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Editor: Rossolini | Section: Mechanisms of Resistance | Designation: T

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Antibiotic Resistance Mechanisms Inform Discovery: Identification and Characterization of a Novel *Amycolatopsis* Strain Producing Ristocetin

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Discovering new antibiotics is a major scientific challenge, made increasingly urgent by the continued development of resistance in bacterial pathogens. A fundamental understanding of the mechanisms of bacterial antibiotic resistance will be vital for the future discovery or design of new, more effective antibiotics. We have exploited our intimate knowledge of the molecular mechanism of glycopeptide antibiotic resistance in the harmless bacterium *Streptomyces coelicolor* to develop a new two-step cell wall bioactivity screen, which efficiently identified a new actinomycete strain containing a previously uncharacterized glycopeptide synthetic gene cluster. The screen first identifies natural product extracts capable of triggering a generalized cell wall stress response and then specifically selects for glycopeptide antibacterials by assaying for the induction of glycopeptide resistance genes. In this study, we established a diverse natural product extract library from actinomycete strains isolated from locations with widely varying climates and ecologies, and we screened them using the novel two-step bioassay system. The bioassay ultimately identified a single strain harboring the previously unidentified biosynthetic gene cluster for the glycopeptide ristocetin, providing a proof of principle for the effectiveness of the screen. This is the first report of the ristocetin biosynthetic gene cluster, which is predicted to include some interesting and previously uncharacterized enzymes. By focusing on screening libraries of microbial extracts, this strategy provides the certainty that identified producer strains are competent for growth and biosynthesis of the detected glycopeptide under laboratory conditions.

Recent years have seen ever increasing numbers of cases of infections caused by drug-resistant bacteria, creating an urgent AQ: A need for new and improved antibiotics (1). Unfortunately, the levels of traditional antibiotic research and development have decreased during this period, leading to reductions in the antibiotic discovery pipeline and a developing public health crisis. Understanding how antibiotics can fail to be active is vital for the development of future novel antimicrobials. Such information is often linked intimately to the drug's mode of action and therefore can provide unique insights that can be used to help discover and design novel compounds or to develop new ways of prolonging the therapeutic usefulness of existing drugs. Such efforts can be assisted by recent advances in genetic engineering and synthetic biology approaches that can facilitate the targeted modification of existing antibiotic structures (2); however, these techniques rely on the supply of a diverse range of well-characterized antibiotic scaffold-modifying enzymes to be called upon to specifically expand the chemical diversity of the natural structures available. The discovery of new enzyme activities can therefore be seen as an important goal in itself, in addition to the larger goal of identifying novel natural antibiotics.

The bacterial cell wall is an important and validated target for antibacterial chemotherapy; it is crucial for bacterial cell growth because it provides a physical protective barrier between the cell and its environment, and it is an important mediator of innate immune responses during bacterial infections. Antibiotics that inhibit bacterial cell wall biosynthesis are therefore clinically important in the treatment of infectious diseases. Understanding bacterial cell wall biosynthesis has been the subject of intensive study from the time of the discovery of penicillin, the first clinically available antibiotic targeting peptidoglycan biosynthesis, up to the present day, in analyses of the distinct mode of action of glycopeptide antibiotics such as vancomycin. Vancomycin inhibits the completion of bacterial cell wall peptidoglycan biosynthesis by noncovalently binding to the terminus of the growing peptidoglycan, D-alanyl–D-alanine (D-Ala-D-Ala) (3). For more than 50 years, vancomycin has been reserved as an antibiotic of last resort for the treatment of infectious diseases caused by Gram-positive bacteria, particularly methicillin-resistant *Staphylococcus aureus* (MRSA). In common with the other antibiotics in mainstream use, however, resistance to glycopeptides has spread through bacterial populations, with the first clinical isolates of vancomycin-resistant enterococci (VRE) being identified in the 1980s and vancomycin-resistant *S. aureus* (VRSA) emerging at the beginning of AQ: B the new millennium (4). It was soon revealed that these resistant

Received 14 May 2014 Returned for modification 15 June 2014
Accepted 6 July 2014
Published ahead of print 14 July 2014

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.03349-14.

doi:10.1128/AAC.03349-14

strains have responded to the challenge of vancomycin through replacement of the D-Ala-D-Ala terminus of peptidoglycan precursors with D-alanyl-D-lactate (D-Ala-D-Lac), which results in an approximately 1,000-fold lower binding affinity for vancomycin and consequently renders the modified bacteria resistant to the antibiotic (3). This peptidoglycan remodeling requires expression of at least three genes, i.e., *vanH*, *vanA*, and *vanX*. Transcription of these genes is usually regulated by VanS and VanR, two-component system regulatory proteins, and genes encoding these proteins are often found adjacent to *vanHAX*.

