Glycopeptide Sulfation Evades Resistance

Lindsay Kalan, Julie Perry, Kalinka Koteva, Maulik Thaker, Gerard Wright

M. G. DeGroote Institute for Infectious Disease Research, Department of Biochemistry and Biomedical Sciences, DeGroote School of Medicine, McMaster University, Hamilton, Ontario, Canada

The incidence of antibiotic resistance among pathogenic microorganisms is increasing at an alarming rate. Resistance against front-line therapeutics such as the glycopeptide antibiotic vancomycin has emerged and has spread to highly virulent pathogens, including *Staphylococcus aureus*. Glycopeptide antibiotics are natural products from the *Actinomycetes* that have a characteristic heptapeptide core. The chemical diversity of the class is achieved through glycosylation, halogenation, methylation, and acylation of the core, modifications that are implicated in improved solubility, stability, or activity of the molecule. Sulfation is yet another modification observed infrequently in glycopeptides, but its role is not known. Although glycopeptide sulfotransferases are found in the environmental metagenome and must therefore serve an evolutionary purpose, all previous studies have reported decreased antibiotic activity with sulfation. We report that sulfation of glycopeptides has little effect on the compound's ability to bind its target, the D-Ala-D-Ala peptidoglycan precursors of the bacterial cell wall. However, sulfation does impact glycopeptide dimerization, and importantly, sulfated glycopeptides are significantly less potent inducers of the resistance gene cluster *vanHAX* in actinomycetes. Our results begin to unravel the mystery of the biological role of glycopeptide sulfation and offer a potential new strategy for the development of new antibiotics that avoid resistance.

Antibiotic-resistant infectious organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA) are a major public health problem of global concern. The glycopeptide antibiotics vancomycin and teicoplanin are used as front-line therapy to treat serious infections by Gram-positive bacteria such as MRSA, but glycopeptide-resistant organisms have emerged in response. A problem of particular significance is the spread of vancomycin-resistant enterococci (VRE) in hospitals and other health care facilities. The genes that confer glycopeptide resistance in VRE have been passed to MRSA on several occasions, presenting an even greater threat due to the resulting increase in the virulence and drug resistance of this organism.

Vancomycin resistance occurs via a tightly regulated five-gene cassette (*vanRSHAX*). The *vanHAX* genes encode enzymes that reengineer peptidoglycan precursors to terminate in D-alanyl-D-lactate (D-Ala-D-Lac) rather than D-alanyl-D-alanine (D-Ala-D-Ala); this change disrupts glycopeptide antibiotic binding by elimination of a key drug-binding hydrogen bond (Fig. 1) (1–3). Expression of these genes is under the genetic control of a two-component regulatory system consisting of the response regulator VanR and the membrane-spanning receptor histidine kinase VanS (4, 5). Previous work in our laboratory using a vancomycin photoaffinity probe has shown that vancomycin directly binds to the VanS histidine kinase in *Streptomyces coelicolor* to induce expression of the *vanHAX* resistance cluster (4).

Glycopeptide antibiotics are bacterial natural products and are categorized according to their core heptapeptide backbone into two general classes: type I (vancomycin, with an N-terminal Leu-β-hydroxy-3-chloroTyr-Asn tripeptide) and type II (teicoplanin, with an N-terminal hydroxyphenylglycine [HPG]-3-chloroTyr-3,5-dihydroxyphenylglycine [DHPG] tripeptide) (6, 7) (Fig. 2). Within each class, individual antibiotic structures differ based on the degree and nature of modification of the backbone by glycosylation, halogenation, methylation, acylation, and sulfation. Modifications serve to alter the activity of the compound. Natural product modification by sulfation is relatively uncommon. Sulfated glycopeptides that have been described in the literature are teicoplanin-like, with sulfate added to phenolic hydroxyls (8–10). However,

environmental metagenome sequencing has uncovered a number of glycopeptide sulfotransferases with various regiospecificities of compound modification, indicating that glycopeptide sulfation may be more widespread (11, 12). Previous work has shown that sulfation decreases the antimicrobial activity of the molecule, regardless of the residue sulfated (8, 11, 12). Why would nature evolve the mechanism of sulfation if the effect of the modification is a reduction in biological activity?

