthesis of a thicker murein layer, we performed transmission electron microscopy and measured the cell wall thickness in *A. balhimycina* WT and in the $\Delta vanHAX_{Ab}$ mutant. However, in contrast to *S. aureus* COL, where the increase in cell wall thickness and the

DOI: 10.1021/acsinfecdis.5b00011
ACS Infect. Dis. 2015, 1, 243−252

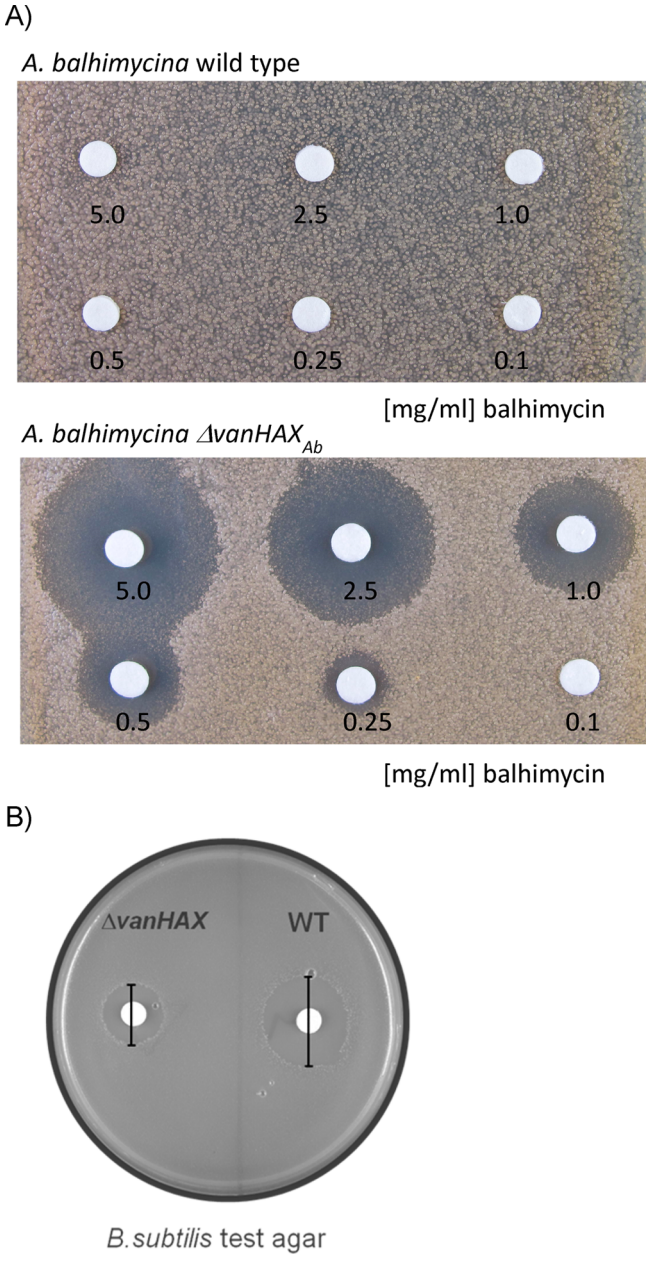


Figure 1. Characterization of balhimycin resistance and balhimycin production in *A. balhimycina* WT and the Δ*vanHAX*$_{Ab}$ mutant. (A) Determination of the balhimycin MIC concentration. (B) Production of balhimycin: culture filtrate of the strains tested in a bioassay with *Bacillus subtilis* as a test organism.

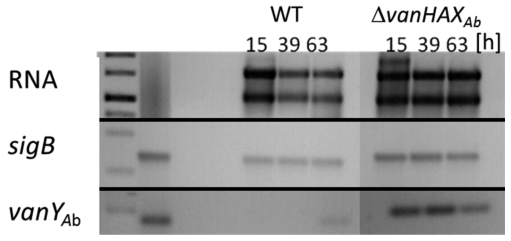


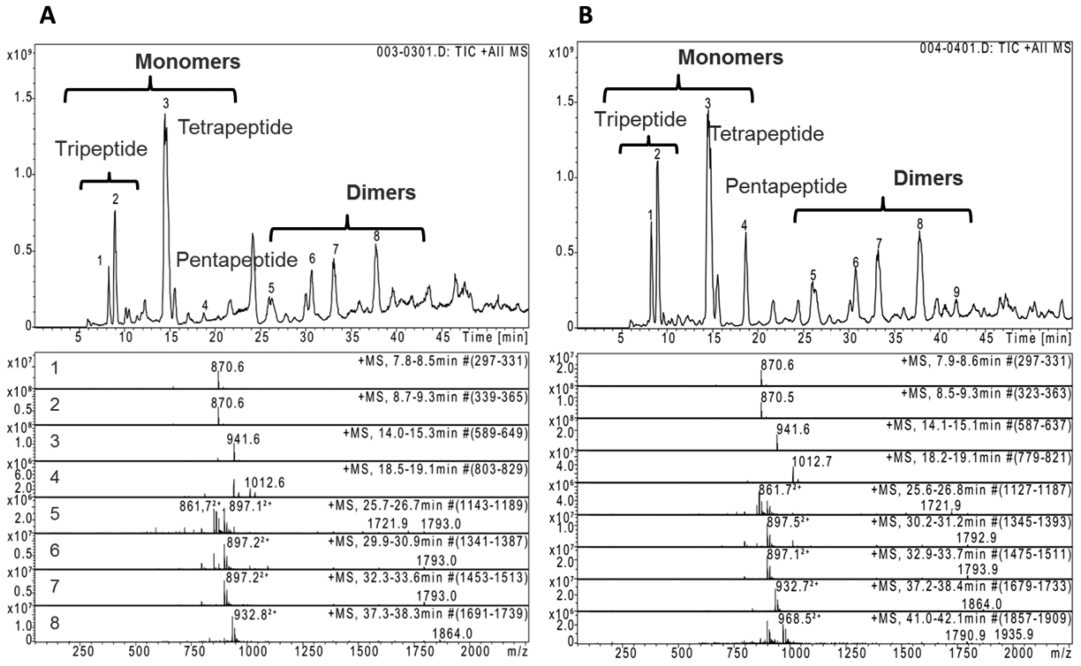
Figure 2. Transcription of *vanY*$_{Ab}$ in *A. balhimycina* WT and the Δ*vanHAX*$_{Ab}$ mutant. RNA: isolated RNA from *A. balhimycina* WT and Δ*vanHAX*$_{Ab}$ after 15, 39, and 63 h of cultivation in R5 medium.*sigB*: transcription of housekeeping gene *sigB*. *vanY*$_{Ab}$: transcription of *vanY*$_{Ab}$.

