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# Trichlorination of a Teicoplanin-Type Glycopeptide Antibiotic by the Halogenase StaI Evades Resistance

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**ABSTRACT** Glycopeptide antibiotics (GPAs) include clinically important drugs used for the treatment of infections caused by Gram-positive pathogens. These antibiotics are specialized metabolites produced by several genera of actinomycete bacteria. While many GPAs are highly chemically modified, A47934 is a relatively unadorned GPA lacking sugar or acyl modifications, common to other members of the class, but which is chlorinated at three distinct sites. The biosynthesis of A47934 is encoded by a 68-kb gene cluster in *Streptomyces toyocaensis* NRRL 15009. The cluster includes all necessary genes for the synthesis of A47934, including two predicted halogenase genes, *staI* and *staK*. In this study, we report that only one of the halogenase genes, *staI*, is necessary and essential for A47934 biosynthesis. Chlorination of the A47934 scaffold is important for antibiotic activity, as assessed by binding affinity for the target N-acyl-D-Ala-D-Ala. Surprisingly, chlorination is also vital to avoid activation of enterococcal and *Streptomyces* VanB-type GPA resistance through induction of resistance genes. Phenotypic assays showed stronger induction of GPA resistance by the dechlorinated compared to the chlorinated GPA. Correspondingly, the relative expression of the enterococcal *vanA* resistance gene was shown to be increased by the dechlorinated compared to the chlorinated compound. These results provide insight into the biosynthesis of GPAs and the biological function of GPA chlorination for this medically important class of antibiotic.

**KEYWORDS** A47934, VanS, antibiotic biosynthesis, antimicrobial resistance, chlorination, glycopeptide, halogenation, *vanHAX*

Glycopeptide antibiotics (GPAs) are clinically essential molecules used to treat a variety of infections caused by Gram-positive pathogens, such as staphylococci, streptococci, and enterococci. Marketed GPAs include the natural products vancomycin and teicoplanin and the semisynthetic derivatives oritavancin, dalbavancin, and telavancin. These complex molecules are specialized metabolites synthesized by bacteria (vancomycin and teicoplanin) or are chemically derivatized versions of microbial products (dalbavancin, telavancin, and oritavancin).

GPAs are heptapeptides synthesized by mega-enzyme nonribosomal peptide synthetases (NRPS). NRPS systems are not constrained by the genetic code and allow microbes to synthesize peptides using not only the 20 canonical amino acids but also a plethora of nonproteinogenic amino acids, such as 4-hydroxyphenylglycine (Hpg) and 3,5-dihydroxyphenylglycine (Dpg), as is the case with GPAs. There are two clinically relevant GPA peptide scaffolds, vancomycin and teicoplanin types, differing by the presence of aliphatic or aromatic amino acids at positions 1 and 3, respectively, at the N-terminal end of the peptide. The NRPS-synthesized heptapeptide scaffolds are decorated by modifying/tailoring enzymes to create their final products. Modifications include oxidative cross-linking, forming three intramolecular rings in the case of vancomycin and four in teicoplanin derivatives, chlorination, glycosylation, sulfonation,

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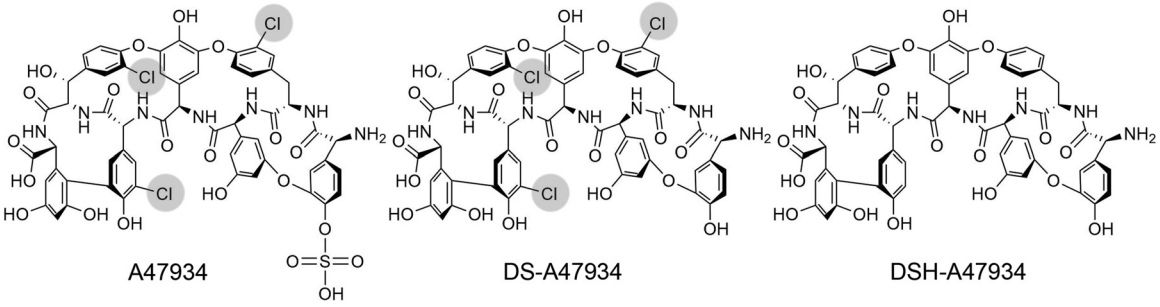


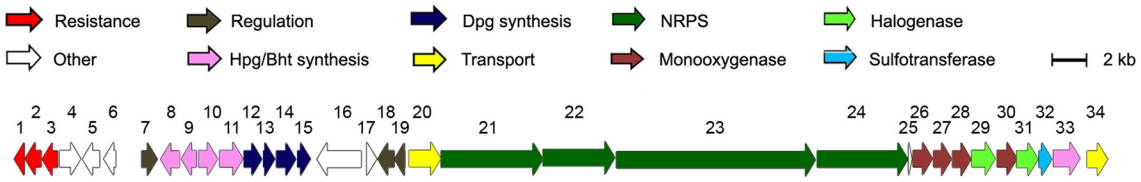

**FIG 1** Structures of the GPAs A47934, DS-A47934, and DSH-A47934. Chlorines are highlighted with shaded circles.

methylation, acylation, etc. Most modifications occur when the peptide has been released from the NRPS (1), but oxidative cross-linking and chlorination occur while amino acids are tethered to the NRPS (2).