We set out to deduce the role of glycopeptide sulfation in both the mechanism of action and the induction of resistance. We chose to use the sulfated glycopeptide A47934 and its mutant desulfo derivative (DS-A47934) to investigate environmental glycopeptide resistance using the soil actinomycete *Streptomyces coelicolor* as a model organism. We report that A47934 and DS-A47934 had comparable inhibitory activities against *S. coelicolor*. While cell wall binding was unchanged by sulfation, the ability of the molecule to dimerize was compromised by the presence of a sulfate residue. We hypothesized that this inability to dimerize due to sulfation would alter the antibiotic's affinity for VanS and would therefore serve as a strategy to evade resistance. Indeed, sulfation has a profound effect on induction of resistance gene expression in *S. coelicolor*. Furthermore, we show that the repression of resistance induction appears to be a widespread phenomenon in vancomycin-resistant environmental organisms. This work resolves the long-standing question as to the evolutionary role of glycopeptide sulfation in the environment and suggests a role for sulfation in next-generation glycopeptide synthesis.

Received 30 August 2012 Accepted 22 October 2012

Published ahead of print 26 October 2012

Address correspondence to Gerard Wright, wrightge@mcmaster.ca.

L.K. and J.P. contributed equally to this article.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JB.01617-12>.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.
doi:10.1128/JB.01617-12

FIG 1 Regulation and mechanism of glycopeptide resistance. Vancomycin binds VanS, causing self-phosphorylation and dimerization. VanS phosphorylates VanR, which activates transcription of *vanHAX*. VanH reduces pyruvate to D-lactate, which is then used as a substrate by the ATP-dependent D-Ala-D-lactate depsipeptide ligase VanA. VanX specifically hydrolyzes the existing D-Ala-D-Ala peptide pool, resulting in the incorporation of peptidoglycan precursors terminating in D-Ala-D-Lac rather than D-Ala-D-Ala at the growing cell wall. This leads to a 1,000-fold decrease in vancomycin binding and high-level resistance.

MATERIALS AND METHODS

Purification of A47934 and DS-A47934. A47934 and its desulfo derivative were purified from *Streptomyces toyocaensis* and *S. toyocaensis* *staL::aac3(IV)* (8) cells after fermentation in Streptomyces Antibiotic Media

(13) for 6 days. The mycelia were harvested, and the glycopeptide antibiotic was extracted with 1 ml of 1% NH_4OH per gram (wet weight) of cell paste. The extract was retained and dried by lyophilization followed by resuspension in distilled water (dH_2O) (1/20), applied to a 26-gauge re-

FIG 2 Structures of glycopeptides used in our study.

verse-phase C₁₈ gold column (Teledyne Isco, Lincoln, NE), and purified by flash chromatography with a linear gradient of 0% to 100% acetonitrile. Fractions were monitored by liquid chromatography-mass spectrometry (LC/MS), and antibiotic-containing fractions were pooled and lyophilized. For A47934, the pooled fractions were applied to a 5.7-gauge SAX anion exchange column (Teledyne Isco, Lincoln, NE), whereas DS-A47934 was applied to a 4.3-gauge reverse-phase C₁₈ column (Teledyne Isco, Lincoln, NE).

ITC. Isothermal titration calorimetry (ITC) was used to monitor the interaction of antibiotics with cell wall precursors as previously described (4), with the following modifications: 1,000 μ M *N,N*-diacetyl-L-Lys-D-Ala-D-Ala tripeptide solution was placed in the calorimetric syringe, and 50 μ M A47934 or DS-A47934 in 20 mM sodium citrate buffer (pH 5.1) was placed in the cell.