reduction of cross-linking leads to vancomycin resistance,[6] the *A. balhimycina* cell wall ultrastructure did not show a visible difference between WT and the Δ*vanHAX*$_{Ab}$ mutant. We conclude that the resistance in the Δ*vanHAX*$_{Ab}$ mutant is based on a different mechanism. One possibility is the production of alternative resistant cell wall precursors such as described for *Nonomuraea,* which synthesizes a peptidoglycan with tetrapeptide side chains.[21] To investigate whether *A. balhimycina* uses such a resistance mechanism, we analyzed the composition of the mature cell wall of *A. balhimycina* by HPLC/MS. Both the cell wall of the wild type as well as of the Δ*vanHAX*$_{Ab}$ mutant revealed peaks corresponding to the masses of GlcNAc-MurNAc-pentapeptide monomers in both strains. In addition, the analyzed dimer fraction showed masses corresponding to tetra-/pentapeptide dimers as well as tri/pentapeptide and tri/tetrapeptide dimers (Figure 3). The occurrence of the tri/tetrapeptide dimers can be explained only as a reaction of L,D-transpeptidase (Ldt). Ldts catalyze the linkage of the third amino acid of each peptidoglycan stem peptide (3−3 cross-links) instead of the 3−4 cross-links catalyzed by common D,D-transpeptidases. The substrates of the Ldts are tetrapeptides[27] which can be provided by VanY$_{Ab}$. In silico analyses revealed the presence of at least three genes encoding putative Ldts in *A. balhimycina*. Besides VanY$_{Ab}$, VanY$_n$ from *Nonomuraea* is the second-most-characterized carboxypeptidase of a glycopeptide producer. The genome of *Nonomuraea* includes only a homologue to *vanY*$_n$ but no *vanHAX* genes. Binda et al.[28] characterized VanY$_n$ as a new enzyme which possesses both D,D-carboxypeptidase and D,D-dipeptidase activity, removing the endstanding D-Ala from the pentapeptide precursor as well as from the D-Ala-D-Ala dipeptide. The L,D-transpeptidase, encoded in the genome of *Nonomuraea* ATCC 39727, subsequently cross-links the tetrapeptide acyl donors at the third amino acid. This results in peptidoglycan 3−3 cross-linked tetra- and tripeptides, devoid of the D-Ala-D-Ala ending peptides, which can no longer serve as a target of glycopeptides.[27]

Because in *A. balhimycina* variations of peptidoglycan dimers (tetra-/pentapeptide, tri/pentapeptide, and tri/tetrapeptide) are present, we concluded that a peptidoglycan based on tetrapeptides is not the main resistance mechanism in the Δ*vanHAX*$_{Ab}$ mutant.

To analyze whether the *vanHAX*$_{Ab}$-independent resistance mechanism in *A. balhimycina* is based on the synthesis of an alternative resistant cell wall precursor, these were enriched in the Δ*vanHAX*$_{Ab}$ mutant by blocking lipid II synthesis with bacitracin, extracted and analyzed by HPLC-MS. From previous analyses,[25] we know that cell wall precursors ending with D-Ala elute with a retention time (rt) of 10 to 11 min and have a molecular mass of 1193 Da whereas cell wall precursors terminating in D-Lac have a rt of 15−16 min and a molecular mass of 1194 Da. HPLC-MS analyses of cell wall precursors isolated from the *A. balhimycina* Δ*vanHAX*$_{Ab}$ mutant (cultivated in balhimycin production medium) revealed the presence of a D-Ala-D-Lac terminus (peak at rt of 15−16 min and a molecular mass of 1194 Da) (Figure 4). This result is consistent with the presence of unknown genes/enzymes that enable the synthesis of lipid II with terminal D-Lac in the absence of *vanHAX*$_{Ab}$. To identify candidate genes we surveyed the genome of *A. balhimycina* for *vanA*$_{Ab}$ and *vanX*$_{Ab}$ paralogues. The third missing gene in Δ*vanHAX*$_{Ab}$ mutant *vanH*$_{Ab}$ encodes a dehydrogenase, which catalyzes the conversion of pyruvate to D-Lac, the substrate of VanA. In

DOI: 10.1021/acsinfecdis.5b00011
ACS Infect. Dis. 2015, 1, 243−252



Calculated and detected mass values of the fragments obtained after digestion of the peptidoglycan of *A. balhimycina* WT und *ΔvanHAX*$_{Ab}$ mutant

	calculated	detected	Peak-No	Rt [min]
GlcNAc-MurNAc-tripeptide	869,4	869,5 665	1;2	~8; ~9
GlcNAc-MurNAc-tetrapeptide	941,47	940,5	3	~14,5
GlcNAc-MurNAc-pentapeptide	1011,72	1011,65	4	~19
GlcNAc-MurNAc-tri-tripeptide	1721	1721,4	5	~26
GlcNAc-MurNAc-tri-tetrapeptide	1792	1792,4	6;7	~30,5;~33
GlcNAc-MurNAc-tetra-tetrapeptide	1863	1863,6	8	~37,5
GlcNAc-MurNAc-tri-pentapeptide	1863	1863,6	8	~37,5
GlcNAc-MurNAc-tetra-pentapeptide	1934,15	1935	9	~41,5

Figure 3. HPLC-MS analyses of the peptidoglycan components in *A. balhimycina* wild type (A) and Δ*vanHAX*$_{Ab}$ mutant (B) (cell wall after mutanolysin digestion) and the corresponding mass values.