Vancomycin was first introduced into the clinic in 1958, and resistance did not appear until approximately 30 years later in enterococci (3). Resistance mechanisms in producer organisms and pathogenic bacteria are mediated by the *vanHAX* genes that remodel the peptidoglycan target. The expression of this three-gene cassette is regulated by a two-component system comprised of a membrane-bound sensor His-kinase, VanS, and its cognate response regulator, VanR (4). There are two common types of vancomycin-resistant enterococci (VRE), A and B, which differ in the signal that triggers the sensor kinase. The VanS of VRE A ($VanS_A$) responds to both vancomycin and teicoplanin type GPAs. While the VanS of VRE B ($VanS_B$) strains senses vancomycin type GPAs and not teicoplanin, this resistance pattern is called VanB-type resistance. Producer organisms such as *Streptomyces toyocaensis* and related organisms such as *S. coelicolor* are VanB like; their VanS does not respond to teicoplanin but responds to other GPAs, such as vancomycin, chloroeremomycin, ristocetin, A47934, and balhimycin (5).

The three-dimensional structure of GPAs enables them to form a tight and intricate interaction with peptidoglycan precursors, such as lipid II, through a hydrogen bond network with the acyl-D-Ala-D-Ala terminus. This binding sequesters the substrates required for two essential cell wall biosynthetic enzymes, transglycosylases and D,D-transpeptidases, resulting in an inability to grow and rigidify the cell wall. Lipo-GPAs such as teicoplanin, telavancin, and oritavancin, which have lipophilic acyl or aromatic tails, have a second mode of action. The lipophilic groups anchor the compound in the cell membrane, increasing their local concentration and aiding binding to lipid II (6) as well as disrupting membrane integrity (7, 8). The *vanHAX* genes encode three enzymes that remodel the *N*-acyl-D-Ala-D-Ala termini of non-cross-linked peptidoglycan and its precursor, lipid II, to the depsipeptide D-Ala-D-Lac. This remodeling of the dipeptide termini results in an ~1,000-fold reduction in affinity of the GPA for its target, effectively preventing the GPA from binding (9, 10). Most GPAs are chlorinated at various positions on the aromatic amino acids; however, the function of this modification is not clear. Earlier studies suggested a role in locking the three-dimensional structure into a conformation that is relatively rigid and conducive to cell wall binding (11); however, the potential importance of chlorination for VanS activation and, consequently, GPA resistance has not been determined.

A47934 is an unusual GPA in that it lacks the eponymous sugar modification of the GPA class and is sulfonated on the phenolic oxygen of the N-terminal Hpg (Fig. 1). This makes A47934 a good candidate for synthetic biology studies that explore the impact of various modifying reactions (12). The antibiotic is produced by *Streptomyces toyocaensis* and is encoded by a 68-kb biosynthetic gene cluster (Fig. 2) that includes two predicted halogenase-encoding genes, *stal* and *staK* (13). A47934 is chlorinated at three distinct sites, C3 of the aryl rings of the beta-hydroxy-Tyr and Tyr residues and the Hpg at amino acid position 5 (Fig. 1). Here, we report the deciphering of the biosynthetic




| Orf | Protein | Proposed Function |
|---|---|---|
| 1 | VanH | D-Ala-D-Ala dipeptidase |
| 2 | VanA | D-Ala-D-lactate ligase |
| 3 | VanX | Lactate dehydrogenase |
| 4 | MurX | D-Ala-D-Ala adding enzyme |
| 5 | StaO | FemABX homologue |
| 6 | StaP | Putative membrane protein |
| 7 | StaQ | Transcriptional regulator |
| 8 | Hmo | *p*-Hydroxymandelate oxidase |
| 9 | HmaS | *p*-Hydroxymandelate synthase |
| 10 | Pdh | Prephenate dehydrogenase |
| 11 | HpgT | *p*-Hydroxyphenylglycine transaminase |
| 12 | DpgA | 3,5-Dihydroxyphenylacetyl-CoA synthase |
| 13 | DpgB | Enhances DpgA activty |
| 14 | DpgC | 3,5-Dihydroxyphenylacetyl-CoA oxygenase |
| 15 | DpgD | Enhances DpgA activity |
| 16 | StaR | Putative flavoprotein |
| 17 | StaS | Putative DNA binding protein |
| 18 | VanS | Transmembrane histidine kinase |
| 19 | VanT | Two-domain response regulator |
| 20 | StaU | ABC transporter |
| 21 | StaA | Peptide synthetase (modules 1-2) |
| 22 | StaB | Peptide synthetase (module 3) |
| 23 | StaC | Peptide synthetase (module 4-6) |
| 24 | StaD | Peptide synthetase (module 7) |
| 25 | StaE | Hypothetical protein |
| 26 | OxyA | P450-related oxidase |
| 27 | OxyE | P450-related oxidase |
| 28 | OxyB | P450-related oxidase |
| 29 | StaI | Halogenase |
| 30 | OxyC | P450-related oxidase |
| 31 | StaK | Non-functional halogenase |
| 32 | StaL | Sulfotransferase |
| 33 | StaM | Bht biosynthesis |
| 34 | StaN | Integral membrane ion transporter |

**FIG 2** Schematic representation of the A47934 biosynthetic gene cluster. Adapted from reference 13 (Copyright [2002] National Academy of Sciences).

roles of *staI* and *staK* and explore the impact of chlorination on target binding and resistance induction.