Research into the mechanism of glycopeptide antibiotic resistance initially focused on pathogenic bacterial strains (5–7), but the complexity of the resistance system uncovered soon led to speculation that the resistance genes might have originated from an antibiotic-producing organism, where they had coevolved for self-protection. *Streptomyces coelicolor* is a model organism for the actinomycetes, the organisms responsible for the production of two-thirds of clinically important antibiotics (8). It does not synthesize any glycopeptide antibiotics but it does possess a vancomycin resistance cluster (*vanRSJKHAX*) (9). *S. coelicolor* has consequently emerged as a safe and convenient model system for the *in vivo* study of glycopeptide resistance, and it has provided detailed information on the specific mechanisms of resistance and how these are integrated with the more-generalized mechanisms of bacterial cell wall homeostasis (9–17). This information has allowed us to develop some simple but effective bioassay tools for identification of antimicrobial agents that are active against cell wall biosynthesis, and here we report their use to screen a large library of natural product extracts for novel glycopeptide antibiotics.

MATERIALS AND METHODS

Bacterial strains, media, oligonucleotides, and growth conditions. Bacterial strains, plasmids, oligonucleotides, and media for this study are described in Tables S1 to S3 in the supplemental material. Except where described here, media and culture conditions were as described previously (18).

Construction of natural product extract library. To establish the extract library, soil samples were collected from diverse sites around southeastern Asia, focusing on environments with unusual weather conditions and ecologies, e.g., alpine, tropical, polar, and desert regions. One gram of each soil sample was suspended in 10 ml of 0.85% NaCl solution and serially diluted. The serial dilutions were spread on humic acid-vitamin (HV) agar (19) to isolate actinomycete strains (see Table S2 in the supplemental material). After incubation at 30°C for 7 days, any single colony displaying representative actinomycete morphology was isolated by streaking on modified Bennett's (BN) agar or Gauze's no. 1 (GN1) agar (20) (see Table S3 in the supplemental material). This also provided the opportunity to confirm the actinomycete colony morphology. After incubation at 30°C for an additional 7 days, the surface of each plate was scraped with sterile cotton wool soaked in 20% glycerol solution. The cotton wool was then squeezed into an empty sterile tube to collect the filtered spores or mycelium suspension of the selected actinomycete strain; samples were stored at −80°C.

For the preparation of crude metabolite extracts, 0.5 ml of the spore (or aerial mycelium) glycerol stock solution of each isolate was inoculated into 30 ml each of three different liquid media, i.e., Bennett's (BN) medium, glucose-soybean starch (GSS) medium, and dextrin-yeast-corn steep liquor (DYC) medium (see Table S3 in the supplemental material). After incubation at 30°C and 280 rpm for 6 days in a 250-ml sterile flask containing a 30-cm coiled stainless steel spring baffle, each culture broth was centrifuged for 5 min at 1,500 × *g*. The supernatant was poured into 30 ml of ethyl acetate and extracted for 5 min by vigorous vortex-mixing. Following phase separation, the ethyl acetate layer was evaporated to dryness and redissolved in 3 ml methanol. The aqueous phase was mildly heated (60°C) for 30 min (to remove ethyl acetate remaining in the aqueous phase), freeze-dried, and then redissolved in 3 ml water. The cell pellet was extracted with 3 ml of methanol by vigorous vortex-mixing for 20 min and then was centrifuged for 5 min at 1,500 × *g* to remove cellular debris. Thirty-microliter aliquots of each extract were added to 96-well plates, freeze-dried, and stored at 4°C. Each actinomycete isolate consequently generated nine different extracts. The full extract library currently consists of approximately 150,000 natural product extracts from over 15,000 different actinomycete strains. The library, the Extract Collection of Useful Microorganisms (ECUM), is further described at www.ecum.or.kr.