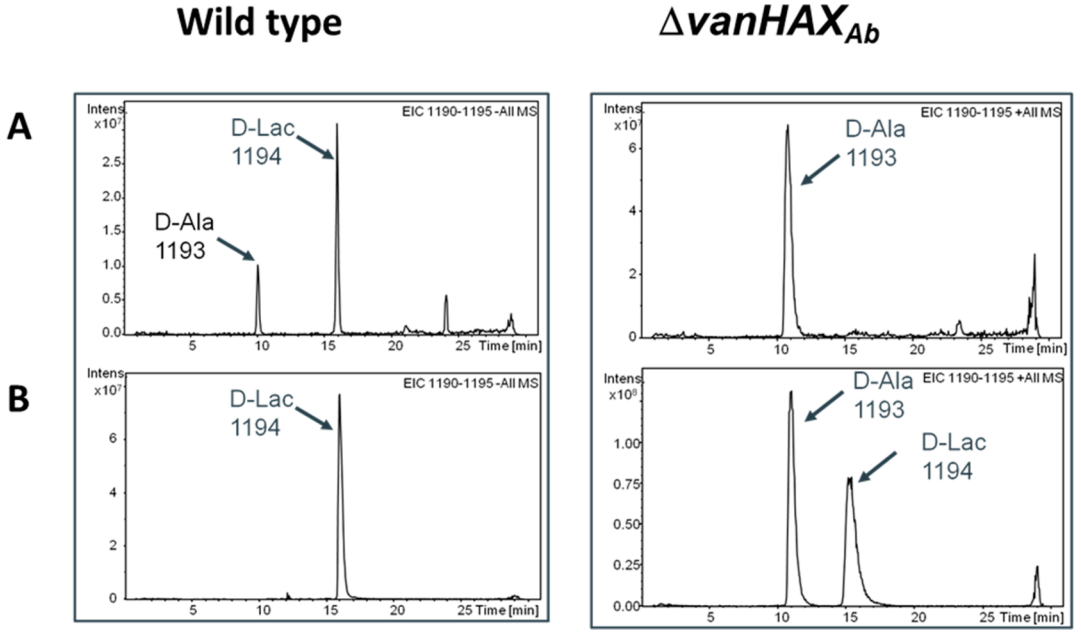


Figure 4. Cell wall precursor profile of *A. balhimycina* wild type and Δ*vanHAX*$_{Ab}$ under (A) nonproduction conditions (cultivation in TSB medium) and (B) production conditions (cultivation in R5 medium).

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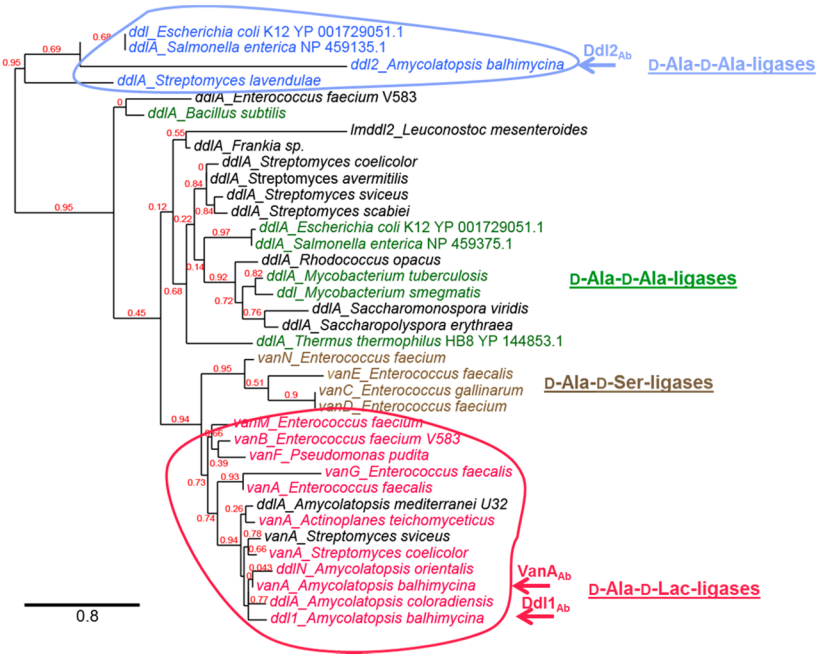

Figure 5. Phylogenetic tree for known Ddl-like enzymes. Phylogenetic inference of enzymes was made using 37 complete sequences of different bacteria.

the Δ*vanHAX*$_{Ab}$ mutant, D-Lac can be provided by different basic metabolic pathways, which are encoded in the genome (such as cell wall recycling via the *nagZ*, *ybbc*, *amiE*, *murR9*, and *murQ* genes[29]).

