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Biosynthesis of Sulfated Glycopeptide Antibiotics by Using the Sulfotransferase StaL

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Summary

The unique glycopeptide antibiotic A47934, produced by *Streptomyces toyocaensis*, possesses a nonglycosylated heptapeptide core that is sulfated on the phenolic hydroxyl of the N-terminal 4-hydroxy-*L*-phenylglycine residue. Genetic and biochemical experiments confirmed that StaL is a sulfotransferase capable of sulfating the predicted crosslinked heptapeptide substrate to produce A47934 both in vivo and in vitro. Incubation of purified His_6-StaL with various substrates in vitro revealed substrate specificity and yielded two sulfo-glycopeptide antibiotics: sulfoteicoplanin aglycone and sulfo-teicoplanin. Quantification of the antibacterial activity of desulfo-A47934, A47934, teicoplanin, and sulfo-teicoplanin demonstrated that sulfation slightly increased the minimum inhibitory concentration. This unique modification by sulfation expands glycopeptide diversity with potential application for the development of new antibiotics.

Introduction

Glycopeptide antibiotics such as vancomycin and teicoplanin have been commonly termed the "drugs of last resort" for treating infections caused by multidrug-resistant bacteria (e.g., methicillin-resistant *Staphyloccoccus aureus*). However, the increasing prevalence of nosocomial infections, and the imminent emergence of community-acquired infections due to vancomycin-resistant *S. aureus* and *Enterococcus* spp., continues to drive the development of novel antibiotics, including diverse semisynthetic derivatives of members of this class.

Glycopeptide antibiotics consist of a heptapeptide core that is assembled via nonribosomal peptide synthesis, followed by enzymatic modifications of the core peptide that can include aryl crosslinking, glycosylation, methylation, hydroxylation, halogenation, acylation, and sulfation [1, 2]. *Streptomyces toyocaensis* NRRL 15009 produces the glycopeptide antibiotic A47934 [3–5], which is unique for two reasons. First, A47934 possesses the characteristic heptapeptide core, but it is not glycosylated; thus, it is an "aglyco"-glycopeptide antibiotic. Second, the phenolic hydroxyl of the N-terminal 4-hydroxy-*L*-phenylglycine (*L*-HPG) residue of A47934 is sulfated [5], resulting in the molecule having an overall negative charge at physiological pH.

Naturally occurring "aglyco"-glycopeptide antibiotics are rare since they are converted to their glycosylated form by glycosyltransferases encoded by the producing organism. To date, only wild-type *S. toyocaensis* NRRL 15009 and *S. virginiae* NRRL 15156 have been reported to produce "aglyco"-glycopeptide antibiotics as major products (i.e., A47934 and A41030A, respectively) [3, 6]. A41030A is the predominant compound of a complex called A41030 that consists of three glycopeptide antibiotics and four "aglyco"-glycopeptide antibiotics. However, *S. toyocaensis* lacks glycosyltransferase-encoding genes in the A47934 biosynthetic cluster [7], and, as a result, it exclusively produces A47934, an attractive and proven scaffold for novel antibiotic development both in vivo and in vitro [8].

Sulfotransferases utilize the sulfate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to catalyze the transfer of a sulfate group to a hydroxyl acceptor. Eukaryotic cytosolic sulfotransferases often can recognize a variety of molecules as substrates and are known to play an important role in the detoxification, metabolism, and regulation of biomolecules [9]. Membrane-associated sulfotransferases are involved in several signaling and recognition processes and therefore exhibit stricter substrate specificity toward complex carbohydrates and proteins [9]. Several eukaryotic PAPS-dependent cytosolic sulfotransferases have been well characterized, but the putative role of bacterial homologs in the detoxification of phenols remains to be determined [10]. In fact, the biological significance of only three bacterial PAPS-dependent sulfotransferases has been established. The sulfotransferases NodH and NoeE of *Sinorhizobium meliloti* are essential for establishing symbiosis [11, 12], and the sulfotransferase Stf0 of *Mycobacterium tuberculosis* has been implicated in virulence [13]. These three sulfotransferases recognize specific carbohydrate moieties as substrates, and not aryl compounds.

To date, only two sulfated glycopeptide antibiotics have been reported: A47934 and UK-68,597 [3, 14]. The biosynthesis of UK-68,597 in *Actinoplanes* ATCC 53533 has not been characterized, nor has a putative sulfotransferase been identified. However, we have previously annotated *staL* as a putative PAPS-dependent sulfotransferase from analysis of the A47934 biosynthetic gene cluster sequence from *S. toyocaensis* [7], and we proposed the sulfation reaction shown in Figure 1. It has been hypothesized that sulfation occurred prior to scaffold formation in A47934 biosynthesis [4]. However, analysis of the A47934 nonribosomal peptide synthetases confirmed that the modules incorporating the N-terminal amino acid (module 1) and the two central amino acids (modules 4 and 5) contained adenylation domains that were highly similar to HPG activation domains [15]. Therefore, sulfation may occur during synthesis of the antibiotic or after the mature crosslinked peptide has been synthesized. Precedence for sulfation after antibiotic assembly can be found in the biosynthesis of the sulfated carbapenem antibiotic C-19393 [16].

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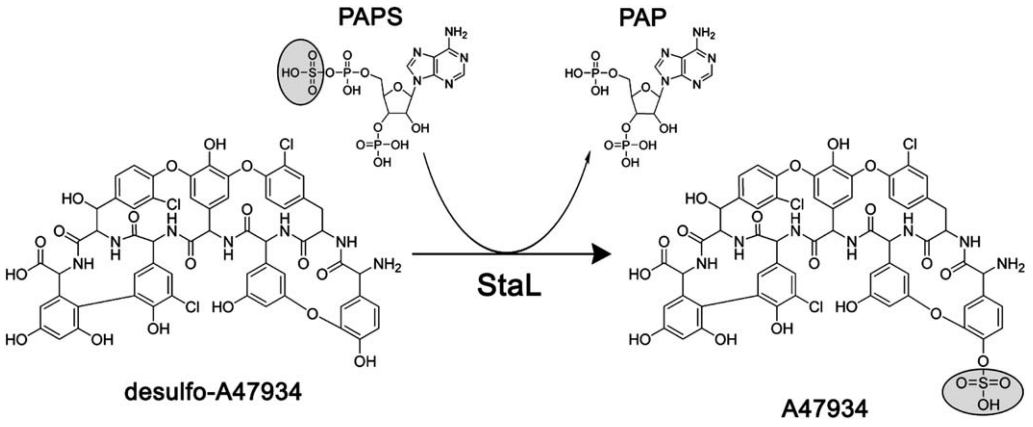

Figure 1. StaL Catalyzes Glycopeptide Antibiotic Sulfation

The predicted de novo substrate desulfo-A47934 is sulfated by the putative sulfotransferase StaL by using the cofactor 3′-phosphoadenosine-5′-phosphosulfate (PAPS), resulting in the formation of 3′-phosphoadenosine-5′-phosphate (PAP) and the sulfo-product A47934.

Characterization of StaL will aid in identifying its natural substrate and thus elucidate the timing of sulfation in A47934 biosynthesis.