Antibiotic activity. MIC values for 3 *Streptomyces* spp. were determined according to guidelines specified by the Clinical and Laboratory Standards Institute (Wayne, PA). *Streptomyces* MICs were determined on solid-phase media in a 96-well plate format inoculated with 2×10^4 spores/well. The plates were incubated at 30°C for 72 h and scored visually. Solid-phase checkerboard assays of *S. coelicolor* and a sampling of 5 vancomycin-resistant environmental actinomycetes isolates chosen at random from our collection of frozen spore stocks were performed using a 1/1,000 dilution. A larger sample size of 23 vancomycin-resistant environmental isolates from frozen spore stocks were then spotted at a 1/1,000 dilution onto Bennet's agar containing teicoplanin at 1 μ g/ml with or without A47934 or DS-A47934 at 0.1 μ g/ml or 0.25 μ g/ml, grown for 72 h at 30°C, and scored visually.

Gene expression analysis. Transcriptional analysis of *vanA* expression in *S. coelicolor* exposed to glycopeptide antibiotics was conducted by quantitative reverse transcription-PCR (qRT-PCR). Cells were inoculated at 2×10^4 onto Bennett's agar containing vancomycin, A47934, and DS-A47934 (all at concentrations of 0.1, 0.25, and 1 μ g/ml) in 20-well plates. Cells were grown at 30°C for 72 h and harvested by gently scraping cells off the surface of the agar. Total RNA was extracted from harvested cells using an RNeasy Mini RNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer's directions for Gram-positive bacteria. Contaminating genomic DNA was removed from RNA samples using RNase-free DNase (Fermentas, Burlington, Ontario, Canada), which was subsequently inactivated by heat/EDTA according to the manufacturer's directions. DNA-free RNA samples were subjected to reverse transcription using a SuperScript variable input, linear output (VILO) cDNA synthesis kit (Invitrogen, Carlsbad, CA). Real-time RT-PCRs were carried out using Phusion High Fidelity DNA polymerase and 1 $\times$ SYBR green I DNA stain (Invitrogen, Carlsbad, CA) in a Bio-Rad C1000 Thermocycler. Cycling conditions were as follows: 98°C for 30 s, 45 cycles of 98°C for 10 s, 54°C for 30 s, and 72°C for 10 s, and 72°C for 10 min. A standard curve was plotted with cycle threshold (Ct) values obtained from amplification of known quantities of cDNAs. The standard curve was used to determine the efficiency (*E*) of primer set binding and amplification using the formula $E = 10^{-1/\text{slope}}$. Comparisons of expression of the *vanA* gene in Bennett's medium without antibiotic (control) and expression in the presence of antibiotic (condition) were quantified using the following formula: ratio = $(E_{\text{vanA}})^{\Delta\text{Ct}(\text{control} - \text{condition})} / (E_{16S \text{ rRNA}})^{\Delta\text{Ct}(\text{control} - \text{condition})}$. The 16S rRNA gene was used as an internal reference. All assays were performed in triplicate with RNA isolated from three independent experiments.

Determination of dimerization constants. Dimerization constants were determined using the method described in reference 15 with the following modifications. Each antibiotic was analyzed on a Bruker microTOF ESI instrument. Serial 2-fold dilutions of each molecule were prepared to give concentrations ranging from 1 μ g/ μ l to 8 ng/ μ l, and the ability to dimerize was recorded at each concentration.

RESULTS AND DISCUSSION

Sulfation does not affect the activity of glycopeptide antibiotics. Evolution of resistance to natural-product antibiotics in the envi-

TABLE 1 Summary of MICs, D-Ala-D-Ala binding constants determined by isothermal titration calorimetry, and dimerization constants for the glycopeptides used in this study

Assay ^a	Value for glycopeptide ^b			
	Vancomycin	DS-A47934	A47934	Teicoplanin
<i>S. coelicolor</i> MIC (μ g/ml)	512	256	512	4
WAC1380 MIC (μ g/ml)	32	8	8	4
WAC1386 MIC (μ g/ml)	32	8	8	4
K_D (μ M)	2.94 ± 5.2	10.4 ± 0.6	4.76 ± 1.6	ND
K_{dim}	$2.7 \times 10^2 \text{ M}^{-1}$	$2.6 \times 10^2 \text{ M}^{-1}$	N.A	$1.6 \times 10^3 \text{ M}^{-1}$

^a K_D , D-Ala-D-Ala binding constant; k_{dim} , dimerization constant.