Identification of Alternative D-Ala-D-Lac-Ligase(s) in *A. balhimycina*. *Phylogenetic Analyses of the VanA-like Enzymes.* A BLAST search of the *A. balhimycina* genome identified two genes encoding putative D-Ala-D-Lac or D-Ala-D-Ala-ligases named Ddl1$_{Ab}$ (with 72% identity to VanA) and Ddl2$_{Ab}$ (with 28% identity to VanA). Ddl1$_{Ab}$ and Ddl2$_{Ab}$ were next analyzed through a phylogenetic analysis[30] that included VanA-like proteins from different glycopeptide-producers (*A. balhimycina*, *A. orientalis*, *Amycolatopsis mediterranei*, *Amycolatopsis coloradiensis*, and *A. teichomyceticus*). In total, 37 members of the D-Ala-D-Ala/D-Ala-D-Lac ligase family were included in this analysis using the tool phylogeny analysis (muscle analyses, Gblocks curation, PhyML, TreeDyn). The sequence comparison yielded caterpillar-like trees with five subclades. Ddl2$_{Ab}$ builds its own subclade, which is related to the subclade including the ligases from *Escherichia coli* K12 and *Salmonella enterica* that exclusively synthesize D-Ala-D-Ala. In contrast, VanA$_{Ab}$ and Ddl1$_{Ab}$ belong to the VanA-group, characterized as a group of enzymes with D-Ala-D-Lac-ligase activity (Figure 5), suggesting that Ddl1$_{Ab}$ might be a VanA$_{Ab}$ substitute implied by the cell wall precursor analysis of the Δ*vanHAX*$_{Ab}$ mutant.

The Δ*ddl1*$_{Ab}$ deletion mutant showed no phenotype concerning balhimycin production, resistance, or cell wall precursor pattern. In this mutant, the formation of the D-Ala-D-Lac depsipeptide is most likely catalyzed by the expression of VanA in the *vanHAX*$_{Ab}$ operon. Additional proteome analyses of the wild type and the Δ*vanHAX*$_{Ab}$ mutant revealed equivalent Ddl1$_{Ab}$ expressions in both strains (data not shown). These results suggested that Ddl1$_{Ab}$ is also active in the wild type together with VanA$_{Ab}$ and that both enzymes may substitute for each other in the corresponding mutants.

Biochemical Characterization of the VanA Homologues. Ddl1$_{Ab}$ and Ddl2$_{Ab}$ ligases were purified and characterized by steady-state kinetics. The results indicate (Table 1) that D-Lac is the preferred substrate for VanA$_{Ab}$ and Ddl1$_{Ab}$. The catalytic efficiencies (k_{cat}/K_M) of VanA$_{Ab}$ and Ddl1$_{Ab}$ for D-Lac were 176- and 121-fold higher than for D-Ala, respectively. Saturable conditions for D-Ala were not achievable for either VanA$_{Ab}$ or Ddl1$_{Ab}$ even at concentrations exceeding 100 mM D-Ala. Ddl2$_{Ab}$ was not functional under all conditions tested.

Table 1. Kinetic Characterization of D-Ala-D-X Ligases Using the Coupled Pyruvate Kinase/Lactate Dehydrogenase (PK/LDH) Assay

enzyme	product	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
VanA$_{Ab}$	D-Ala-D-Ala			1.7×10^1
	D-Ala-D-Lac	0.13 ± 0.0098	0.38 ± 0.0078	3.0×10^3
	ATP	0.06 ± 0.041	0.38 ± 0.011	6.7×10^3
Ddl1$_{Ab}$	D-Ala-D-Ala			2.8×10^1
	D-Ala-D-Lac	0.13 ± 0.014	0.43 ± 0.014	3.4×10^3
	ATP	0.12 ± 0.0092	0.40 ± 0.014	3.4×10^3

The enzymes bind two molecules of D-amino/hydroxy acids in two distinct binding sites. The first is always D-Ala, thus we are measuring the kinetic parameters for the second binding site using the coupled assay. Although the active site discriminates which hydroxy amino acid it prefers, in the case of VanA's some D-Ala-D-Ala is concurrently being formed in addition to D-Ala-D-Lac. Unfortunately, it is difficult to resolve the rate and the effect on D-Ala-D-Lac formation using the spectrophotometric assay used to determine steady-state parameters. The markedly large increase in k_{cat}/K_M is a good indicator of the discrimination of D-Lac in the active site of the enzyme; however, this assay is unable to quantify directly the exclusive formation of D-Ala-D-Lac. Therefore, a direct TLC assay involving radiolabeled substrates was employed.

DOI: 10.1021/acsinfecdis.5b00011
ACS Infect. Dis. 2015, 1, 243−252

The amino acid specificity of each ligase was qualitatively determined using [U−^{14}C]- D-Ala and unlabeled D-amino and D-hydroxy acid substrates followed by separation of the products by thin-layer chromatography. DdlB, the D-Ala-D-Ala-ligase from *E. coli*, and $VanA_{Ao}$, the D-Ala-D-Lac-ligase from the vancomycin producer *A. orientalis* C329.2, were used as positive controls. Even with this more sensitive assay, the D-Ala-D-Ala-ligase from *A. balhimycina* was not active. The formation of D-Ala-D-Lac is preferred over D-Ala-D-Ala for DdlN, $VanA_{Ab}$ and $Ddl1_{Ab}$ while DdlB produce only D-Ala-D-Ala. These results were confirmed by the shift in the retention factor (Rf) between the depsipeptide and dipeptide (Figure 6).