There is much interest in expanding natural product diversity by using tailoring enzymes or combinatorial approaches [1, 2, 17–19]. "Aglyco"-glycopeptide antibiotics represent minimal structures, and both A47934 and A41030A have been proven to be effective scaffolds for developing novel hybrid glucosyl derivatives by using cloned vancomycin glycosyltransferases [8]. In a similar experiment, aglycone derivatives of vancomycin and teicoplanin yielded novel hybrid antibiotics by using glycosyltransferases cloned from different antibiotic-producing organisms [20]. This substrate promiscuity exhibited by glycosyltransferases has been exploited by using various chemoenzymatic approaches [20–22] and glycorandomization strategies [23, 24] to create a large number of glycosylated variants of vancomycin, teicoplanin, and their derivatives. Investigating the substrate specificity of StaL could ultimately provide an opportunity to expand glycopeptide antibiotic diversity through sulfation, resulting in unique sulfo-glycopeptide antibiotics.

Using both genetic and biochemical approaches, we have characterized the putative sulfotransferase StaL of *S. toyocaensis*. The gene inactivation strategy utilizing λ-Red recombinase in *Escherichia coli* [25] has been modified to successfully create precise in-frame gene replacements in *Streptomyces coelicolor* [26]. We applied this approach to facilitate the replacement of *staL* in *S. toyocaensis* in order to determine the in vivo role of StaL and to identify its natural substrate. Additionally, the in vitro sulfotransferase activity and substrate specificity of purified His_6-StaL was investigated. These experiments have resulted in the creation of two novel sulfo-glycopeptide antibiotics and provide insight into the sulfation of glycopeptide antibiotics.

Results

A47934 Production In Vivo Requires *staL*

A *staL* replacement strain, *S. toyocaensis ΔstaL::aac(3)IV*, was constructed by optimization of the established approach [26] and was confirmed by using multiple PCR amplifications (Figure 2) and Southern analysis (Figure S1; see the Supplemental Data available with this article). Fermentation of the strains followed by LC/ESI-MS analysis of the antibiotic-containing extracts revealed that the mutant strain no longer synthesized intact A47934, but, instead, produced an antibacterial compound with an *m/z* of 1232.8 (Figure 3). This value is

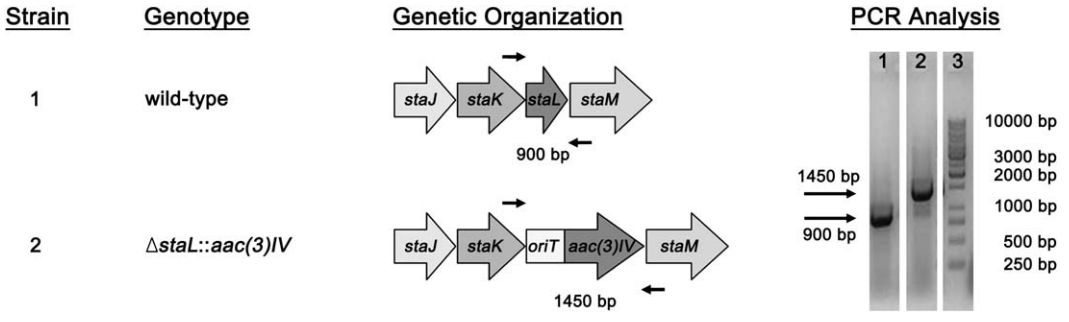

Figure 2. Confirmation of the *S. toyocaensis staL* Replacement Strain

The genetic organization at the *staL* locus is depicted in block arrows for each strain, namely, *S. toyocaensis* wild-type (Strain 1) and *S. toyocaensis ΔstaL::aac(3)IV* (Strain 2). Oligonucleotide primers are shown as black arrows above and below the gene locus. For each strain (lanes 1 and 2), PCR amplification generated a product corresponding to the size indicated below each gene locus and to the left of the gel (arrows). Lane 3 contains a GeneRuler 1 kb DNA Ladder (sizes are shown in bp to the right of the gel). Note: Both samples and the DNA ladder were run on the same gel, but they are shown here as cropped lanes for clarity.

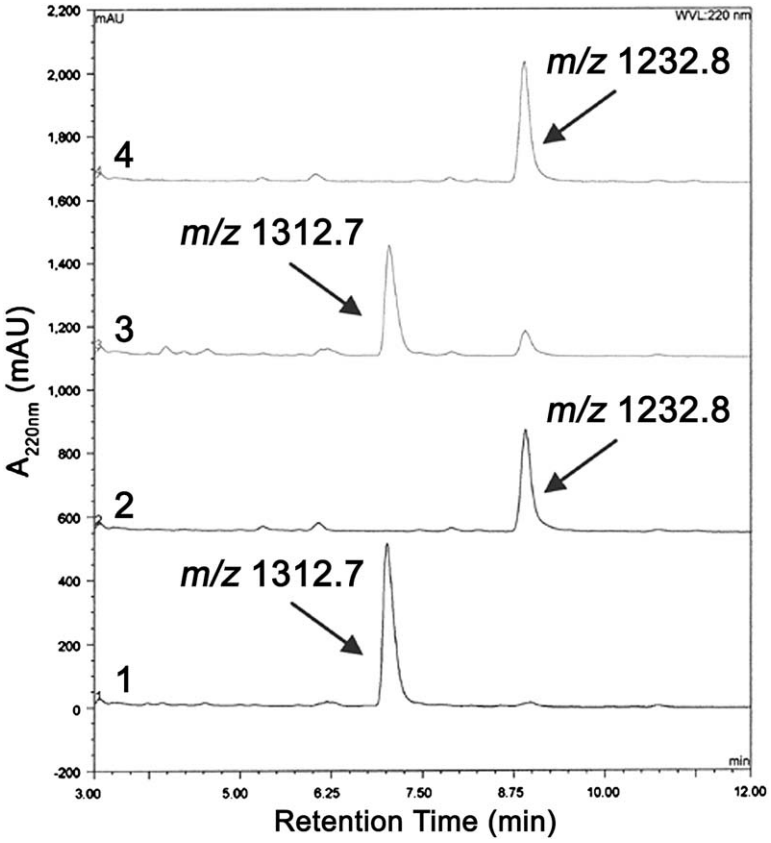

Figure 3. StaL Is Required for the In Vivo Sulfation of Desulfo-A47934

A RP-HPLC trace of the pH-adjusted extracts from the following strains: (1) *S. toyocaensis* wild-type, (2) *S. toyocaensis ΔstaL::aac(3)IV*, (3) *S. toyocaensis ΔstaL::aac(3)IV* ϕC31::*staL*/pIJ8600, and (4) *S. toyocaensis ΔstaL::aac(3)IV* ϕC31::pIJ8600. For each sample, the major antibiotic peak corresponding to either A47934 (R_t = 7.0 min) or desulfo-A47934 (R_t = 8.9 min) was confirmed by LC/ESI-MS (Table S2). The arrows denote the *m/z* $[M-H]^-$ for the compounds of interest.

consistent with the desulfo-antibiotic $[M-H]^-$, a compound previously termed A41030A that was isolated from a complex mixture of glycopeptides from *S. virginiae* [6]. No detectable amount of A47934 was present in the extract from *S. toyocaensis ΔstaL::aac(3)IV* or from the negative control strain, indicating the exclusive production of desulfo-A47934 in the absence of *staL*.

Complementation of *S. toyocaensis ΔstaL::aac(3)IV* was accomplished by integration of *staL*/pIJ8600 into the chromosome *in trans* at the ϕC31 *att* integration site. The complemented strain, *S. toyocaensis ΔstaL::aac(3)IV* ϕC31::*staL*/pIJ8600, now produced almost exclusively an antibiotic with an *m/z* of 1312.7, a difference of 80, which is consistent with monosulfation (Figure 3). The same *m/z* was observed for A47934 $[M-H]^-$ produced by wild-type *S. toyocaensis*.