Screening of extract library and isolation of MJM2582. Each freeze-dried actinomycete extract aliquot from the 96-well plates was dissolved in 30 μl dimethyl sulfoxide (DMSO), and 10 μl was used for the screening assay. For the first-round screening using the *sigEp-neo* fusion reporter system, approximately 10^7 spores of *S. coelicolor* M600 carrying pIJ6880 (a multicopy promoter probe plasmid, pIJ486, carrying *sigEp-neo* fusion DNA) (see Table S1 in the supplemental material) were spread on minimal medium plus casamino acids, glucose, and tiger milk (MMCGT) agar plates (12 cm by 12 cm) containing 100 μg/ml kanamycin; 6-mm sterile paper discs impregnated with the 10-μl DMSO extract samples were applied to the surface of the freshly spread plates, and activity was scored after incubation at 30°C for 2 to 4 days.

Positive inducers of the *sigE* promoter raise the level of expression of the *neo* gene and hence produce a halo of kanamycin-resistant growth around the paper disc. The number of isolates tested for the first-round screening was over 5,300, with ~260 strains (about 5%) giving positive responses. These positive extracts were subjected to a second round of screening using the Δ*femX* mutant strain to identify extracts containing glycopeptide antibiotics capable of inducing *van* gene expression (explained in detail in Results). Approximately 10^7 spores of *S. coelicolor* Δ*femX* mutant strain J3130 (see Table S1 in the supplemental material) were spread on MMCGT agar containing no antibiotic, and 10-μl DMSO solutions of the selected inducer extracts were applied on paper discs to the plates. Plates were incubated at 30°C for 2 to 4 days. Positive inducers produced a halo of glycopeptide-dependent growth of the *S. coelicolor* Δ*femX* mutant strain around the disc.

A single strain, designated *Amycolatopsis* species MJM2582, was isolated from the 260 strains tested in the first round of screening. Among the 9 different extracts from MJM2582 that were tested, very clear induction halos were observed for all extracts prepared from the aqueous and methanol phases. In contrast, negligible halos were observed for extracts prepared from the ethyl acetate layer. This is consistent with the water solubility and organic solvent insolubility of glycopeptide antibiotics.

Genomic DNA extraction, sequencing, and annotation. The genomic DNAs of MJM2582 and *Amycolatopsis lurida* were isolated by a modification of the Kirby mix procedure, according to the method described previously (18). Each strain was cultured in 10 ml tryptic soy broth (TSB) (Oxoid) at 30°C at 280 rpm for 2 days. The harvested mycelia were washed twice with an equal volume of 10.3% sucrose and then were lysed in 1 ml of lysozyme solution (2 mg/ml lysozyme in 10.3% sucrose, 25 mM Tris [pH 8], 25 mM EDTA [pH 8]) by incubation at 30°C for 10 min. The lysate was extracted with 1 ml of 2× Kirby mixture (2 g sodium triisopropylnaphthalene sulfonate, 12 g sodium 4-amino-salicylate, 5 ml 2 M Tris-HCl [pH 8], and 6 ml phenol mixture, made up to 100 ml with water), 2 ml of phenol-chloroform solution (phenol/chloroform, 1:1 [vol/vol], equilibrated with 0.1 M Tris [pH 8]), and 1 ml of phenol-chloroform. The nucleic acids in the upper phase were transferred to a fresh tube and precipitated for 10 min at room temperature with 1/10 volume of 3 M sodium acetate (pH 6) and an equal volume of isopropanol. The pellet of nucleic acids obtained by centrifugation was then resuspended in RNase solution (50 μg/ml RNase I in 500 μl Tris-EDTA [TE] buffer) and incubated for 30 min at 37°C to remove all RNAs. The genomic DNA sample was cleaned by extraction with 200 μl of phenol-chloroform and isopro-

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panol precipitation as described above. The DNA pellet obtained after centrifugation was washed with 70% ethanol and dissolved in TE buffer solution (25 mM Tris [pH 7.5], 2 ml EDTA).

Whole-genome sequencing was performed by the DNA sequencing facility at the Department of Biochemistry, University of Cambridge, using a combined Illumina MiSeq and Roche 454 sequencing approach. Assembly using the 454 GS De Novo Assembler (v.2.8) produced a draft sequence of 9.3 Mb in 149 contigs. Anti-SMASH 2.0 (http://www.secondarymetabolites.org) (21, 22) was used to identify a single glycopeptide gene cluster in each genomic sequence. Sequence comparison studies with previously reported glycopeptide biosynthetic clusters, i.e., teicoplanin, balhimycin, A47934, chloroeremomycin, dalbavancin, and vancomycin, were performed using the ACT web comparison tool (23). Artemis (24) and Glimmer 3.02 (25) were used for prediction and annotation of open reading frames (ORFs) in the cluster sequence.