^b ND, not done; N.A, not applicable.

ronment is assumed to be counteracted by concurrent evolution of new strategies by the producing microorganisms to overcome resistance. Given the recent discovery of glycopeptide sulfotransferases in environmental metagenome sequences (11, 12), we set out to investigate the role of sulfation from an evolutionary perspective in the environment. We chose to use the well-described sulfated glycopeptide A47934 and its desulfo form (DS-A47934) to investigate environmental resistance using *S. coelicolor* as a model organism. *S. coelicolor* is not a producer of any glycopeptide antibiotic but expresses the canonical *vanHAX* resistance cassette and exhibits VanB-type resistance (induction by vancomycin but not teicoplanin).

Sulfation has been shown to decrease the antimicrobial activity of glycopeptides, regardless of the residue sulfated (8, 11, 12). We tested A47934 and DS-A47934 against *S. coelicolor* and two additional vancomycin-resistant (*vanA* positive by PCR) environmental strains (WAC1380 and WAC1386) of the genus *Streptomyces* chosen at random from our in-house collection. Consistent with previous reports, the MICs of A47934 and DS-A47934 were equivalent in WAC1380 and WAC1386 and differed by 2-fold in *S. coelicolor* (Table 1); in other words, sulfation has little effect on antibiotic activity in environmental organisms. To directly assess the affinity of the glycopeptides for their target, we used isothermal titration calorimetry (ITC) to determine the dissociation constant (K_D) of A47934 and DS-A47934 for N_{α},N_{ϵ} -diacetyl-L-Lys-D-Ala-D-Ala. In keeping with our MIC results, K_D values for the sulfated and desulfo forms of the glycopeptide differed only 2-fold to 3-fold (4.76 and 10.4 μ M, respectively; Table 1), indicating no significant differences in affinity for the antibiotic target. ITC is a cell-free system and is therefore unbiased by resistance genotype.

Sulfation blocks induction of glycopeptide resistance. Vancomycin resistance occurs via activation of the histidine kinase VanS and the response regulator VanR and results in transcription of the *vanHAX* operon (4, 5). VanS is variable in its ability to bind different classes of glycopeptides; VanS in "VanA-type" organisms recognizes vancomycin and teicoplanin, while VanS in "VanB-type" organisms recognizes vancomycin but not teicoplanin. VanA-type organisms are therefore resistant to vancomycin and teicoplanin, while VanB-type organisms are sensitive to teicoplanin (16). We hypothesized that glycopeptide sulfation might change the affinity of the antibiotic for VanS and therefore serve as a strategy to overcome resistance. To test our hypothesis, we used the teicoplanin-sensitive vancomycin-resistant VanB-type organism *S. coelicolor*; induction of resistance to teicoplanin in VanB-

FIG 3 Solid-phase checkerboard analysis of *S. coelicolor* with teicoplanin in combination with DS-A47934 and A47934. Vancomycin served as a positive control for VanS activation. The concentration of teicoplanin in each row is indicated on the y axis, and the concentration of DS-A47934, A47934, or vancomycin in each column is indicated on the x axis. Dark coloring indicates growth.

type organisms can be used to indicate VanS binding and activation (17, 18).

Using *S. coelicolor* as an indicator system and the nonsulfated glycopeptide vancomycin as a positive control, we induced teicoplanin resistance at concentrations ranging from 0.1 to 1 µg/ml as previously reported (17, 19) (Fig. 3). DS-A47934 induced teicoplanin resistance at a slightly higher concentration than vancomycin (0.25 µg/ml) but appeared to be readily recognized by a VanB-type VanS. Importantly, the sulfated glycopeptide A47934 did not induce teicoplanin resistance at 0.25 µg/ml but did induce resistance at the next highest concentration tested (1 µg/ml), indicating that the sulfated form of the molecule is a weaker inducer of resistance. The difference between A47934 and DS-A47934 in the ability to induce resistance to teicoplanin is important, given that the only distinction between these two molecules is a single sulfate modification of the phenolic hydroxyl group of the N-terminal 4-HPG.