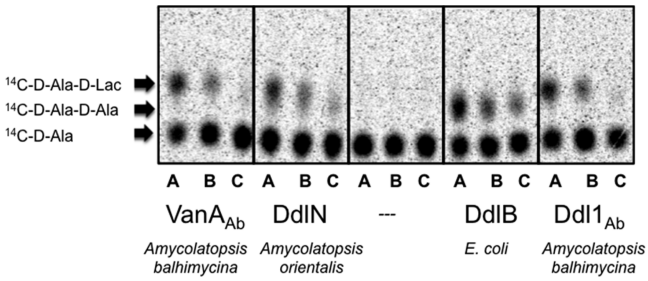

Figure 6. Substrate specificity of D-Ala-D-X ligases. Substrate specificity was examined using [U−^{14}C]-D-Ala and either D-Ala or D-Lac. The products of each reaction were separated by TLC and exposed to a phosphor-storage screen. All reactions contain 0.1 uCi [U−^{14}C]-D-Ala. DdlN and DdlB represent positive controls for D-Ala-D-Lac and D-Ala-D-Ala-ligases, respectively. (A) ^{14}C-Ala +2 mM D-Lac. (B) ^{14}C-Ala +2 mM D-Lac +1 mM D-Ala. (C) ^{14}C-Ala +2 mM D-Ala +1 mM D-Ala.

Analysis of the Alternative Carboxypeptidase in *A. balhimycina*. The dipeptidase $VanX_{Ab}$ cleaves the D-Ala-D-Ala dipeptide in order to enhance the synthesis of resistant cell wall precursors. Since this peptidase is missing in the Δ*vanHAX*$_{Ab}$ mutant we screened the *A. balhimycina* genome for homologous genes. The genome does not harbor any *vanX*$_{Ab}$ homologue. However, RT-PCR experiments revealed that *vanY*$_{Ab}$ transcription is increased in the Δ*vanHAX*$_{Ab}$ mutant. The carboxypeptidase $VanY_{Ab}$ generates tetrapeptides and contributes thereby to resistance through providing cell wall building units with reduced affinity for balhimycin. This represents a separate but complementary event to formation of D-Ala-D-Lac.

However, although in the Δ*vanHAX*$_{Ab}$ mutant $Ddl1_{Ab}$ and $VanY_{Ab}$ are active, cell wall analysis of the mutant showed a significant fraction of D-Ala-D-Ala ending precursors. Using cell extracts of *A. balhimycina* wild type and the Δ*vanHAX*$_{Ab}$ mutant, we determined biochemically the internal D-Lac pool in each strain by a D-Lac assay (Megazyme, Ireland). The concentration of D-Lac in the Δ*vanHAX*$_{Ab}$ mutant was about 5-fold decreased in comparison to the wild type. The lower provision of the D-Lac substrate may be the reason for the synthesis of a mixture of resistant and sensitive cell wall precursors.

CONCLUDING REMARKS

The sensitivity of the Δ*vanHAX*$_{Ab}$ mutant toward balhimycin confirmed the function of the *vanHAX*$_{Ab}$ genes as resistance determinants of *A. balhimycina*. The ability of the mutant to produce balhimycin and to synthesize a resistant cell wall in the absence of the *vanHAX*$_{Ab}$ genes suggested the existence of alternative resistance enzymes with $Ddl1_{Ab}$ as a key enzyme. We demonstrate that the chromosomally encoded ligase $Ddl1_{Ab}$ has robust D-Ala-D-Lac synthesis activity and is able to take over the function of $VanA_{Ab}$ in the Δ*vanHAX*$_{Ab}$ mutant. To ensure that only resistant cell wall precursors were used for cell wall synthesis, $VanY_{Ab}$ generates tetrapeptide precursors that do not bind glycopeptides.

These findings suggest that the synthesis of the resistant peptidoglycan precursors was originally based on a mechanism where the endogenous D-Lac pool was used to provide the substrate for the ligase that formed the D-Ala-D-Lac-depsipeptide. Because the specificity of the ligase is determined by only a few conserved amino acids, the D-Ala-D-Lac ligase is most likely evolved from a D-Ala-D-Ala-ligase. Subsequently, the *vanHAX* genes clustered together with the accessory (*vanY*) and regulatory genes (*vanRS*) were captured, forming the glycopeptide resistance cassette. Besides the canonical resistance mechanism mediated by the *vanHAX* genes and the alternative way to synthesize D-Ala-D-Lac-precursors in the absence of the *vanHAX* genes, glycopeptide producers seem to develop a third resistance mechanism. This mechanism is based on the elimination of the target by synthesizing a peptidoglycan with 3−3 cross-linked tetrapeptide stems.

METHODS

Strains and Media. For all experiments, *Amycolatopsis balhimycina* was precultured from R5-agar plates in 20 mL of TSB medium (Difco) or R5 medium for 48 h at 28−30 °C at 120 or 180 rpm. For the main cultures, 50 mL of TSB (nonproduction conditions) or 50 mL of R5 medium (production conditions) was inoculated with 2 mL of preculture and grown for 48 h for the cell wall precursor and DNA extraction or for 5 days for production assays. Resistance assays were performed on HA-agar.[31] The production of balhimycin was assayed against *B. subtilis* spores on antibiotic test agar[32] at 37 °C.

E. coli was grown in Luria-Bertani broth at 37 °C. Liquid cultures were shaken at 180 rpm. Media were supplemented with appropriate antibiotics.

Plasmid Construction and Transformation. Genomic DNA for PCR from *A. balhimycina* was isolated from 48- to 72-h-old liquid cultures using the peqGOLD bacterial total DNA kit by the manufacturer's instructions. Genomic DNA for Southern blot was isolated using the lysozyme-based Kirby-Mix procedure described in Kieser.[33] Plasmid DNA from *E. coli* was prepared by alkaline lysis[34] (small scale) or the Promega PureYield system (large scale). Plasmid DNA for sequencing was isolated using the Qiagen QiaPrep spin miniprep kit.