Extraction and purification of glycopeptide antibiotics. Aerial mycelia (or spores) of each glycopeptide-producing strain of interest (*Amycolatopsis* species MJM2582, *Amycolatopsis lurida*, *Streptomyces toyocaensis*, and *Amycolatopsis balhimycina*) (see Table S1 in the supplemental material) were prepared from 7-day cultures on soy flour-mannitol (SFM) agar or BN agar at 30°C (see Table S2 in the supplemental material). MJM2582 was tested for preparative glycopeptide production in media previously reported for the production of A47934 (26), teicoplanin (27), vancomycin (28), and ristocetin (29). Bioassays using *S. coelicolor* Δ*femX* and *S. coelicolor* Δ*vanRS* mutant strains showed that the only medium that reliably yielded glycopeptides was *Streptomyces* antibiotic medium (SAM), used for A47934 biosynthesis (26). Seed cultures of MJM2582 were prepared by transferring mycelia (or spores) to 25 ml glucose-yeast extract-malt extract (GYM) liquid medium (20) in a 250-ml Erlenmeyer flask containing a 30-cm stainless steel spring wire and incubating the mixture at 30°C for 48 h with shaking at 250 rpm (see Table S3 in the supplemental material); 2.5 ml of dense seed culture was then used to inoculate 50 ml SAM in 250-ml Erlenmeyer flasks containing spring wire baffles. After incubation at 30°C for 6 days with shaking at 250 rpm, the culture broth was centrifuged and the cell pellet was extracted with 1% NH_4OH (1 ml per 1 g wet pellet). This mixture was centrifuged, and the alkaline supernatant was harvested and neutralized with HCl. This pellet extract was used for initial bioassay screening, and a similar process was used to obtain glycopeptide extracts from *S. toyocaensis* and *A. balhimycina*.

The MJM2582 glycopeptide was further purified by combining the pellet extract with the culture supernatant, which was then applied to Diaion HP20 resin. The resin was washed with a stepwise gradient of water and methanol (50 to 80% methanol) to elute the bound glycopeptides. Fractions containing glycopeptides were pooled and further purified using a D-Ala-D-Ala affinity resin. This was prepared and used as described previously (30) and yielded a mixture of glycopeptides. Individual glycopeptides were obtained by preparative high-performance liquid chromatography (HPLC) (Agilent 1200 system) using a Phenomenex Luna $C_{18}(2)$ column (250 mm by 21.2 mm; particle size, 10 μm) eluted with a linear gradient of 5 to 95% acetonitrile (plus 0.1% trifluoroacetic acid [TFA]) in water (plus 0.1% TFA) over 35 min, with a flow rate of 20 ml/min. UV detection was performed at 280 nm. AQ: D

Additional antibiotic bioassays. All other antibiotic susceptibility, resistance, and induction tests using *S. coelicolor* strains were performed on minimal medium plus casamino acids, glucose, and tiger milk (MMCGT) agar (see Table S2 in the supplemental material). Approximately 10^7 spores of *S. coelicolor* strains were spread on square plates (12 cm by 12 cm), or 10^5 spores were spread on round plates (9-cm diameter); 10 to 30 μl of antibiotic solution (containing 30 μg antibiotic) was soaked into sterile 6-mm paper discs, which were applied to the freshly spread spore lawns, and the plates were incubated at 30°C for 2 to 4 days before scoring. For antibiotic susceptibility tests using *Bacillus subtilis*, *Enterococcus faecalis*, and *Staphylococcus aureus*, plates were prepared by mixing a 200-μl aliquot from an overnight liquid culture of each strain with 20 ml of just-molten LB soft agar (18) and setting at room temperature. Plates were scored after incubation at 37°C for 18 h. For bioassays using antibiotic solution made from commercially available antibiotic powder, 30 μg of antibiotic was applied. For bioassays performed using glycopeptide antibiotics extracted from producer strains, 20 μl of a neutralized NH_4OH extract solution was used. All tested antibiotics were purchased from either Sigma-Aldrich or Oxoid.