In Vitro Sulfotransferase Activity of StaL

A *staL* expression vector was constructed in pET28a, and His_6-StaL was successfully purified as a 30 kDa protein via Ni-NTA affinity chromatography (Figure 4A). Subsequent gel filtration chromatography indicated that purified His_6-StaL was a homodimer of approximately 75 kDa (results not shown). This result is consistent with the reported quaternary structure of most eukaryotic sulfotransferases [9].

The following phenolic compounds were assessed as potential substrates of His_6-StaL: desulfo-A47934, teicoplanin aglycone, teicoplanin, vancomycin, ristocetin, 3,5-dihydroxybenzoic acid, phenol, 4-nitrophenol, rifamycin SV, *L*-HPG, *D*-HPG, and *L*-tyrosine (Tyr). The linear heptapeptide core of A47934 (H_2N-*L*-HPG-*L*-Tyr-*D*-DHPG-*D*-HPG-*L*-HPG-*D*-Tyr-*D*-DHPG-COOH; DHPG = 3,5-dihydroxy-*D*-phenylglycine) was synthesized and was also tested as an in vitro substrate of His_6-StaL. RP-HPLC analysis of the reactions and controls revealed that only the desulfo-A47934, teicoplanin aglycone, and teicoplanin reactions yielded products (Figure 5). These products exhibited the predicted *m/z* for A47934, sulfo-teicoplanin aglycone, and sulfo-teicoplanin when analyzed by LC/ESI-MS (Table S1; see the Supplemental Data available with this article online). In addition to confirming the in vitro sulfotransferase activity of His_6-StaL, these experiments produced two novel sulfoglycopeptide antibiotics: sulfo-teicoplanin aglycone and sulfo-teicoplanin.

The regiospecificity of the sulfate transfer was examined by using high-field NMR (700 MHz) and LC/ESI-MS/MS. Assignments for A47934 and desulfo-A47934 via NMR were complicated by peak overlap and potential aggregation of desulfo-A47934. However, the MS/MS fragmentation patterns of the sulfo- and desulfo-antibiotics (Table S2) confirmed that monosulfation occurred on the phenolic hydroxyl of the N-terminal *L*-HPG residue of desulfo-A47934 (observed *m/z* of 163.2) to give the corresponding sulfated fragment in the A47934 spectrum (observed *m/z* of 245.3). Sulfo-teicoplanin followed similar fragmentation patterns to those observed for A47934 and led to the identification of a common fragment (observed *m/z* of 686.1), corresponding to a monosulfated derivative of the N-terminal tetrapeptide. Subsequent fragmentation resulted in loss of the sulfate ion. Although we could not unambiguously identify the sulfated residue of sulfo-teicoplanin, the results

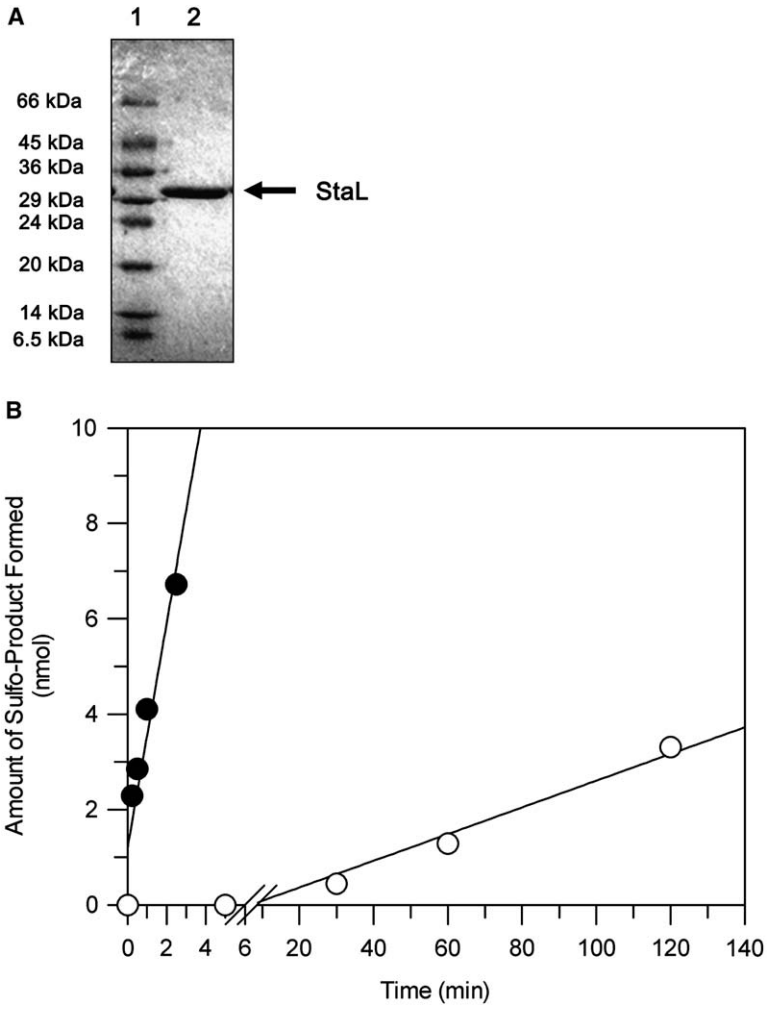

Figure 4. Purification and Kinetic Characterization of His_6-StaL

(A) SDS-polyacrylamide gel of purified His_6-StaL revealed a 30 kDa protein. Lane 1 contains a SigmaMarker Low Range protein standard (sizes are shown in kDa to the left of the gel), and a purified His_6-StaL sample is in lane 2.

(B) Kinetic analysis of the in vitro sulfation reaction with His_6-StaL and the substrates desulfo-A47934 and teicoplanin (both at 0.1 mM final concentration). The amount of A47934 (solid circle) and sulfo-teicoplanin (open circle) produced at each time point was quantified by RP-HPLC analysis. The initial velocity (v_0) was calculated to be 2.4 nmol/min and 2.8×10^{-2} nmol/min for reactions with desulfo-A47934 and teicoplanin, respectively.

suggest sulfation on either the N-terminal *L*-HPG residue or the third amino acid residue (i.e., *D*-DHPG) of teicoplanin. However, based on the precedent with the A47934 fragmentation, we conclude that His_6-StaL preferentially sulfates these glycopeptide antibiotics on the N-terminal *L*-HPG residue.

Steady-state kinetic studies were performed with Ni-NTA-purified His_6-StaL and either purified desulfo-A47934 or commercially available teicoplanin as substrates. At high substrate concentrations, the enzyme exhibited significant inhibition by an unknown mechanism. In an effort to quantify the production of substrate, we determined the initial velocity (v_0) of the sulfotransferase reaction by using an antibiotic substrate concentration of 0.1 mM, at which this anomalous kinetic behavior was not observed (Figure 4B). Under these conditions, the v_0 of the reaction was calculated to be 2.4 nmol/min and 2.8×10^{-2} nmol/min for desulfo-A47934 and teicoplanin, respectively, indicating a 100-fold preference for desulfo-A47934 over teicoplanin.

Quantification of the Antibacterial Activity of Sulfo- and Desulfo-Glycopeptides

Construction of the mutant *S. toyocaensis ΔstaL::aac(3)IV* strain allowed facile access to desulfo-A47934 via fermentation and subsequent purification by RP-MPLC. This procedure facilitated the use of desulfo-A47934 in the substrate specificity studies detailed in the previous section, as well as for the comparison of the antibacterial activity of this compound to A47934. Similarly, access to the overexpressed His_6-StaL permitted large-scale reactions with teicoplanin that resulted in the RP-HPLC purification of microgram quantities of the novel sulfo-derivative. The minimum inhibitory concentration (MIC) was determined for desulfo-A47934 and sulfo-teicoplanin according to the NCCLS guidelines against both vancomycin-sensitive and vancomycin-resistant bacterial strains (Table 1). A 2-fold increase in the MIC was observed upon sulfation of desulfo-A47934. Similar results were obtained for teicoplanin, where sulfation resulted in at least a 2-fold increase the in MIC.