Up- and downstream regions of genes of interest were amplified by PCR using either the Proofstart polymerase (Qiagen) or the Kapa-Hifi-proofreading polymerase with buffers for GC-rich templates from *A. balhimycina* genomic DNA. Fragments of expected size were gel extracted (GE Healthcare Gel extraction Kit) and cloned blunt end into pJET1.2 (Thermo Scientific) by the supplier protocol and transferred into *E. coli* XL1 blue $CaCl_2$ competent cells. Clones were checked for correct insertion by minilysis and restriction analysis with *Bgl*II. Plasmids with expected restriction patterns were selected for sequencing.

Sequence-verified sequences were subcloned in two steps with appropriate restriction enzymes into pSP1.[35] Restriction-verified pSP1 derivatives were transferred to methylation-

DOI: 10.1021/acsinfecdis.5b00011
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Table 2. List of Primers and Plasmids Used in These Studies

primer list:	
RT-PCR	
sigB for	CGTAGGTCGAGAACTTGAAC
sigB rev	GTGTCTACCTCAACGGTATC
ddlAForward	GGTGTCCAGCAAGGAAGAAC
ddlAReverse	ACCCACCGGTCGATCATCTC
ddlBForward	TGCTGCCGCACAGCATGTTC
ddlBReverse	AAGTCGTACACGCCGCTCTC
D-2-hydroxyForward	TGTCGGCCACCGGGTACATC
D-2-hydroxyReverse	GGTTCTGGCTCCAGGCAATC
D3-G-DHForward	TCGGCGCCTTCGGGTTCAGC
D3-G-DHReverse	CGCCGACGCCATCTCCATCC
vanY_RT_rev	TTCACGCACAGTTCG
vanY_RT_fwd	TCGGCACGAGGATTG
vanH1	GGGACAAGCCCATCAAGAAC
vanA2	GAGCGGACTTGACGGAGATG
vanA3	GGGTGGACCTGTTCCTCAAG
vanX4	TTGGCGTCCCAGCGAATACC
Cloning Primers for Gene Deletions	
vanY-down SphI	ATGCATGCGAGTCTTCTCCGCTCCGAAC
vanY-down XbaI	ATTCTAGATGTATGCCGACCCGAGTCAC
vanY-up XbaI	ATTCTAGACCGGGCCGACTCGCGGTAGG
vanY-up *Eco*RI	ATGAATTCGTCCGGCGGCGTGGGCAGTG
ddl1 down *Eco*RI	ATGAATTCCCGAATATTGCCGTCGAAGC
ddl1 down XbaI	ATTCTAGATACTGATTCCCGCTTTCGCA
ddl1 up XbaI	ATTCTAGACGCGAGGTAACGATTCTCC
ddl1 up SphI	ATGCATGCTTCATCGCCTGGGCGTACAC
deltavanHAX1_1	TTTATAGAGCTCTCACGCGAGTTACCCATCCG
deltavanHAX1_2	AATAATTCTAGATAACCGAAGTGGGCCGTGCT
deltavanHAX2_1	TTTATATCTAGAGCCCGGCAGTATAGGCAGCGCGGTGA
deltavanHAX2_2	AATAATCTGCAGCGGCGTGCCGTCCGTATATC
vanYdelproof1	TCGAGGAGCGACTCTTTCAC
vanYdelproof2	CGGAAGCGACGATCCGTTTG
ddl1delproof1	GACTACGTACGGTACGGTAACGGTCGACAG
ddl1delproof2	CGTGAACGATGACCGGAATGGTGTGGTCTC
vanHAX-delproof1	ATCATAACCACCGCGTTGCCGGAC
vanHAX-delproof2	GAGGAACGTCGTCTCGTACCAGTC
ddl1extern	TCGGCATCTACTCGCTCTTC
vanHAX extern	CCCGATCACGCGAGTTACCC
vanHupstream1	GGTCATGCCCGGCAGTATAG
vanHupstream2	GTCATGCTTCCCGTGTACCG
Others	
pJet1.2 forward	CGACTCACTATAGGGAGAGCGGC
pJet1.2 reverse	AAGAACATCGATTTTCCATGGCAG
Eryright	AAGGGAGAAAGGCGGACAGG
Eryleft	GTCGCTTCTGCGCAAGTACC
Plasmid for Gene Deletion	
pSP1	deletion plasmid, *bla, ermE*
pSP1Δddl1	plasmid used for *ddlX* deletion
pSP1ΔvanY	plasmid used for $vanY_{Ab}$ deletion
pSP1ΔvanHAX	plasmid used for deletion of the $vanHAX_{Ab}$-operon

deficient *E. coli* ET12567[36] and reisolated on a large scale for direct transformation of *A. balhimycina*.[33,35]

The plasmid pSP ΔvanHAX was constructed to inactivate $vanHAX_{Ab}$ by an in-frame deletion. A upstream fragment (frΔ$vanHAX_{Ab}$-left) and a downstream fragment (frΔ$vanHAX_{Ab}$-right) of $vanHAX_{Ab}$ were amplified from genomic DNA by PCRs using primer pairs deltavanHAX1_1-deltavanHAX1_2 and deltavanHAX2_1- deltavanHAX2_1 (Table 2), respectively. The primers contained restriction sites at the 3′ and 5′ ends (*Eco*RI/XbaI and XbaI/SphI sites, respectively). Both fragments were cloned into the pSP1 vector to generate pSPΔvanHAX.

For the in-frame deletion of $vanY_{Ab}$, plasmid pSPΔvanY was constructed. A downstream fragment (frΔvanY-left) and an upstream fragment (frΔvanY-right) of $vanY_{Ab}$ were amplified from cosmid DNA by PCRs using primer pairs vanY-down SphI-vanY-down XbaI and vanY-up XbaI-vanY-up *Eco*RI (Table 2), respectively. The primers contained restriction

DOI: 10.1021/acsinfecdis.5b00011
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sites at the 5′ and 3′ ends (SphI/XbaI and XbaI/*Eco*RI sites, respectively). Both fragments were cloned into the pSP1 vector to generate pSPΔvanY.