Mass spectrometric analysis of glycopeptide antibiotics. Glycopeptide antibiotics were analyzed by liquid chromatography-mass spectrometry (LC-MS) (Agilent 1100 HPLC system coupled to a Thermo Scientific LCQ instrument with an electrospray ionization [ESI] source in positive-ion mode) using a Phenomenex Luna $C_{18}(2)$ column (250 mm by 2.0 mm; particle size, 5 μm) eluted with a linear gradient of 5 to 95% acetonitrile (plus 0.1% TFA) in water (plus 0.1% TFA) over 25 min, with a flow rate of 0.3 ml/min. UV detection was performed at 280 nm. The major components after D-Ala-D-Ala resin purification were found at *m/z* 1,773.3, 1,759.3, 1,034.3, and 1,027.3. High-resolution mass spectrometry was performed with a Thermo Scientific LTQ Orbitrap instrument and yielded the major component with an isotopic pattern of *m/z* 1,034.3386, 1,034.8396, 1,035.3406, 1,035.8416, and 1,036.3429. This is identical to the pattern generated from authentic ristocetin A ($[M+2H]^{2+}$; $C_{95}H_{112}N_8O_{44}^{2+}$), i.e., *m/z* 1,034.3385, 1,034.8395, 1,035.3404, 1,035.8415, and 1,036.3427.

Nucleotide sequence accession numbers. The DNA sequences of the clusters from MJM2582 and *A. lurida* were deposited in the GenBank database under accession numbers KF882511 and KJ364518, respectively. AQ:E-F

RESULTS

Extensive screening of microbial extract library using novel two-step bioassay system, with identification of *Amycolatopsis* strain producing glycopeptide antibiotic. For this study, we initially established a geographically diverse collection of actinomycete strains. Each actinomycete strain was then cultured in three different liquid media and extracted with three different solvents, generating a total of nine different natural product extracts. A randomly selected subset of this library, covering ~5,300 actinomycete isolates, was screened using a two-step procedure, i.e., an initial bioassay using a *sigEp-neo* fusion reporter strain to identify extracts capable of triggering a generalized cell wall stress response (10), followed by a growth assay using a Δ*femX* mutant strain to identify specific glycopeptide antibiotic activity (11, 12) (Fig. 1A). F1 *sigE* encodes an extracytoplasmic function (ECF) sigma factor, σ^E, which is part of a signal transduction system that senses and responds to cell wall stress and therefore is required for normal cell wall integrity in *S. coelicolor* (31). Expression of *sigE* is induced by a wide variety of agents that stress the cell wall, and wild-type *S. coelicolor* harboring a multicopy *sigEp-neo* fusion reporter plasmid (pIJ6880) (see Table S1 in the supplemental material), in which the aminoglycoside phosphotransferase gene (*neo*) is expressed under the control of the *sigE* promoter, exhibits kanamycin-resistant growth only in the presence of such compounds (Fig. 1B). About 5% of the tested extracts strongly activated expression of the *sigEp-neo* fusion reporter, and these were subjected to a second round of screening using a Δ*femX* mutant bioassay system. FemX adds a single branched glycine to the stem pentapeptide of peptidoglycan precursors during cell wall biosynthesis and is essential for growth under normal conditions. During exposure to vancomycin, however, induction of the *vanH*, *vanA*, and *vanX* genes remodels peptidoglycan biosynthesis to produce precursors with pentapeptide chains terminating with D-Ala-D-Lac instead of D-Ala-D-Ala. This modification confers resistance to vancomycin, but FemX cannot efficiently use D-Ala-D-Lac-containing precur-