Glycopeptide sulfation depresses transcription of *vanA*. To directly address the impact of glycopeptide sulfation on induction of resistance, we determined the effect of each antibiotic on levels of *vanA* expression in *S. coelicolor*. As expected, the nonsulfated glycopeptide vancomycin induced *vanA* expression in a dose-dependent manner: 0.1 µg/ml and 1 µg/ml increased expression of *vanA* 1.76-fold and 20.3-fold, respectively (Fig. 4). The nonsulfated glycopeptide DS-A47934 induced the greatest increase in *vanA* expression, with a nearly 70-fold increase compared to the expression seen with the no-drug control in the presence of 0.1 µg/ml DS-A47934. Curiously, 1 µg/ml DS-A47934 increased *vanA* expression only 9-fold. Importantly, in keeping with our hypothesis that sulfation blocks resistance induction, 0.1 and 1 µg/ml A47934 resulted in modest 2.35-fold and 4.32-fold increases in *vanA* gene expression and appeared to be the weakest inducer of resistance gene expression of all three molecules tested. Taken together, these results show not only that the desulfo form of the antibiotic is a more powerful inducer of *vanHAX* than the sulfated form but also that low concentrations of DS-A47934 are more powerful inducers of resistance than higher concentrations (Fig. 4). This result is intriguing in the context of several reports that suggest that antibiotics behave as signaling molecules at low, subinhibitory concentrations (20). An organism that produces a nonsulfated antibiotic such as DS-A47934 risks triggering induc-

FIG 4 qRT-PCR analysis of *S. coelicolor* *vanA* upon exposure to vancomycin, DS-A47934, and A47934. The nonphosphorylated glycopeptides vancomycin and DS-A47934 induced high-level *vanA* expression, as expected. In contrast, A47934 induced only low-level *vanA* expression. DS-A47934 induced the highest levels of *vanA* expression of all three glycopeptides tested when present at a low concentration, which may indicate a signaling effect. Data are normalized to 16S rRNA levels and represent averages of the results of three independent experiments.

tion of resistance in neighboring organisms prematurely. As a result, sulfation becomes an attractive modification to reduce induction of resistance.

Sulfation impairs dimerization of glycopeptide antibiotics.

The differential induction of *vanA* by sulfated and desulfo glycopeptides suggests that sulfation may be interfering with VanS binding. Vancomycin and other type I glycopeptides have been reported to dimerize “back to back” (21, 22), and this property is postulated to enhance their antimicrobial activity. On the other hand, teicoplanin has not been reported to form dimers but the acyl chain imparts favorable properties enhancing activity (23). This difference prompted us to explore whether dimer formation correlates with induction of resistance.

Mass spectrometry was used to determine the glycopeptide dimerization constants (K_{dim}) (Table 1; see also Fig. S1 in the supplemental material). Dimers of the nonsulfated glycopeptide vancomycin were readily quantified, and at higher concentrations the molecule appeared to oligomerize, forming higher-order complexes. DS-A47934 exhibited properties similar to those of vancomycin, but the sulfated antibiotic A47934 remained a monomeric species even at concentrations reaching 4 mg/ml (see Fig. S1 in the supplemental material). “Back-to-back” and “face-to-face” dimers resulting in tetramers have been described in the literature for vancomycin (21) and balhimycin (24), supporting our observations. While the full effect of dimerization on antibiotic action is still not fully understood, in the context of our studies the formation of higher-order oligomers ($n > 2$) correlates with activation of VanS.