For the in-frame deletion of $ddl1_{Ab}$, plasmid pSPΔddl1 was constructed. A downstream fragment (frΔddl1-left) and an upstream fragment (frΔddl1-right) of ddl_{Ab} were amplified from cosmid DNA by PCRs using primer pairs ddl1-down *Eco*RI-ddl1-down XbaI and ddl1-up XbaI-ddl1-up SphI (Table 2), respectively. The primers contained restriction sites at the 5′ and 3′ ends (*Eco*RI/XbaI and XbaI/SphI sites, respectively). Both fragments were cloned into the pSP1 vector to generate pSPΔddl1.

A. balhimycina strains were transformed with pSP1ΔvanHAX, pSP1ΔvanY, or pSP1Δddl1 by use of the direct transformation method as described previously.[36] The integration of the plasmids into the chromosome via homologous recombination was confirmed by PCR screening for the erythromycin resistance cassette using primers Eryright and Eryleft (Table 2).

Double crossover for subsequent gene deletion was provoked by heat-shock (37 °C for 6 h or overnight). Single cells were derived from mycelium by generating protoplasts,[33] which were regenerated on R5-agar at 30 °C. Single colonies were transferred to R5-agar with and without erythromycin. Sensitive clones were checked by PCR using primers vanHAXdelproof1-vanHAXdelproof2 (or vanHAXextern), vanYdelproof1-vanYdelproof2, and ddl1delproof1-ddl1delproof2 (or ddl1extern).

Cell Wall Precursor Isolation. Cell wall precursors were isolated as described previously[25] after 48 h of main culturing under production or nonproduction conditions. Cells were treated twice for 30 min with bacitracin (100 μg/mL final concentration) to enrich cell wall precursors. Cells were harvested and resuspended in H_2O and incubated in a boiling water bath for 30 min. Cell debris was removed by centrifugation, and supernatant was lyophilized and resuspended in 1 mL of H_2O. Unsoluble material was removed by extraction with 200 μL of chloroform prior to LC-MS analysis.

Five microliters of sample was injected into an HPLC-MS instrument (XCT6330 LC/MSD ultra trap system, Agilent Technologies) with a Nucleosil 100 C18 column (3 μm × 100 mm × 2 mm internal diameter). The linear gradient was from 0% eluent A (H_2O, 0.1% HCOOH) to 10% eluent B (0.6% HCOOH in acetonitrile) over 25 min with a flow rate of 400 μL/min. Ionization alternated between positive and negative with a capillary voltage of 3.5 kV at 350 °C.[25]

Muropeptide Preparation from Cell Walls. Cell wall peptidoglycan was prepared as previously described,[25] with the exception that 4% SDS was used to obtain murein sacculi and that sonication for 3 × 2 min was used to disrupt cells prior to DNaseI and RNase treatment.

Peptidoglycan was purified from ∼20 mg of cell walls by incubation with 0.5 mL of 48% hydrofluoric acid for 48 h at 4 °C. HF was removed by repeated washes (13 000 rpm, 10 min) with ice-cold water until pH 7 was reached. Peptidoglycan was subsequently resuspended in water and lyophilized.

Muropeptides were prepared from peptidoglycan by digestion with mutanolysin in 12.5 mM potassium phosphate buffer at pH 5.5 at 37 °C overnight with shaking. Insoluble material was removed by centrifugation (13 000 rpm, 20 min). Supernatant was used for the reduction of muropeptides with borhydrid as described before.[25] HPLC-MS analysis was performed as previously described.[25]

RNA Isolation and RT-PCR. Cells were harvested at 4 °C at 4500 rpm at appropriate time points. Pellets were resuspended in TE buffer and transferred to a 2 mL reaction tube containing 200 μL of glass beads (Ø 0.17−0.18 mm) to yield a total of ∼250 μL of pellet. TE buffer was replaced with 0.5 mL of 2× Kirby-Mix,[33] and cells were disrupted by bead beating at 6500 rpm over a 2 × 20 s−30 s break. Phenol/chloroform (1:1, 0.5 mL) was added, and disruption was repeated two more times. Cell lysates were centrifuged at 14 800 rpm at 4 °C, and the supernatant (∼1 mL) was transferred to a new 2 mL tube. Protein was removed by repeated extraction with 1 mL of phenol/chloroform until no interphase was visible. Total nucleic acid was precipitated by the addition of a 1/10 volume of 3 M NaAc pH 6 and 1 volume of isopropanol. Pellet was washed twice with 70% ethanol before dissolving in H_2O. Total nucleic acid was digested twice with DNaseI and was tested by PCR to ensure absence of genomic DNA. cDNA was synthesized from 1.5 or 3 μg of RNA with the RevertAid First Strand cDNA Synthesis Kit using random hexamer primers.

Balhimycin Production Assay. 10−20 mL of R5-medium were inoculated with ∼1 cm^2 *A. balhimycina* mycelium from an R5-agar plate and grown for 2 days at 30 °C at 180 rpm. Two milliliters of preculture was used to inoculate 2× 500 mL of baffled Erlenmeyer flasks with 50 mL of R5-medium. Main cultures were grown for 5 days at 27 °C at 120 rpm. Samples (3 × 1 mL) were taken. Supernatant from one sample was used to check production against *B. subtilis* and to determine the balhimycin concentration in the culture filtrate by HPLC. Two samples were lyophilized to determine the complete culture dry weight for normalization.