## RESULTS

**Deletion of two candidate halogenase genes reveals the importance of StaI in A47934 biosynthesis.** The biosynthetic gene cluster of the GPA A47934 (Fig. 2) includes two predicted halogenase-encoding genes, *staI* and *staK* (13). The structure of A47934 and its simpler derivative lacking a sulfate group, desulfo-A47934 (DS-A47934), have three chlorines located on the second amino acid, Tyr, the fifth amino acid, Hpg, and the sixth amino acid, beta-hydroxy-Tyr (Bht) (Fig. 1). To determine which of the predicted halogenases is responsible for chlorinating each of the three positions, each halogenase gene was deleted from an *Escherichia coli-Streptomyces* P1-derived artificial chromosome (PAC) plasmid carrying the DS-A47934 biosynthetic cluster, pA47934 Δ*staL* (12), and the cluster heterologously expressed in the specialized metabolite production-optimized *S. coelicolor* strain M1146 (14). Deletions were made from the PAC plasmid in *E. coli*, since genetic manipulation is more facile in this organism than in the A47934 producer strain *S. toyocaensis*.

When *staK* was deleted from the DS-A47934 biosynthetic gene cluster, the main peak detected in the fermentation of the M1146 pA47934 Δ*staKL* strain was DS-A47934 (Fig. 3B), with a profile similar to that of the strain with all halogenases present (Fig. 3A). On the other hand, following deletion of *staI* from the DS-A47934 biosynthetic cluster to generate plasmid pA47934 Δ*staL* Δ*staI*::*aac*(3)-*iv*, the primary fermentation product detected was DS-A47934 lacking detectable chlorination, DSH-A47934 (Fig. 3C). When the pA47934 Δ*staL* Δ*staI*::*aac*(3)-*iv* strain was complemented with the *staI* gene integrated in *trans* in another location on the genome, production of DS-A47934 was restored (Fig. 3D). Taken together, this suggested that StaI is sufficient for all three chlorination reactions on DS-A47934 and that StaK is not required for DS-A47934 biosynthesis.

**Purification and structural characterization of DSH-A47934.** DSH-A47934 was isolated from the fermentation of the *S. coelicolor* M1146 pA47934 Δ*staL* Δ*staI*::*aac*(3)-*iv* strain. The structure of DSH-A47934 (Fig. 1) was elucidated by comparing its high-resolution electrospray ionization mass spectrometry (HRESIMS) and one-dimensional nuclear magnetic resonance (1D NMR) spectra (see Fig. S1 to S6 and Table S1 in the supplemental material) to those obtained for DS-A47934 (Fig. S28 in reference 12). 2D NMR spectra further confirmed the structure. The molecular formula of DSH-A47934 was determined as $C_{58}H_{47}N_7O_{18}$ according to the HRESIMS (1128.2905 $[M-H]^-$, cal

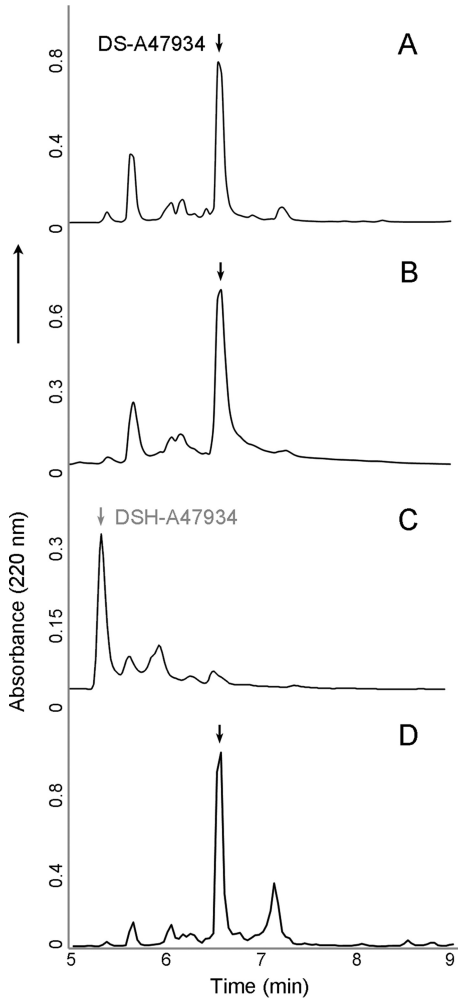



**FIG 3** Reverse-phase HPLC chromatograms of fermentations after affinity chromatography of GPAs heterologously produced in *S. coelicolor* M1146 carrying the following integrated plasmids: pA47934 Δ*staL* (A), pA47934 Δ*staKL* (B), pA47934 Δ*staL* Δ*stal*::*aac*(3)-*iv* (C), and pA47934 Δ*staL* Δ*stal*::*aac*(3)-*iv stal*::*aph*(4) (D). Black arrows indicate the peak corresponding to DS-A47934, and the gray arrow indicates the peak corresponding to DSH-A47934.

1128.2905; Fig. S6), suggesting that all three chlorine atoms in DS-A47934 were replaced with three protons in DSH-A47934. In the $^{1}$H NMR spectrum of DSH-A47934, there are three extra aromatic protons at $\delta$ 7.35, 7.11, and 7.05 ppm, corresponding to the 2c, 5e, and 6c protons (Fig. S5 and Table S1), showing strong $^{1}$H-$^{1}$H correlation (by correlation spectroscopy) with the 2d, 5f, and 6d protons, respectively (Fig. S1 and Table S1). In the distortionless enhancement by polarisation transfer (DEPTq) $^{13}$C spectrum of DSH-A47934, there were three fewer quaternary carbons and three new aromatic methine carbons at $\delta$ 122.4, 117.5, and 121.7 ppm, which showed strong correlations by heteronuclear single quantum correlation (HSQC) spectroscopy (Fig. S2) to the three new proton signals.