Discussion

Natural product diversity arises first from the synthesis of polypeptide, polyketide, or alkaloid scaffolds. These chemical scaffolds are then modified, sometimes in combinatorial fashion, through in vivo biosynthetic approaches that access a variety of modifying enzyme catalysts. Some of the most prevalent modifications include glycosylation, halogenation, acylation, and alkylation. These alterations impact the water solubility, size, and

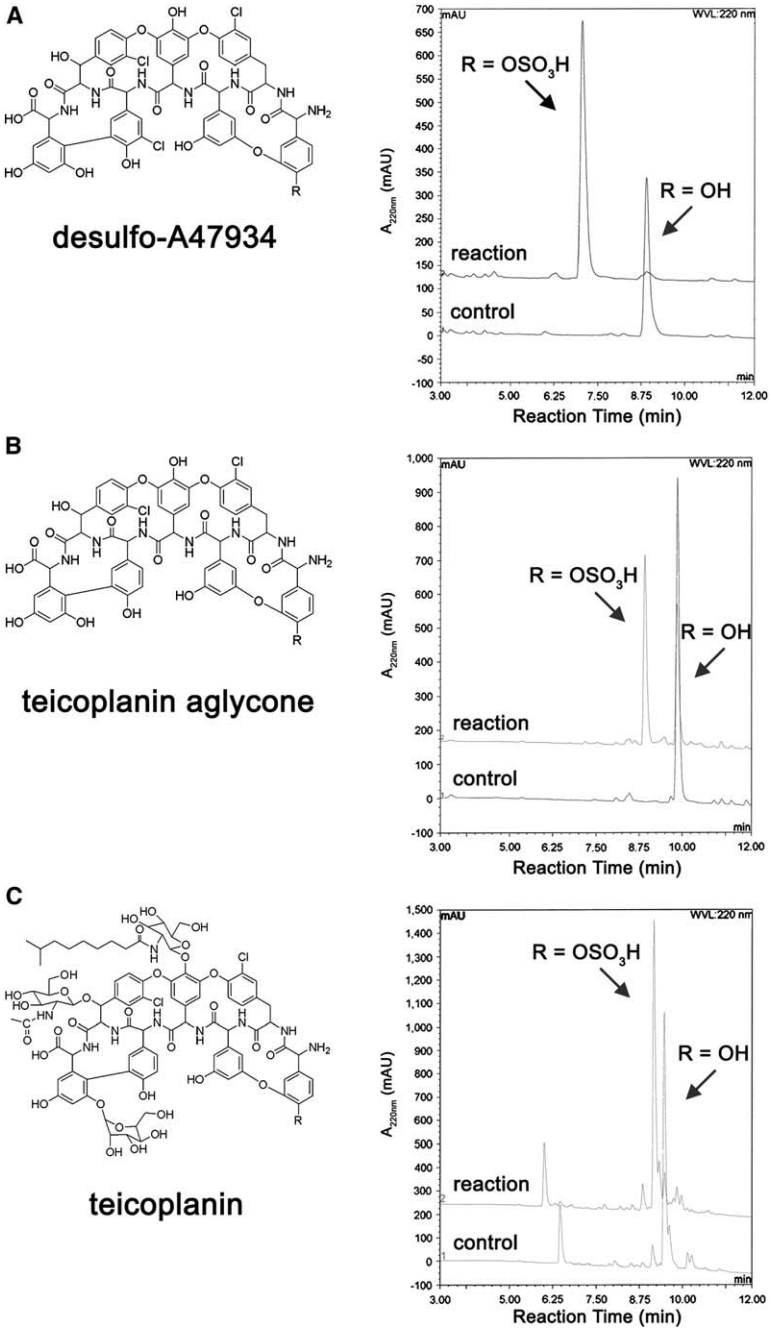

Figure 5. Characterization of His_6-StaL In Vitro

(A–C) The structures of the substrates (A) desulfo-A47934, (B) teicoplanin aglycone, and (C) teicoplanin are shown to the left of each RP-HPLC trace (R_t = 8.9 min, 9.8 min, and 9.5 min, respectively). Reactions were set up for each substrate as described in the Experimental Procedures and contained purified His_6-StaL, whereas the controls did not. LC/ESI-MS analysis (Table S2) confirmed that the product of each reaction was (A) A47934, (B) sulfo-teicoplanin aglycone, and (C) sulfo-teicoplanin (R_t = 7.0, 8.9, and 9.2 min, respectively).

receptor affinity of the molecules, in addition to the pharmacological properties of the subgroup of compounds that find use as drugs. In some cases, such as the sugar moieties of the coumarin and glycopeptide antibiotics, the modifying agents are, in fact, key pharmacophores of drug activity.

With the exception of aminoglycosylation, these modifications of natural product scaffolds rarely alter the charge of the natural product. Modification by sulfation, however, does provide the means to modify not only steric bulk and hydrophobicity, but also to introduce a negative charge. While sulfation is a common solvating strategy for xenobiotic detoxification in eukaryotes, assessing the biological role of sulfation in prokaryotes has generally been stalled due to the lack of information regarding natural substrates, with the exception of three bacterial carbohydrate sulfotransferases [11–13].

The "aglyco"-glycopeptide A47934 is exceptional not only in the lack of glycosyl modification that is characteristic of this class of antibiotic, but also in the presence of a sulfate group linked to the phenolic hydroxyl of the N-terminal *L*-HPG residue. As a result, and unlike other antibiotics of the glycopeptide class such as vancomycin and teicoplanin, A47934 possesses an overall negative charge at pH 7.

Annotation of the A47934 biosynthetic gene cluster identified a putative PAPS-dependent sulfotransferase, StaL, encoded by a gene located at the extreme 3′ terminus of the gene cluster [7]. The role of this enzyme in A47934 biosynthesis was confirmed here by in-frame

Table 1. Antibacterial Activity of Sulfo- and Desulfo-Glycopeptide Antibiotics

	MIC (μg/ml)			
Antibiotic	*S. aureus*[a]	*E. faecalis*	VRE VanB	VRE VanA
Vancomycin	0.5	2	8–16	>16
Ristocetin	8	4	>16	>16
Teicoplanin	1–2	1	1	>16
Sulfo-teicoplanin	8	2	4	>16
Teicoplanin aglycone	0.25	1	4	>16
Desulfo-A47934	0.5	1–2	2	>16
A47934	1	4	4	>16

[a] Strain abbreviations (left to right) for *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *E. faecalis* ATCC 51299, and an *E. faecalis* clinical isolate (see Experimental Procedures).

gene replacement of *staL*, which resulted in the exclusive production of desulfo-A47934. Reintroduction of *staL in trans* recapitulated A47934 production, confirming that the phenotype of the mutant strain was not the result of polar genetic effects due to the incorporation of the *aac(3)IV* resistance marker during gene replacement. This mutant strain provided access to large quantities of the predicted StaL substrate desulfo-A47934, which was shown to retain antibiotic activity in the absence of sulfation. This modification is therefore not essential to the antibacterial properties of the compound, but it does result in a 2-fold increase in the MIC.