HPLC was performed using an Agilent 1260 infinity chromatography system equipped with a thermocontrolled 1260 infinity autosampler with a Nucleosil 100 C18 column (5 μm 125 × 3 mm ID + precolumn 20 × 3 mm^2). Gradient ranged from 95.5% eluent A (0.1% phosphoric acid) plus 4.5% eluent B (acetonitrile) to 100% eluent B over 15 min and remained at 100% eluent B for an additional 3 min. The injection volume was 5 μL. For detection, a 1260 infinity diode array detector with a 10 mm flow cell was used at wavelengths of 210, 230, 260, 280, 310, 360, 434, and 500 nm.

Determination of the Minimal Inhibition Concentration against Balhimycin. A 24 h culture of *A. balhimycina* strains from the TSB medium was harvested and homogenized to yield disperse mycelium. One hundred milligrams of cell wet weight was resuspended in 1 mL of HA medium. HA-agar at the appropriate temperature was inoculated 1:100 with this cell suspension, and plates were cast normally. Filter discs with defined amounts of balhimycin were placed on the plates after solidification, the plates were incubated for 3 days at 30 °C, and the zones of growth inhibition were inspected.

Cloning and Expression of D-Ala-D-X Enzymes. Two putative D-Ala-D-Lac ($VanA_{Ab}$ and $pVanA_{Ab}$) and D-Ala-D-Ala-ligase (Ddl_{Ab}) genes from *A. balhimycina* were synthesized with codon optimization for expression in *E. coli* and incorporated 5′ NdeI and 3′ *Hin*dIII restriction enzyme sites (GenScript, Piscataway, NJ, USA). The resulting genes were subcloned from pUC57 into expression vector pET28a with an N-terminal His6 tag for downstream purification. Constructs were confirmed by sequencing and were subsequently propagated in *E. coli* BL21 (DE3) (Novagen, Darmstadt, Germany) for expression. The three *A. balhimycina* constructs produced enough soluble protein for enzyme analysis. High-level protein

DOI: 10.1021/acsinfecdis.5b00011
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expression was achieved by growing cells in 1 L of Luria−Bertani broth to an optical density of 0.6 at 600 nm. Protein expression under the T7 promoter was induced by the addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1 mM, followed by incubation at 16 °C for 16−18 h. Postharvest, cells were washed in 0.85% NaCl (w/v), and the pellet was stored at −20 °C or prepared for protein purification. Enzymes were purified as His6-tagged proteins as above with the following modifications. Cell pellets were resuspended in lysis buffer containing 50 mM HEPES, 500 mM NaCl, 20 mM imidazole, 10 mM $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride, and 1 mM DNase at pH 7.5 and lysed by three passes on a continuous cell disruptor (Constant Systems Ltd., Daventry, U.K.) at 30 kPSI. After elution in imidazole from a Nickel-NTA-immobilized metal affinity column, fractions containing pure protein were pooled and dialyzed into 50 mM HEPES, 150 mM NaCl, pH 7.5 at 4 °C.

E. coli BL21(DE3) harboring pET28b*vanA*$_{Ao}$ from vancomycin producer *Amycolatopsis orientalis* C329.2 was prepared as previously described[13] and purified as a His6-tagged protein as described above. *E. coli* W3110 harboring pTB2 for the expression of D-Ala-D-Ala-ligase DdlB was previously reported.[38]

Ddl Assays. For the qualitative determination of ligase substrate specificity, initial enzymatic characterization was carried out using the pyruvate kinase/lactate dehydrogenase coupled assay to monitor ADP formation.[39] Amino and hydroxy acid substrate specificity was determined by thin-layer chromatography and using radiolabeled substrates. [U−^{14}C]-L-Alanine was isomerized to a racemic mixture of [^{14}C]-L/D-Alanine with one unit of *Bacillus stearothermophilus* alanine racemase (SigmaAldrich). Ligase reactions contained 50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 40 mM KCl, 6 mM ATP, 2 μM enzyme; 0.1 μCi [U−^{14}C]-L/D-Ala, 1 mM D-Ala, and 10 mM D-X substrate. Reactions were quenched with 50% methanol and applied to a PEI-cellulose TLC plate (SigmaAldrich). The plates were developed in 12:3:5 butanol/acetic acid/water, dried overnight, and exposed to a phosphor-storage imaging screen. The screens were imaged using a Typhoon variable mode imager, and relative radioactive intensity was quantified using ImageQuant 5.2 software.

Michaelis−Menten kinetics were determined by using software program GraFit, version 4.0.21 (Erithacus software), and initial rates were determined using the nonlinear least-squares method and eq 1.[40]

$$v = \frac{(k_{cat}/E_t)[S]}{(K_M + [S])}$$

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Anna-Maria Puglia and Guiseppe Gallo for providing proteome data. E.S. was supported by the DFG (SFB 766 TP-A03). H.-J.F. and R.K. were financed by grants from the DFG (SFB 766 TP-A03). We thank Iris Maldener for transmission electron microscopy and Mulugeta Nega for his help in peptidoglycan isolation. We are grateful to Guenther Muth and Wolfgang Wohlleben for helpful discussions, Stefanie Günther for the construction on the Δ*vanY*$_{Ab}$ mutant, Philipp Steimle for the construction of the Δ*ddl1*$_{Ab}$ mutant, and Sigrid Stockert for technical support. G.D.W was funded by the Canadian Institutes of Health Research (CIHR; MT-14981) and by a Canada Research Chair award. L.K. was funded by the CIHR with a Canada Graduate Fellowship.

ABBREVIATIONS:

D-Ala-D-Ala, acyl-D-alanyl-D-alanine; D-Ala-D-Lac, acyl-D-alanyl-D-lactate

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DOI: 10.1021/acsinfecdis.5b00011
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DOI: 10.1021/acsinfecdis.5b00011
ACS Infect. Dis. 2015, 1, 243−252