**Antibiotic activity of dechloro- and desulfo-A47934 against GPA-sensitive and -resistant bacteria.** The antibiotic activity of DSH-A47934 was tested against *B. subtilis* 168, vancomycin-sensitive enterococci (VSE; *Enterococcus faecalis* ATCC 29212, *E. faecalis* JH2-2, and *E. faecalis* ATCC 49383) and vancomycin-resistant clinical strains VRE A (*E. faecium* ATCC 700221 with inducible resistance by vancomycin and teicoplanin) and VRE B (*E. faecalis* ATCC 51299, 36875, and CO559 with inducible resistance by vancomycin but not teicoplanin) (Table 1). Based on MIC determination of the GPA-sensitive strains, *B. subtilis* and VSE, DSH-A47934 is a less active antibiotic (MIC increased 8- to

**TABLE 1** MIC of GPAs measured against vancomycin-sensitive and -resistant strains

| | MIC ($\mu$g/ml) | | | |
|---|---|---|---|---|
| **Compound** | **Vancomycin** | **A47934** | **DS-A47934** | **DSH-A47934** |
| *B. subtilis* 168 (GPA sensitive) | 0.25 | 0.125 | 0.063 | 4 |
| *E. faecalis* ATCC 29212 (GPA sensitive) | 4 | 0.5 | 0.5 | 16 |
| *E. faecalis* ATCC 49383 (GPA sensitive) | 2 | 0.5 | 0.25 | 8 |
| *E. faecalis* JH2-2 (GPA sensitive) | 2 | 1 | 0.5 | 8 |
| *E. faecium* VRE A (ATCC 700221) | >128 | 128 | >128 | >128 |
| *E. faecalis* VRE B (ATCC 51299) | 64 | 0.5 | 0.5 | 64 |
| *Enterococcus* sp. strain VRE B CO559 | >128 | 1 | 0.25 | >128 |
| *Enterococcus* sp. strain VRE B 36875 | 64 | 0.5 | 0.25 | >128 |

64-fold) compared to the chlorinated GPAs DS-A47934 and A47934. Like other GPAs, all compounds tested induce resistance in the VRE A strain and have high MIC values. Interestingly, when comparing the MIC ratios of DSH-A47934 to DS-A47934 and A47934 in the VRE B and VSE strains, the DSH-A47934 MIC is 128 to over 512× higher than those of DS-A47934 and A47934 in the VRE B strain. For example, the MIC ratio of DS-A47934 to DSH-A47934 in VSE JH2-2 is 16-fold, whereas in the VRE B strain 36875 the MIC ratio is over 512. Additionally, the MICs of DS-A47934 and A47934 were very similar between the various VSE and VRE B strains, between 0.25 and 1 $\mu$g/ml. The MICs of DSH-A47934 ranged from 64 to greater than 128 $\mu$g/ml, at least 4-fold greater in the VRE B strains than the VSE strains. This significant difference in the ratio of MICs and higher MIC values of DSH-A47934 in the VRE B strains suggests that chlorination plays a significant role in recognition by $VanS_B$, since previous experiments suggested that direct binding of the GPA induces VanB-type resistance to $VanS_B$ (15).

**Dechlorination of A47934 has a modest impact on D-Ala-D-Ala binding.** The binding affinity of DSH-A47934 and DS-A47934 to the GPA target for bacterial growth inhibition, the D-Ala-D-Ala termini of peptidoglycan, was determined by isothermal titration calorimetry (ITC) using the *N,N*-$(Ac)_2$-L-Lys-D-Ala-D-Ala tripeptide. The dissociation constant ($K_d$) values for DS-A47934 and its dechlorinated derivative were 1.7 $\pm$ 0.3 $\mu$M and 5.1 $\pm$ 0.9 $\mu$M, respectively. The slightly weaker $K_d$ of the dehalo-antibiotic correlates with the lower MICs of DSH-A47934 compared to those of DS-A47934 in the VSE strains and *B. subtilis* (Table 1).

**Impact of chlorination on the induction of GPA resistance.** Since the relative MIC of DSH-A47934 compared to those of DS-A47934 and A47934 in VRE B suggested that DSH-A47934 was a more potent inducer of the *vanHAX* genes and ITC suggested that DSH-A47934 has only slightly diminished binding than fully chlorinated DS-A47934, we explored the impact of halogenation on induction of GPA resistance. A teicoplanin resistance induction assay is essentially a *vanHAX* reporter experiment measuring induction of GPA resistance. In this assay, agar plates containing the GPA teicoplanin swabbed with a test organism with inducible GPA resistance grows in response to the inducer compound spotted on a filter disk (12) (Fig. 4A). Two types of test organisms, VRE B (Fig. 4B and 5) and *S. coelicolor* (Fig. 4C), were used. Since all strains have VanB-type resistance, their VanS is not triggered by teicoplanin and consequently growth is inhibited by teicoplanin in the agar plate unless resistance is induced by a suitable compound applied in *trans*. *S. coelicolor* is a better test organism for this genus than the producer of A47934, *S. toyocaensis*, since it has a *vanRSHAX* cluster but lacks a GPA biosynthetic gene cluster, thus A47934 production will not complicate results. In this plate-based assay, DSH-A47934 is a stronger inducer of the *vanHAX* genes than DS-A47934/A47934 in both the pathogenic VRE B strains and producer-like organism *S. coelicolor* (Fig. 4 and 5). As might be expected from the low MICs of DS-A47934 and A47934 in VRE B (Table 1), DS-A47934 and A47934 do not appear to induce teicoplanin resistance in VRE B (Fig. 4B and 5) but do induce some resistance in *S. coelicolor* (Fig. 4C). As expected, vancomycin resistance induction is strong in all tester strains. This suggests that chlorination reduces recognition by both VRE B and *S. coelicolor* VanS sensor proteins.