Sulfation masks resistance in VanB-type environmental isolates. Our observations support our hypothesis that sulfation downregulates induction of resistance in *S. coelicolor*, but is this phenomenon apparent in other species of *Streptomyces* that are vancomycin resistant? Strains from a collection of VanB-type environmental strains isolated in our laboratory from soil samples from geographically distinct locations were tested for induction of teicoplanin resistance using A47934 or DS-A47934. Antibiotic checkerboard assays were performed initially on a subset of 5 iso-

lates to determine test concentrations, which were found to be identical to those established for *S. coelicolor* (Fig. 3). We then expanded our sample size to include a total of 23 vancomycin-resistant environmental strains. Importantly, 52% (12/23) of the strains exhibited a phenotype consistent with our results in *S. coelicolor*; DS-A47934 induced resistance to teicoplanin at a lower concentration than A47934 (0.1 µg/ml DS-A47934 versus 0.25 µg/ml A47934). Of the remaining strains tested, we found that 31% (7/23 strains) were susceptible to teicoplanin and that neither A47934 nor DS-A47934 was capable of inducing resistance at the concentrations tested. Both A47934 and DS-A47934 were able to induce resistance to teicoplanin at the lowest concentration tested in 17% of strains (4/23; resistance induced at 0.1 µg/ml). These results support our hypothesis that sulfation of glycopeptide antibiotics blocks induction of antibiotic resistance and suggests that this phenomenon is present in many environmental actinomycetes.

Conclusions. In this study, we have elucidated a previously unknown role of glycopeptide sulfation in the environment. Sulfation does not affect the binding affinity of the molecule to its target D-Ala-D-Ala, but inducible resistance mediated by *vanA* is significantly repressed in the environmental model organism *S. coelicolor* with the addition of a sulfate molecule. Furthermore, addition of sulfate appears to block dimerization of the glycopeptide which may also result in altered binding to the VanS sensor peptide loop.

The glycopeptides remain one of the most important classes of antibiotics used clinically. The continual expansion of this antibiotic scaffold is important as resistance inevitably arises. The explosion in available microbial genomes and metagenomes has led to the identification of new glycopeptide biosynthetic clusters with tailoring enzymes that can be exploited to create new chemical diversity. Blocking resistance rather than overcoming the mechanism of resistance is a novel approach for fighting resistant organisms. Understanding the molecular interactions involved in induction of resistance is the first step in overcoming said resistance. Using nature's toolbox of glycopeptide-modifying enzymes provides an excellent starting point both for understanding natural evolutionary forces in environmental antibiotic-producing organisms and for the future engineering of novel therapeutics.

ACKNOWLEDGMENTS

This research was funded by a Canadian Institutes of Health Research (CIHR) grant (MT-14981) and by a Canada Research Chair in Biochemistry (to G.W.). L.K. and J.P. are recipients of CIHR Fellowships.