Most sulfotransferases are functional as homodimers [9], and, in this study, recombinant His_6-StaL was purified as a homodimer. StaL also demonstrated the predicted sulfotransferase activity in vitro, generating A47934 when incubated with desulfo-A47934 (purified from the *staL* replacement strain). Therefore, both the in vivo and in vitro experiments support sulfation as the final step in A47934 biosynthesis. Complete in vitro conversion of desulfo-A47934 to A47934 was accomplished by using the recombinant catalyst His_6-StaL, demonstrating the utility of using this enzyme for the in vitro synthesis of sulfo-compounds. This potential was exploited with the in vitro synthesis of two novel sulfoglycopeptide antibiotics: sulfo-teicoplanin aglycone and sulfo-teicoplanin. Purification of microgram quantities of sulfo-teicoplanin permitted analysis of its antibacterial activity and the observation that sulfation of teicoplanin resulted in at least a 2-fold increase in the MIC. Thus, modification of glycopeptide antibiotics via sulfation results in modest increases in the MIC values.

Vancomycin, which lacks an N-terminal *L*-HPG residue, was not a substrate for StaL, despite the presence of free *para*- and *meta*-hydroxyls in the C-terminal region of the molecule. Ristocetin was also not a substrate, even though it possesses a similar peptide backbone as A47934 and teicoplanin, including an N-terminal *L*-HPG residue. However, ristocetin contains a methylated amino acid (i.e., 3,5-dihydroxyl-4-methylphenylglycine) that is crosslinked to the N-terminal HPG residue, as well as an expansion of glycosylation of the central *D*-HPG residue. These results demonstrate the unique discriminatory capacity of StaL for glycopeptide antibiotics (Figure 6). Furthermore, the linear uncrosslinked heptapeptide core and both HPG isomers were not substrates for the enzyme. StaL is therefore unlike other glycopeptide modifying enzymes (e.g., vancomycin crosslinking oxidases, [27]) that recognize precursors often still covalently linked to nonribosomal peptide synthetases, but not the free peptide or polyketide scaffolds.

To date, only three other sulfotransferases from bacteria have been characterized. NodH and NoeE are essential for the symbiosis of *S. meliloti* [11, 12], and Sft0 is implicated in *M. tuberculosis* pathogenesis [13]. These enzymes transfer sulfate to carbohydrate substrates by using PAPS as a sulfate donor. This study adds StaL as a bacterial PAPS-dependent sulfotransferase with a role in natural product biosynthesis. The function of sulfation in *S. toyocaensis* is unclear at this time. Sulfation in eukaryotes is often associated with detoxification [9], but the antibacterial activity of desulfo-A47934 is only marginally better than that of the mature sulfo-antibiotic A47934. Therefore, detoxification is likely not a main function of sulfation in A47934 biosynthesis. Alternatively, the advantage of sulfation might be increased solubility since A47934 lacks glycosylated residues. Ultimately, the utility of this sulfating reaction may have more impact on the pharmacological properties (e.g., ADME) of sulfo-glycopeptide antibiotics. This novel reaction adds to the expanding repertoire of engineering possibilities for glycopeptide antibiotics. In particular, the ability to manipulate biosynthetic genes for the fermentation of new glycopeptide scaffolds, coupled with the in vitro application of modifying enzyme action, can provide leverage for the development of new antibiotics.

Significance

StaL is a novel sulfotransferase from *S. toyocaensis* with the regiospecific capacity to modify glycopeptide antibiotic scaffolds. This modification expands the reaction set available for accessorizing glycopeptide antibiotics with the potential to develop new compounds with sulfated N-terminal *L*-HPG residues, thus altering the properties of these compounds (e.g., overall charge). Glycopeptide antibiotics can already be modified by glycosylation, acylation, methylation, halogenation, and oxidation. The availability of a sulfation reaction via StaL adds another level of diversity to this class of antibiotics that can now be explored in structure-activity and ADME studies.

The successful combination of genetic and biochemical approaches to generate desulfo-antibiotics and new sulfo-antibiotics further broadens the significance of this work to include a generic strategy for the synthesis of novel glycopeptide antibiotics. Selective combinations of in vivo and in vitro approaches will increase the chemical diversity of this class and can be applied to other groups of antibiotics. Overall, the use of a genetically malleable strain, such as *S. toyocaensis*, with state-of-the-art gene inactivation and replacement strategies demonstrates the power of combining genetic and biochemical methods in natural product research.

Experimental Procedures

Compounds and Media

Chemicals were purchased from Sigma-Aldrich Canada, Ltd. or from BioShop Canada, Inc.; dehydrated media were bought from Becton

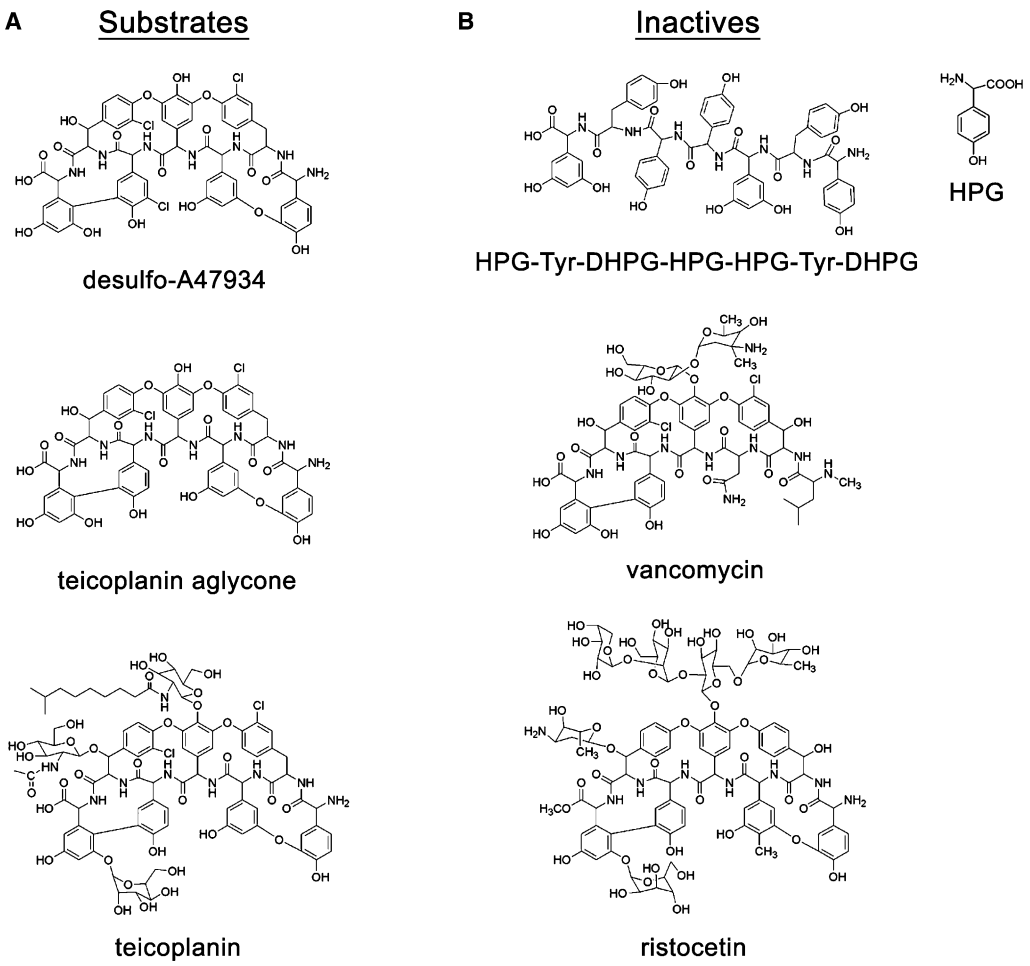

Figure 6. Substrate Specificity of His_6-StaL

(A) Substrates (top to bottom) are desulfo-A47934, teicoplanin aglycone, and teicoplanin.