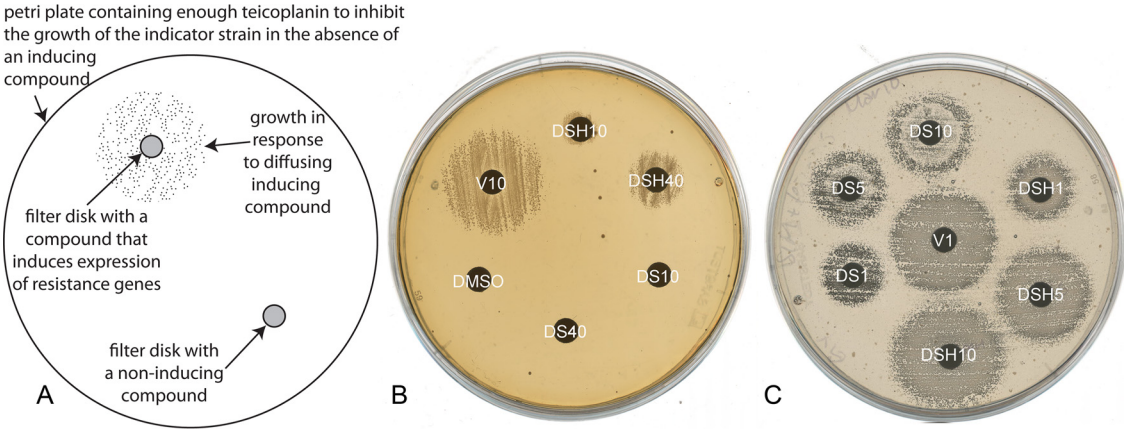



**FIG 4** Teicoplanin resistance induction assays. Filter discs containing 1, 5, 10, or 40 $\mu$g (as indicated) of the GPAs DSH-A47934 (DSH), DS-A47934 (DS), and vancomycin (V) and the solvent control (DMSO) induce teicoplanin resistance and growth of the tester organism as inducing GPA compounds diffuse away from the disc. (A) Representative illustration of the assay. (B) The tester organism *E. faecalis* VRE B ATCC 51299 grown on BHI agar containing 1.0 $\mu$g/ml teicoplanin. (C) The tester organism *S. coelicolor* M1146 grown on SIM agar plates containing 2.5 $\mu$g/ml teicoplanin.

Since teicoplanin resistance induction experiments implied that DSH-A47934 was a better inducer of *vanHAX* expression than chlorinated DS-A47934, *vanA* expression in VRE B was also measured in response to vancomycin, DSH-A47934, and DS-A47934 by reverse transcription-PCR (RT-PCR). Consistent with the trend observed with the teicoplanin resistance induction assay, DSH-A47934 was a better inducer of *vanA* gene expression than DS-A47934, and neither GPA displayed a response as strong as that of control vancomycin (Fig. 6).

## DISCUSSION

Sequenced GPA biosynthetic clusters include up to two halogenases, and known GPA structures can have up to four chlorinations. When one halogenase gene is present in the biosynthetic gene cluster, the corresponding GPA has one to two chlorinations (16, 17). If two halogenase genes are present in the biosynthetic gene clusters, the corresponding GPA has three to four chlorinations (1). If no halogenase genes are present in the biosynthetic gene cluster, the corresponding GPA (e.g., ristocetin) has no chlorinations (18, 19). For example, deletion of the sole balhimycin halogenase from the vancomycin-type GPA biosynthetic gene cluster resulted in detection of a product by mass spectrometry that lacked its two cognate chlorine atoms, suggesting that this

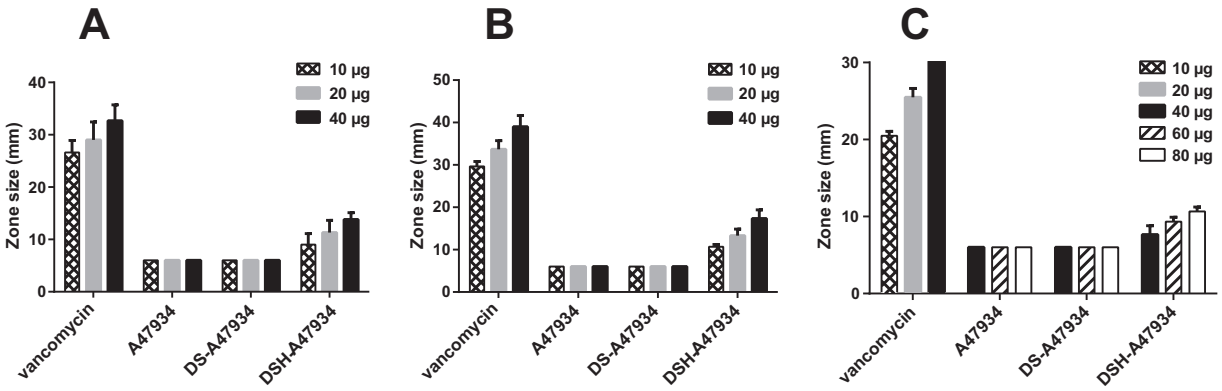



**FIG 5** Dose response of GPA resistance to A47934, DS-A47934, DSH-A47934, and vancomycin in VRE B strains. Filter discs containing 10, 20, 40, 60, or 80 $\mu$g (as indicated) of DSH-A47934, DS-A47934, A47934, and vancomycin induce teicoplanin resistance and a zone of growth of the VRE B tester organisms ATCC 51299 (A), 36875 (B), and CO559 (C) as inducing GPA compounds diffuse away from the disc. A value of 6 mm is the size of the filter disc and indicates that there was no observable growth and no observable resistance induction at that concentration of inducer. The results are an average of at least three biological replicates. Error bars indicate one standard deviation.