REFERENCES

- Arthur M, Molinas C, Bugg TD, Wright GD, Walsh CT, Courvalin P. 1992. Evidence for in vivo incorporation of D-lactate into peptidoglycan precursors of vancomycin-resistant enterococci. *Antimicrob. Agents Chemother.* 36:867–869.
- Bugg TD, Wright GD, Dutka-Malen S, Arthur M, Courvalin P, Walsh CT. 1991. Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. *Biochemistry* 30: 10408–10415.
- Bugg TD, Dutka-Malen S, Arthur M, Courvalin P, Walsh CT. 1991. Identification of vancomycin resistance protein VanA as a D-alanine:D-alanine ligase of altered substrate specificity. *Biochemistry* 30:2017–2021.
- Koteva K, Hong HJ, Wang XD, Nazi I, Hughes D, Naldrett MJ, Buttner MJ, Wright GD. 2010. A vancomycin photoprobe identifies the histidine kinase VanS as a vancomycin receptor. *Nat. Chem. Biol.* 6:327–329.
- Wright GD, Holman TR, Walsh CT. 1993. Purification and characterization of VanR and the cytosolic domain of VanS: a two-component regulatory system required for vancomycin resistance in *Enterococcus faecium* BM4147. *Biochemistry* 32:5057–5063.
- Kahne D, Leimkuhler C, Lu W, Walsh C. 2005. Glycopeptide and lipoglycopeptide antibiotics. *Chem. Rev.* 105:425–448.
- Nicolaou KC, Boddy CN, Brase S, Winssinger N. 1999. Chemistry, biology, and medicine of the glycopeptide antibiotics. *Angew. Chem. Int. Ed. Engl.* 38:2096–2152.
- Lamb SS, Patel T, Koteva KP, Wright GD. 2006. Biosynthesis of sulfated glycopeptide antibiotics by using the sulfotransferase StaL. *Chem. Biol.* 13:171–181.
- Pootoolal J, Thomas MG, Marshall CG, Neu JM, Hubbard BK, Walsh CT, Wright GD. 2002. Assembling the glycopeptide antibiotic scaffold: the biosynthesis of A47934 from *Streptomyces toyocaensis* NRRL15009. *Proc. Natl. Acad. Sci. U. S. A.* 99:8962–8967.
- Skelton NJ, Williams DH. 1990. Structure elucidation of the novel glycopeptide antibiotic UK-68,597. *J. Org. Chem.* 55:3718–3723.
- Banik JJ, Brady SF. 2008. Cloning and characterization of new glycopeptide gene clusters found in an environmental DNA megalibrary. *Proc. Natl. Acad. Sci. U. S. A.* 105:17273–17277.
- Banik JJ, Craig JW, Calle PY, Brady SF. 2010. Tailoring enzyme-rich environmental DNA clones: a source of enzymes for generating libraries of unnatural natural products. *J. Am. Chem. Soc.* 132:15661–15670.
- Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA. 2000. *Practical streptomyces genetics*, 2nd ed. John Innes Foundation, Norwich, England.
- Reference deleted.
- Mirgorodskaya OA, Olsufyeva EN, Kolumbe DE, Joergensen TJD, Røpstorff P, Pavlov AY, Miroschnikova OV, Preobrazhenskaya MN. 2000. The dimerization of semisynthetic eremomycin derivatives studied by the electrospray ionization mass spectrometry and its effect on their antibacterial activity. *Russ. J. Bioorg. Chem.* 26:566–574.
- Baptista M, Depardieu F, Courvalin P, Arthur M. 1996. Specificity of induction of glycopeptide resistance genes in *Enterococcus faecalis*. *Antimicrob. Agents Chemother.* 40:2291–2295.
- Hong HJ, Hutchings MI, Hill LM, Buttner MJ. 2005. The role of the novel Fem protein VanK in vancomycin resistance in *Streptomyces coelicolor*. *J. Biol. Chem.* 280:13055–13061.
- Hong H-J, Hutchings MI, Neu JM, Wright GD, Paget MSB, Buttner MJ. 2004. Characterization of an inducible vancomycin resistance system in *Streptomyces coelicolor* reveals a novel gene (*vanK*) required for drug resistance. *Mol. Microbiol.* 52:1107–1121.
- Hong HJ, Hutchings MI, Buttner MJ. 2008. Vancomycin resistance VanS/VanR two-component systems. *Adv. Exp. Med. Biol.* 631:200–213.
- Goh EB, Yim G, Tsui W, McClure J, Surette MG, Davies J. 2002. Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. *Proc. Nat. Acad. Sci. U. S. A.* 99:17025–17030.
- Nitanai Y, Kikuchi T, Kakoi K, Hanamaki S, Fujisawa I, Aoki K. 2009. Crystal structures of the complexes between vancomycin and cell-wall precursor analogs. *J. Mol. Biol.* 385:1422–1432.
- Schäfer M, Schneider TR, Sheldrick GM. 1996. Crystal structure of vancomycin. *Structure* 4:1509–1515.
- Tesarová E, Tuzar Z, Nesmerak K, Bosakova Z, Gas B. 2001. Study on the aggregation of teicoplanin. *Talanta* 54:643–653.
- Lehmann C, Bunkoczi G, Vertesy L, Sheldrick GM. 2002. Structures of glycopeptide antibiotics with peptides that model bacterial cell-wall precursors. *J. Mol. Biol.* 318:723–732.