(B) Compounds that are not His_6-StaL substrates (top right to bottom) are the linear heptapeptide (H_2N-HPG-Tyr-DHPG-HPG-HPG-Tyr-DHPG-COOH), HPG, vancomycin, and ristocetin.

Dickinson and Company. Oligonucleotide primer synthesis and DNA sequencing were performed at The MOBIX Lab Central Facility (McMaster University). To induce the λ-Red genes, *L*-arabinose was used as reported in [26], and 1 mM isopropyl-β-*D*-1-thiogalacto-pyranoside (IPTG) was used to induce expression of His_6-StaL. Antibiotics were used at the following final concentrations (μg/ml): ampicillin (Amp, 100), kanamycin (Kan, 50; 200 on R2YE), apramycin (Apr, 50), chloramphenicol (Cam, 25), nalidixic acid (Nal, 25), and thiostrepton (Tsr, 10). Stock solutions of Nal and Tsr were made up in 0.3 M NaOH and dimethylformamide, respectively.

The following liquid media for *S. toyocaensis* contained a spring baffle to aid in mycelial dispersion: *Streptomyces* Vegetative Medium (SVM) [28], *Streptomyces* Antibiotic Medium (SAM) as reported in [29] plus 1 g yeast extract per liter, Tryptone Soya Broth CM0129 (TSB) (Oxoid, Ltd.) supplemented with 0.5% glycine (stock solution 20% [w/v]), and 2xYeast-Tryptone (2xYT) [30]. Solid media for *S. toyocaensis* were the following: Mannitol Soyaflour Agar (MSA; also known as Soya Flour Medium [SFM]) [30, 31] and R2YE Agar [30, 32]. Bennett's Agar (BA) was used to facilitate spore formation and was modified from the original reference [33] to contain the following components per liter: 10 g potato starch, 2 g casamino acids, 1.8 g yeast extract, 15 g agar, and 2 ml Czapek mineral mix (per 100 ml: 10 g KCl, 10 g $MgSO_4 \bullet 7H_2O$, 12 g $NaNO_3$, 0.2 g $FeSO_4 \bullet 7H_2O$, and 200 μl concentrated HCl). LB or SOC [34] were used as growth media for *E. coli* and *B. subtilis*, whereas BHI and CA-MHB [35] facilitated growth of *S. aureus* and *E. faecalis* strains.

Bacterial Strains

E. coli BL21(DE3) (EMD Biosciences, Inc.) was used as an expression strain, and *E. coli* SURE2 (Stratagene) and *E. coli* TOP10 (Invitrogen Canada, Inc.) were used for cloning purposes. The REDIRECT technology kit (John Innes Centre) [26] employs *E. coli* BW25113 as a recombination host strain, and *E. coli* ET12567 was used for intergenic conjugation with *S. toyocaensis* NRRL 15009. Dr. E.D. Brown (Department of Biochemistry and Biomedical Sciences, McMaster University) provided the test organism *B. subtilis* 168 *trpC2* (*B. subtilis* 1A1; *Bacillus* Genetic Stock Center). An *E. faecalis* VanA clinical isolate, donated by Dr. A.K. Petrich (Department of Pathology and Molecular Medicine, McMaster University), and the following American Type Culture Control (ATCC) strains were employed as test organisms for the quantification of antibacterial activity: *Staphylococcus aureus* ATCC 29213, *E. faecalis* ATCC 29212, and *E. faecalis* ATCC 51299.

B. subtilis 1A1 and *S. toyocaensis* strains were grown at 30ºC; all other bacteria were incubated at 37ºC. *E. coli* pIJ790/BW25113 strains were grown at 30ºC or 37ºC as required [26]. *S. toyocaensis* strains were maintained in 20% glycerol at −80ºC, and spore suspensions [30] and frozen stocks of all others strains were preserved

in 15% glycerol at −80ºC. Single colonies of *S. toyocaensis* strains from either BA or R2YE agar plates were seeded in 25 ml SVM in a 50 ml Erlenmeyer flask for 2–3 days prior to subculturing into other media.

Construction of the *staL* Replacement Cosmid and Strain

The gel-purified EcoRI-HindIII fragment of pIJ773 (see [26]) was used as a template for the PCR amplification of the 1460 bp *oriT aac(3)IV* replacement cassette by using the following primers: 5′-GCTGCCCGCCGCGTCGAAGGAACGAGAGGACGCCCG**A*TGA***ACATTCCGGGGATCCGTCGACC-3′ and 5′-AACATCGAGCGGTGTCGGACTGTTCAAGCGGGGAATT**CA**TGTAGGCTGGAGCTGCTTC-3′. Underlined sequences anneal to pIJ773, and the nucleotide extensions include either the start or stop codon (shown in bold type) of *staL* plus flanking DNA. An additional three nucleotides (italics) were included due to the overlap of the *staK* TGA and the *staL* start codons. PCR conditions were as follows: 3 min at 94ºC, 30 cycles (1 min at 94ºC, 1 min at 55ºC, 1.5 min at 72ºC), and 10 min at 72ºC with 1 U BIOTOOLS DNA Polymerase (Intersciences, Inc.), 5% DMSO, and 4.5 mM exogenous $MgCl_2$.

The protocol as reported [26] was adhered to with the following modifications. The pIJ773 EcoRI-HindIII fragment was gel purified once, and the purified PCR product was treated with DpnI and was gel purified. SOC was substituted for SOB, and pIJ790/BW25113-competent cells were induced twice with arabinose. R2YE agar was used in place of DNA plates for assessing phenotypes of *S. toyocaensis* colonies. These protocols facilitated the λ-Red-mediated construction of the recombinant plasmid pCepC1 *ΔstaL*::*aac(3)IV* from pCepC1 [7] and the *oriT aac(3)IV* PCR product. A restriction enzyme digest, PCR, and Southern analyses confirmed the construction of the recombinant cosmid. Introduction of this cosmid into *S. toyocaensis* via conjugation with *E. coli* [30, 36] resulted in the construction of the *S. toyocaensis ΔstaL*::*aac(3)IV* strain, which was confirmed via PCR and Southern analyses. PCR primers were 5′-GGTCTCGCCCGACGGAATGA-3′ and 5′-AGCGGTGTCGGACTGTTCAAG C-3′, and PCR conditions were as follows: 3 min at 94ºC, 30 cycles (1 min at 94ºC, 1 min at 56.5ºC, 1 min at 72ºC), and 10 min at 72ºC with 1 U BIOTOOLS DNA Polymerase, 10% DMSO, and 4.5 mM exogenous $MgCl_2$.

Genomic DNA Isolation, Probe Construction, and Southern Methodology

Wild-type *S. toyocaensis* and *S. toyocaensis ΔstaL*::*aac(3)IV* were seeded in SVM, followed by subculturing 200 μl into 20 ml TSB supplemented with 0.5% glycine. After incubation for 2–3 days, cultures were centrifuged, and the pellets were washed in sterile 10% (w/v) glycerol and then stored at −20ºC. After thawing, cell pellets were resuspended, homogenized, and washed twice in SET buffer, followed by isolation of the genomic DNA as previously reported [37].