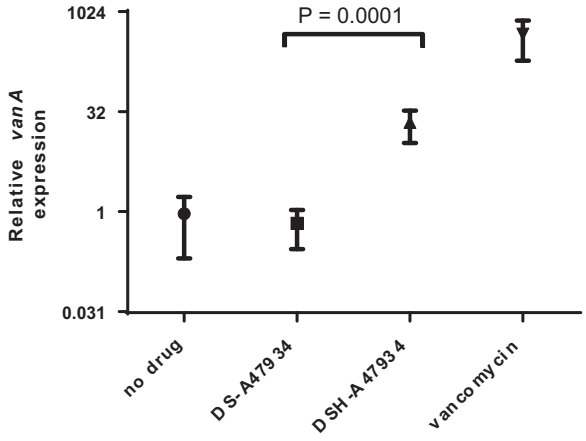



**FIG 6** RT-PCR of *vanA* gene expression relative to that of *gyrA* in *E. faecalis* ATCC 51299 VRE B cells treated with 1/4 MIC GPA. Concentrations were 0.125 $\mu$g/ml of DS-A47934, 16 $\mu$g/ml of DSH-A47934, and 16 $\mu$g/ml of vancomycin in BHI. Results are an average of two biological replicates with four technical replicates each. Error bars represent one standard deviation. The *P* value was determined by an unpaired *t* test.

single halogenase was responsible for the two chlorinations that occur on distinct sites in balhimycin (16). GPAs with more than two halogenases, such as A47934 and UK-68,597, have three and four chlorinations, respectively (1). One logical hypothesis was that one halogenase would chlorinate a maximum of two amino acids. However, data presented here show that only one halogenase, StaI, is necessary and sufficient for halogenation at all three amino acids on the A47934 GPA scaffold.

When the amino acid sequences of StaI and StaK are compared, StaK is missing 49 amino acids at the C-terminal portion of the protein (see Fig. S7 in the supplemental material). All GPA halogenases, including StaI and StaK, are flavin-dependent ($FADH_2$) enzymes with predicted FAD binding sites (Fig. S7). While no crystal structures have been reported yet for any GPA halogenase, crystal structures have been determined for other flavin-dependent natural product halogenases, such as PrnA, RebH, and PyrH, that can modify the amino acid tryptophan (20, 21). When the GPA halogenases StaI and StaK are compared to the tryptophan halogenases, the two residues essential for PrnA activity, K79 and E346 (21), have similar patterns in StaI and StaK. StaI and StaK both possess the equivalent to K79 at K74, but neither possesses E346 (Fig. S7). The biochemical basis for StaK's lack of apparent activity is therefore not trivially due to truncation of conserved halogenase signature motifs.

When an amino acid alignment of StaI and StaK is expanded to include other sequenced GPA biosynthetic clusters, other halogenases missing the same 49 amino acids in the C-terminal region can be identified (Fig. S7). The two sequenced GPA biosynthetic clusters with smaller halogenases, similar to that of StaK, also have a second longer halogenase similar to that of StaI. One cluster corresponds to a known GPA structure with three chlorinations (UK-68,597) (22), and the other cluster (ensnapd15) encodes a predicted GPA. This observation predicts that the StaI homologs in these clusters, Auk23 in the UK-68,597 gene cluster and AGS49782 (GenBank accession no.) of the ensnapd15 GPA gene cluster, are also responsible for all of the halogenations in their respective molecules. Analogously, the StaK homologs in the same clusters, Auk21 and AGS49780, respectively, likely have a role similar to that of StaK, either responsible for the biosynthesis of a chlorinated minor product or nonfunctional.

Recently, the substrate for a GPA halogenase was determined to be the single amino acid bound to its PCP domain (2). The authors demonstrated that the appropriate amino acid, Tyr, loaded onto its cognate Tyr PCP domain (teicoplanin has two chlorinations on its two Tyr residues), as well as Tyr loaded onto a PCP domain corresponding to a nonchlorinated amino acid (Hpg), were able to act as substrates for the teicoplanin halogenase *in vitro*, implying that the PCP domain is not solely responsible for substrate

specificity but also participates in tailoring chemistry (2). Thus, perhaps it is not surprising that a single halogenase is able to perform all three halogenations on three of the aromatic amino acids of the A47934 scaffold. However, the PCP domain must have some role in recognition of the substrate, since all of the aromatic amino acids of A47934 are not chlorinated.

Chlorination is also important for GPA activity in vancomycin-type GPAs (23, 24). Dechlorinated and monochlorinated derivatives of vancomycin (dichlorinated) synthesized by chemical methods reduced activity against bacterial pathogens, including VSE, and further reduced activity against VRE B (25). Specifically, the MICs of fully dichlorinated, monochlorinated, and dechlorinated vancomycin suggested an intermediate phenotype for the monochlorinated vancomycin in terms of activity (25). In terms of *vanHAX* induction, the monochlorinated and dechlorinated vancomycin both appear to have greater increases in VRE B MICs than VSE MICs (25). This is consistent with the evidence shown here for A47934 that chlorination is also crucial for avoiding activation of the enteroccal VanS in vancomycin-type GPAs.

Interestingly, chemical mutagenesis of *Nonomuraea* sp. strain ATCC 39727, the producer of the Lipo-GPA A40926, yielded a strain with a partially inactivated halogenase, leading to the detection of dechlorinated and monochlorinated derivatives of the dichlorinated GPA A40926. In contrast to our results and those of others showing reduced activity of dechlorinated compounds, Beltrametti et al. found that the dechlorinated derivatives had activity similar to that of the chlorinated compound when using VRE A and VSE strains as tester organisms (17). However, these compounds were not tested against VRE B, so the ability of these dechlorinated derivatives to activate $VanS_B$ was not examined. The main difference between A40926 and other GPAs that showed less activity when dechlorinated is the presence of an acylated sugar at amino acid 4 (a Lipo-GPA). Thus, maintenance of activity in the dechlorinated A40926 may be explained by the additional acylated sugar that may aid in dimerization (increasing activity) and alter sensing by the $VanS_A$ (17) and/or membrane anchoring by its lipophilic tail (its secondary mode of action).

Although the VanS proteins share functional similarity and all regulate *vanHAX* genes, $VanS_A$, $VanS_B$, and *S. coelicolor* VanS ($VanS_{sc}$) are quite different, sharing only 20% to 25% amino acid similarity. There are two prevailing hypotheses regarding the signal sensed by the various VanS sensor proteins: (i) VanS is triggered by direct binding by a GPA and (ii) VanS senses GPAs indirectly by binding a cell wall intermediate built up as a result of GPA binding to its target. In VRE A, GPA resistance is induced by GPAs as well as other non-structurally related cell wall inhibitors, such as moenomycin (26). The broad structural diversity of the compounds that trigger $VanS_A$ supports the second hypothesis, but recently *in vitro* studies have demonstrated binding of $VanS_A$ to teicoplanin and vancomycin (27). Since $VanS_B$ only responds to vancomycin-type GPAs but not teicoplanin, $VanS_B$ may bind directly to the GPA, since cell wall intermediates would be built up in both vancomycin and teicoplanin treatment. In producer organisms, the evidence seems to support both hypotheses. $VanS_{sc}$ has been shown to directly bind vancomycin using a vancomycin photoaffinity probe (28), and binding of cell wall intermediates has been shown using a series of *in vivo* experiments (5). These two lines of evidence led to a revised hypothesis that vancomycin bound to lipid II or peptidoglycan intermediates terminating in D-Ala-D-Ala is the signal that $VanS_{sc}$ directly senses (5). Our evidence in VRE B supports the direct binding of GPA to $VanS_B$, since our experiments show that bioactivity and induction of GPA resistance can be decoupled. Chlorination of DS-A47934 had different effects on binding to the target D-Ala-D-Ala and induction of resistance (presumably by sensing by VanS), improving binding to D-Ala-D-Ala but decreasing activation of $VanS_B$, respectively. These results further emphasize the differences between $VanS_A$, $VanS_B$, and $VanS_{sc}$ and reveal a more nuanced role of GPA chlorination than previously understood.

**Conclusions.** In this work, we have characterized the predicted halogenases in the biosynthetic gene cluster of the GPA A47934. We have shown that one halogenase,

StaI, is sufficient for chlorinating the amino acid backbone at three different positions. Halogenation is not only important for antimicrobial activity, as was previously known, but also is essential for avoiding induction of resistance mechanisms in a pathogen, namely, VRE B, as well as in a producer-like organism. At this time, where antibiotics are being rendered increasingly ineffective by resistant pathogens and antibiotic discovery is at a nadir, it is even more vital to understand the biosynthesis of known antibiotics and the mechanisms by which resistance is regulated and triggered.

## MATERIALS AND METHODS

**General methods, culture methods, and reagents.** *E. coli* and *Streptomyces* were cultivated using media and methods described previously (12). The following antibiotics were used to supplement media for selection as needed: hygromycin B (40 $\mu$g/ml in *Streptomyces*, 100 $\mu$g/ml in *E. coli*), kanamycin (50 $\mu$g/ml), chloramphenicol (35 $\mu$g/ml), nalidixic acid (25 $\mu$g/ml), and thiostrepton (50 $\mu$g/ml). *E. faecalis* was grown in BD BBL brain heart infusion (BHI) media (Fisher Scientific, Canada) at 37°C, with shaking for 16 h. All antibiotics and medium components were purchased from Sigma (St. Louis, MO).

Oligonucleotide synthesis and DNA sequencing were performed at The MOBIX Lab Central Facility (McMaster University). Oligonucleotides used in this study are listed in Table S2 in the supplemental material. PCR products for cloning were generated using Phusion polymerase (Invitrogen). Sanger sequencing was used to verify constructs.

The clinical VRE B strains CO559 and 36875 were gifts from Hamilton Health Sciences.

**Deletion and cloning of halogenase genes.** The two putative halogenase genes *staI* and *staK* (13) were deleted from the PAC vector pA47934 Δ*staL* (12) using the lambda red system (29) with the oligonucleotides listed in Table S2. To create the PCR product for *staI* deletion, pSET152 was used as a template carrying apramycin resistance with the oligonucleotides staI apra KO F and staI apra KO R. The PCR product was electroporated into BW25113 carrying the PAC vector pA47934 Δ*staL* and pKD46 to create the plasmid pA47934 Δ*staL* Δ*staI*::*aac*(3)-*iv*. To create the PCR product for *staK* deletion, pKD3 plasmid DNA was used as a template carrying chloramphenicol resistance with the oligonucleotides staK KO for and staL KO rev. The PCR product was electroporated into BW25113 carrying pA47934 Δ*staL* and pKD46. The *cat* resistance cassette was removed using pCP20 as described previously (26) to create the PAC vector pA47934 Δ*staKL*. The plasmid pKD46 was removed from all strains by standard methods (26). Nucleotide sequencing confirmed gene deletions.