The PCR product used to create the *staL* complementation plasmid (see next section) was used to make the *staL* probe. The *aac(3)IV* probe DNA was obtained by PCR by using the EcoRI-HindIII fragment of pIJ773 as a template, the primers 5′-ATTCCGGG GATCCGTCGACC-3′ and 5′-TGTAGGCTGGAGCTGCTTC-3′, and PCR conditions as described for the generation of the linear *aac(3)IV* replacement fragment (see previous section). The Random Primers DNA Labeling System (Invitrogen Canada, Inc.) was used to label 200 ng of each PCR product with α-^{32}P-dATP (PerkinElmer Life and Analytical Sciences). The QIAEX II Gel Extraction kit (QIAGEN, Inc.) was used to purify the labeled probes.

Three individual restriction enzyme digests (i.e., StuI, XhoI, and XmnI) were performed in duplicate on 10 μg of each of the following DNA samples: pCepC1 [7], pCepC1 *ΔstaL*::*aac(3)IV*, and genomic DNA isolated from wild-type *S. toyocaensis* and the *S. toyocaensis ΔstaL*::*aac(3)IV* strain. The ^{32}P-dATP-labeled *staL* and *aac(3)IV* probes were included as controls. Southern hybridization was performed essentially as described in [26, 34]. Phosphor screens were exposed to the *staL* and *aac(3)IV* blots for 1 hr and 24 hr and were developed on a Typhoon 9200 Variable Mode Imager (Molecular Dynamics, Amersham Biosciences, Inc.).

Construction of the *staL* Complementation Vector and Strain

The following primers were used to amplify *staL* from pCepC1 [7]: 5′-GCTCTAGACATATGAACGGG**ATG**TGCTGGAT-3′ and 5′-GCTCTAGATCTGGGGAATTCATTCAGCGTATC-3′, in which the two putative start sites (shown in bold type) and the NdeI and BglII sites (underlined) are shown. PCR conditions were as follows: 3 min at 94ºC, 30 cycles (1 min at 94ºC, 1 min at 50ºC, 1 min at 72ºC), and 10 min at 72ºC with 1 U BIOTOOLS DNA Polymerase, 5% DMSO, and 3 mM exogenous $MgCl_2$.

The 820 bp PCR product (NdeI-BglII digested) was ligated into NdeI-BglII-digested pIJ600 [38]. Restriction digests and sequencing with the primer 5′-CGTGAGGAGGCAGCGTGGAC-3′ confirmed the integrity of *staL*/pIJ8600. Transformation of this plasmid into pUZ8002/ET12567 facilitated its subsequent conjugation and integration [30, 36] into *S. toyocaensis ΔstaL*::*aac(3)IV*, resulting in the complementation strain *S. toyocaensis ΔstaL*::*aac(3)IV* ϕC31::*staL*/pIJ8600. Similarly, the negative control strain *S. toyocaensis ΔstaL*::*aac(3)IV* ϕC31::pIJ8600 was created by using pIJ8600. Confirmation of these two strains was based on phenotype (i.e., Tsr resistance) and the antibiotics present in the extract.

Construction of the *staL* Expression Vector

The following primers were used to amplify *staL* (containing only the second putative start site [bolded]) from pCepC1 [7]: 5′-GC***GAATTC*** CAT**ATG**TGCTGGATCGCCTCCTACCCG-3′ and 5′-GC***GAATTC*** AAGCTT***GAATTC***ATTCAGCGTATCCAT-3′, in which the NdeI and HindIII sites (underlined) and the EcoRI sites (bolded italics) are shown. PCR conditions were as follows: 3 min at 94ºC, 25 cycles (1 min at 94ºC, 1 min at 46ºC, and 1.5 min at 72ºC), and 10 min at 72ºC with 1 U $Vent_R$ DNA Polymerase (New England Biolabs, Ltd.), 2.5% DMSO, and a dNTP mix (70:30; GC:AT). The 820 bp PCR product (EcoRI digested) was ligated into EcoRI-digested pUC18 (Fermentas Life Sciences). The NdeI-HindIII fragment containing *staL* was subcloned from this plasmid and was ligated into similarly digested pET28a (EMD Biosciences, Inc.). Subsequent restriction enzyme digests and sequencing (Robarts Research Institute) of the isolated plasmid confirmed the construction of *staL*/pET28a.

Expression and Purification of His_6-StaL

An overnight culture of *staL*/pET28a/BL21(DE3) was used to inoculate 1 L LB Kan (1:100), which was then grown until an absorbance of 0.6 at 600 nm was reached (~2 hr). After gentle swirling on ice for 5 min, IPTG was added to induce expression. The culture was then incubated at 16ºC for 16–20 hr, after which time the cell pellet was harvested, washed in 20 ml 0.85% NaCl, and stored at −20ºC.

The cell pellet was thawed on ice and resuspended in 20 ml lysis buffer containing 1 mM EDTA (pH 8.0), 0.1 mM dithiothreitol (BioShop Canada, Inc.), and 1 mM phenylmethylsulfonylfluoride in Buffer A (50 mM HEPES, [pH 7.5], 500 mM NaCl, and 20 mM imidazole). After three passages through a French Press, the sample was centrifuged, and the supernatant was loaded onto a 1 ml Ni-NTA Superflow (QIAGEN, Inc.) column. Purification of His_6-StaL was performed at 4ºC with a flow rate of 1 ml/min. Absorbance was monitored at 280 nm. The column was washed with Buffer A, after which the following linear gradient of Buffer B (50 mM HEPES [pH 7.5], 500 mM NaCl, and 250 mM imidazole) was initiated: 0%–50% B over 25 min, 50% B for 5 min, 50%–100% B over 10 min, and 100% B for 5 min. After SDS-PAGE and Coomassie staining, fractions containing pure His_6-StaL were pooled, and the protein concentration was determined by using the Bio-Rad Protein Assay-Dye Reagent Concentrate (Bio-Rad [Canada] Laboratories, Inc.) [39]. The total yield of His_6-StaL was 5 mg (0.3 mg/ml), and the sample was concentrated to 5 mg/ml prior to assessing sulfotransferase activity (see next section).

Gel filtration chromatography was performed by using a Superdex 75 FPLC column (Amersham Biosciences, Inc.) according to the manufacturer's instructions. The buffer consisted of 50 mM HEPES (pH 7.5) and 200 mM NaCl. The log(molecular weight) of the recommended standards was plotted against V_e/V_o in order to determine the molecular weight of purified His_6-StaL.

In Vitro Sulfation Reactions

For the substrate specificity studies, a control and reaction sample (30 μl each) was set up in a microcentrifuge tube and contained the following: 1 mM PAPS, 0.1 mM dithiothreitol, and 25 mM HEPES (pH 7.5). Substrates tested were desulfo-A47934, teicoplanin aglycone, teicoplanin (Haorui Pharma-Chem, Inc.), vancomycin, ristocetin, 3,5-dihydroxybenzoic acid, phenol, 4-nitrophenol, rifamycin SV,

L-HPG, *D*-HPG, *L*-Tyr, and the linear heptapeptide (0.5–0.8 mM final concentration). To each reaction sample, 5 μg purified His_6-StaL (5.2 μM final concentration) was added to initiate the reaction. Both the controls and reactions were incubated at 30ºC for 20 hr and were then placed in a boiling water bath for 10 min, followed by incubation on ice for 10 min and storage at −20ºC. RP-HPLC analysis (10 μl) and LC/ESI-MS (10 μl) were performed on each sample. However, the substrate specificity samples with nonglycopeptide antibiotic substrates were analyzed solely by LC/ESI-MS.