For complementation studies, the gene *staI* (13) was cloned into the integrating and expression vector, pIJ10257, which carries the strong constitutive promoter *ermE*p* and integrates into BT1 *attB* (30). The gene was amplified by PCR using the oligonucleotides described in Table S2 from *S. toyocaensis* NRRL 15009 genomic DNA and cloned using the NdeI and HindIII restriction endonuclease sites.

**Triparental mating and *E. coli-Streptomyces* conjugation.** To create donor strains carrying the biosynthetic gene cluster, PAC vectors were delivered into *E. coli* ET12567 using triparental mating, as described previously, with the driver plasmid pR9406 (31). *Streptomyces* exconjugants were selected with thiostrepton and PCR verified using primers that hybridize the ends and middle of the biosynthetic cluster.

The plasmid carrying *staI* cloned into pIJ10257 was transformed into *E. coli* ET12567/pUZ8002 cells using standard methods (32). Resulting transformants were conjugated into *S. coelicolor* M1146 carrying pA47934 Δ*staL* Δ*staI*::*aac*(3)-*iv* using standard methods (29, 32). Exconjugants were selected with hygromycin and thiostrepton and PCR verified using oligonucleotides that amplify the multiple cloning site of pIJ10257.

**Fermentation and antibiotic purification.** Fermentation of *S. coelicolor* for heterologous production of GPAs was conducted as described previously (12). GPAs were purified by affinity chromatography over a D-Ala-D-Ala column followed by reverse-phase high-performance liquid chromatography (RP-HPLC) as described previously (12).

**Analytical RP-HPLC, LC-ESI-MS, HRESIMS, and NMR analysis.** Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESIMS), HRESIMS, and NMR data were collected and analyzed as described previously (12), except DSH-A47934 was solubilized in NMR solvent using 0.4% $NH_4OH$.

**MIC determination.** Enterococcal MICs were determined using the broth microdilution method in BHI broth according to NCCLS protocols. *B. subtilis* MICs were determined using the broth microdilution method in cation-adjusted Mueller-Hinton broth according to NCCLS protocols. All MICs had a minimum of four replicates.

**Isothermal titration calorimetry.** Heat flow resulting from the binding of the GPA to its target was measured using a high-sensitivity Nano-ITC low-volume instrument (TA Instruments, New Castle, DE). Before use, solutions were degassed under vacuum to eliminate air bubbles and dissolved gas. Three hundred $\mu$l of a 100 $\mu$M GPA was placed in the cell, and 1 mM *N*,*N*-diacetyl-L-Lys-D-Ala-D-Ala tripeptide was placed in the syringe. Both solutions contained 20 mM sodium citrate buffer (pH 5.1) and 1.12% (vol/vol) dimethyl sulfoxide (DMSO). Injections (2.0 $\mu$l) were made every 300 s. Experiments were conducted at 30°C. Each injection produced a heat of reaction which was determined by the integration of the heat flow tracings using NanoAnalyze software from TA Instruments.

**Teicoplanin resistance induction assays.** For enterococcal assays, a BHI overnight culture was diluted to an optical density at 600 nm ($OD_{600}$) of 0.1 and swabbed onto BHI agar plates containing 1 $\mu$g/ml teicoplanin. For *S. coelicolor* assays, starter cultures of M1146 were grown in 2XYT broth supplemented with apramycin for 2 days, homogenized using a sterile glass homogenizer, diluted to an

$OD_{450}$ of 0.3, and swabbed onto *Streptomyces* isolation media (SIM) agar plates containing 2.5 $\mu$g/ml teicoplanin. Sterile 6-mm filter disks (AMD Manufacturing, Mississauga, ON) were spotted with GPAs and placed on top of swabbed plates. BHI plates were incubated at 37°C for 2 to 3 days and then photographed. SIM plates were incubated at 30°C for 4 days and then photographed. Tabulated results are averages from at least three biological replicates.

**RT-PCR measurement of gene expression.** For RNA isolation, an overnight culture of *E. faecalis* ATCC 51299 was diluted 1/100 into a solution of BHI containing 1/4 MIC of vancomycin, DS-A47934, DSH-A47934, or no drug and incubated an additional 16 h before harvesting. All cultures, including the no drug control, had an equal volume of DMSO. After incubation, cells were pelleted and stored at −80°C for later use. RNA was isolated using the PureLink RNA minikit with the following modifications. Cells were thawed and incubated in 100 $\mu$l of 20 mg/ml lysozyme in 10 mM Tris, pH 8.0, and 0.1 mM EDTA for 15 min at 37°C. One ml of TRIzol (Invitrogen, ThemoFisher Scientific, Canada) was added and RNA isolated according to the manufacturer's instructions for RNA isolation using TRIzol. RNA was eluted in 50 $\mu$l RNase-free water. The Maxima first-strand cDNA synthesis kit for quantitative RT-PCR with dsDNase (ThermoFisher Scientific) was used for cDNA synthesis according to the manufacturer's instructions. RT-PCRs contained 5 $\mu$l SYBR Select master mix for CFX (ThermoFisher Scientific), 400 nM each primer, and 1 $\mu$l of cDNA. Cycling was conducted in a Bio-Rad C1000 CFX96 real-time PCR detection system using the following program: 50°C for 2 min and 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 1 min and then melt curve analysis from 65 to 95°C.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01540-18.

**SUPPLEMENTAL FILE 1**, PDF file, 1.7 MB.

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