For the kinetic analysis of His_6-StaL, 100 μl reactions were set up in duplicate and contained the following: 1 mM PAPS, 0.1 mM DTT, 0.1 mM substrate (desulfo-A47934 and teicoplanin), and 15 mM HEPES (pH 7.5). Reactions were initiated with the addition of 1 μg purified His_6-StaL (1.04 μM final concentration) and were then incubated at 30ºC. For desulfo-A47934, reactions were incubated for 0.25, 0.5, 1.0, 2.5, 5.0, 10, and 30 min. Teicoplanin reactions were incubated for 0.08, 0.5, 1, 2, 4, and 8 hr. Reactions were stopped as mentioned for the substrate specificity reactions and then were immediately analyzed by RP-HPLC (100 μl). Additionally, the peak areas of duplicate desulfo-A47934 and teicoplanin samples were used to generate a standard curve for each substrate at 280 nm. The amount of product formed from each substrate was plotted versus time, and the initial velocity (v_0) of each reaction was calculated by using linear regression.

Solid-Phase Synthesis of Linear Heptapeptide Core of A47934

Fmoc-*L*-Tyr(OHBu), Fmoc-*D*-Tyr(OHBu), Wang resin, and N-hydroxytriazole were purchased from EMD Biosciences, Inc. (Novabiochem). *D*-DHPG was prepared as reported in [40]. Prior to use, amino acids were Fmoc protected [41]. The synthesis of the linear heptapeptide (H_2N-*L*-HPG-*L*-Tyr-*D*-DHPG-*D*-HPG-*L*-HPG-*D*-Tyr-*D*-DHPG-COOH) was accomplished by using solid-phase peptide synthesis (0.33 mM scale, 2-[1H-benzotriazole-1-yl]1,1,3,3-tetramethyluroniumtetrafluoroborate/hydroxytriazole/diisopropylamine, alternated by diisopropylcarbodiimide/hydroxytriazole, activation) on Wang resin derivatized with Fmoc-*D*-dimethoxyphenylglycine. Side chain protecting groups were methyl ether for *D*-DHPG, benzyl ether for *L*-HPG, and t-butyl ether for *D*-Tyr and *L*-Tyr. *D*-HPG was used without protection. The assembled peptide was cleaved from the resin by using a mixture of trifluoroacetic acid (TFA):phenol:triisopropylsilane:water (95:0.5:0.5:0.2) for 3 hr at room temperature. RP-HPLC afforded the partially protected peptide in 95% purity. Methyl ether groups were removed by using a method described in the literature [42]. Benzyl ether was cleaved simultaneously. The final product was purified by using RP-HPLC and was verified by LC/ESI-MS.

Separation and Analytical Procedures

RP-HPLC was performed with a C_{18} column (3 μm, 120 Å, 4.6 × 150mm; Dionex Corporation) and a Dionex GP40 Gradient Pump system (flow rate of 1 ml/min). Monitoring was performed at 220 and 280 nm.

The extracts from *S. toyocaensis* and the His_6-StaL controls and reactions were separated by using a dual solvent system consisting of 20 mM ammonium acetate (Solvent A) and 100% acetonitrile (Solvent B). For each antibiotic tested, a linear gradient of Solvent B was optimized to separate the sulfated and nonsulfated compounds. The initial percentage of B (1 min) and the gradient percentage of B (over 9 min) for each antibiotic were as follows: desulfo-A47934 (20%, 20%–25%), teicoplanin aglycone (15%, 15%–30%), teicoplanin (15%, 15%–45%), vancomycin (10%, 10%–15%), and ristocetin (5%, 5%–20%).

To purify sulfo-teicoplanin, separation was optimized by using 0.05% TFA in water (Solvent A) and 0.05% TFA in 100% acetonitrile (Solvent B) and the following program: 20% B for 5 min, 20%–27.5% B over 20 min. The linear heptapeptide was purified by using the same solvent system and the following program: 5% B for 1 min and then an increasing linear gradient to 97% B over 10 min.

LC/ESI-MS data were obtained by using an Agilent 1100 Series LC system (Agilent Technologies Canada, Inc.) and a QTRAP LC/MS/MS System (Applied Biosystems/MDS Sciex). The same RP-HPLC conditions stated for the linear heptapeptide were used for LC/ESI-MS, but formic acid was used in place of TFA. For the fragmentation analysis by LC/ESI-MS/MS, the experimental conditions were as follows. Samples were diluted with 50% acetonitrile in water to a final concentration of 5 mg/ml, and 30 μl was directly injected. Information Dependent Acquisition (IDA) enabled "on the fly" acquisition of the MS/MS spectra during the chromatographic run in a negative ion mode. Run parameters were as follows: scan rate, 4000 amu/s; settling time, 700 ms; LIT fill time, 20.0 ms; dynamic fill time, on; Q3 entry barrier, 8.0 V; CUR, 12.0; CAD, medium; ion spray, 4300.0; temperature, 100.0ºC; GS1, 20.0; DP, −40.0; EP, −10.0; and CE, −10.0 and −20.0.

Antibiotic Extraction from *S. toyocaensis* Strains

Strains were cultivated in SVM, followed by subculturing 500 μl into 50 ml SAM in a 250 ml Erlenmeyer flask. The culture was centrifuged after incubation for 6 days, and the wet weight was determined. For every 1 g of cell mass, 500 μl 1% (v/v) NH_4OH was added to extract the antibiotic; after vortexing and centrifugation, the pH of the supernatant was adjusted to 7.5 with 1 M HCl (modification of a previously described method [3]). The extract from each strain was subjected to analysis by disk-agar diffusion (20 μl) [43], RP-HPLC (10 μl), and LC/ESI-MS (10 μl).

Purification of Desulfo-A47934 and Sulfo-Teicoplanin

An 85 ml column of octadecyl-functionalized silica gel facilitated desulfo-A47934 purification from 3 ml *S. toyocaensis ΔstaL::aac(3)IV* extract. After washing with 100 ml water, a 5%–50% (v/v) acetonitrile gradient was initiated. Fractions exhibiting high absorbance readings at 220 and 280 nm, antibacterial activity via disk-agar diffusion [43], and an RP-HPLC trace corresponding to desulfo-A47934 were pooled and lyophilized. Resuspension in 200 μl water and adjustment to pH 7.5 yielded a 5 mg/ml solution of desulfo-A47934. The concentration was determined by using an A47934 standard curve at 281 nm, and the purity of the sample was confirmed by RP-HPLC and LC/ESI-MS analyses.

Aliquots of the large-scale in vitro reaction (150 μl) with teicoplanin and purified His_6-StaL were subjected to RP-HPLC, and the eluent was collected during elution of sulfo-teicoplanin (A2 component). The collected fractions were concentrated as per desulfo-A47934 procedures and yielded a 1 mg/ml solution of sulfo-teicoplanin. The concentration was determined by using a teicoplanin standard curve at 278 nm.

Qualitative and Quantitative Determination of Antibiotic Activity

The test organism *B. subtilis* 168/1A1 was employed for qualitative disk-agar diffusion assays on LB plates [43]. The 0.5 McFarland standard was prepared by using an inoculated LB plate and the colony suspension method [43]. Plates were incubated at 30ºC for 24 hr. A zone of clearance around a disk was indicative of antibacterial activity in the sample. To quantify the antimicrobial activity, the MIC of each sample was determined by using the microdilution broth method in CA-MHB [35]. The 0.5 McFarland standards were prepared by using the colony suspension method [35] and *S. aureus* or *E. faecalis* strains grown on BHI agar plates. After 24 hr of incubation at 37ºC, the MIC was determined in μg/ml.

Supplemental Data

Supplemental Data including Southern analysis (Figure S1), MS data (Table S1), and MS fragmentation analysis (Table S2) are available at http://www.chembiol.com/cgi/content/full/13/2/171/DC1/.

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