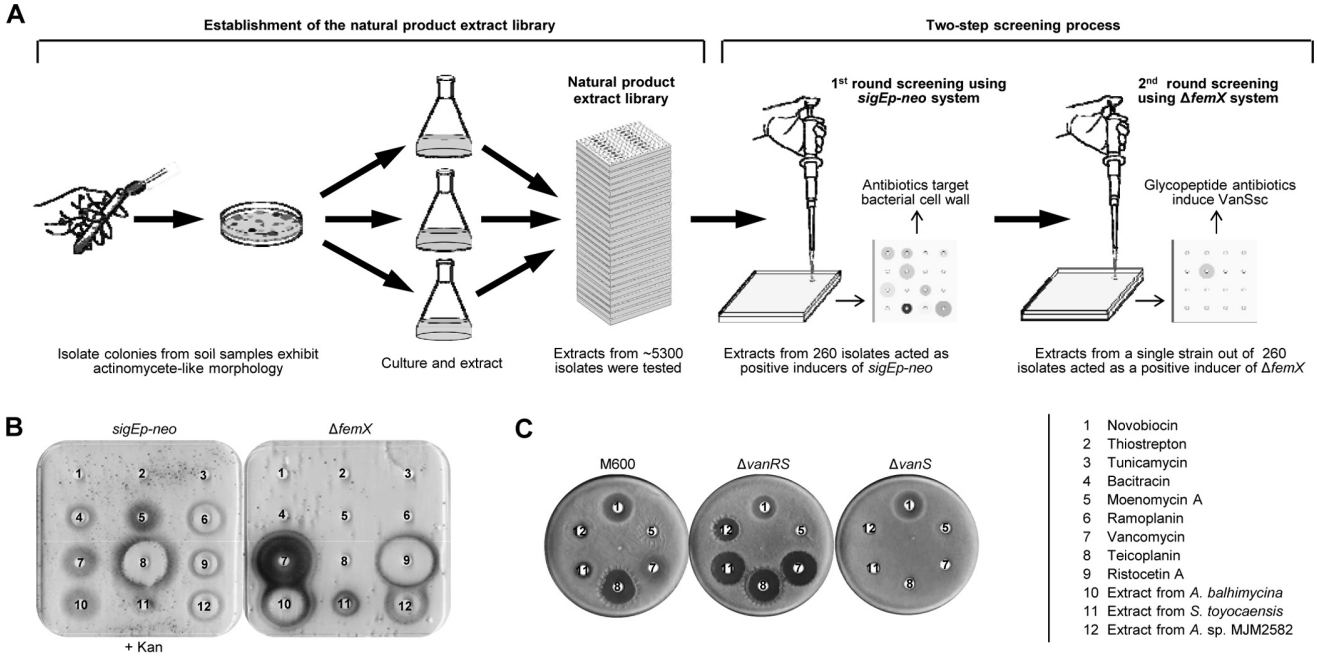


FIG 1 Screening for novel glycopeptide antibiotics. (A) Schematic diagram illustrating the application of a two-step cell wall bioactivity screen to a natural product extract library. Each isolated actinomycete strain was cultured in three different liquid media and extracted with three different solvents, creating a total of nine extracts per strain. Extracts from approximately 5,300 different isolates were tested with the first-round *sigEp-neo* fusion reporter system, and 260 different isolates gave extracts that acted as strong inducers. Extracts from these 260 isolates were then subjected to a second round of screening using Δ*femX* to identify glycopeptide antibiotics, yielding a single positive isolate that was designated MJM2582. (B) Plates demonstrating the responses of the *sigEp-neo* and Δ*femX* reporter systems to antibiotics. Antibiotics that target DNA gyrase (novobiocin), the ribosome (thiostrepton), or early peptidoglycan biosynthesis (tunicamycin) do not induce the *sigEp-neo* system, while those that target late peptidoglycan biosynthesis (bacitracin, moenomycin A, ramoplanin, vancomycin, teicoplanin, and ristocetin) do. The amounts of novobiocin, thiostrepton, and tunicamycin used in the bioassays (30 μg each) are sufficient to produce a halo of growth inhibition when assayed against *S. coelicolor*. Extracts obtained from known glycopeptide-producing strains (*A. balhimycina* and *S. toyocaensis*) and from MJM2582 also induced the *sigEp-neo* system. Only glycopeptide antibiotics (but not teicoplanin) or extracts containing glycopeptides, including MJM2582, acted AQ: L as inducers in the Δ*femX* bioassay. Kan, kanamycin. (C) Bioassay showing the spectrum of activity of the MJM2582 extract against glycopeptide-sensitive (Δ*vanRS* mutant), constitutively resistant (Δ*vanS* mutant), and inducibly resistant (M600) strains of *S. coelicolor*.

sors as substrates; under these growth conditions, this function is instead performed via induction of the homologous enzyme VanK. Therefore, FemX becomes nonessential provided that the *van* genes are expressed (11). As a consequence, the Δ*femX* mutant strain is viable in the presence of vancomycin but nonviable in its absence, which makes growth of the Δ*femX* mutant strain itself a simple drug-dependent bioassay system for screening specifically for glycopeptide antibiotics (Fig. 1B). Of the 260 strains with extracts producing positive responses in the first round of screening, extracts from a single isolate were found to strongly induce Δ*femX* mutant strain growth in the glycopeptide bioassay. Susceptibility tests using vancomycin-sensitive and -resistant (inducible and constitutive) bacterial strains provided further evidence for the presence of a glycopeptide antibiotic in the positive extract (Fig. 1C; see also Fig. S1 in the supplemental material). The 16S rRNA gene sequence of the producing strain was next determined as a filter to guard against the rediscovery of known glycopeptide-producing strains, and the analysis indicated a previously unidentified *Amycolatopsis* species, which we designated *Amycolatopsis* F2 species MJM2582 (Fig. 2; see also Fig. S2 in the supplemental material).

Determination that *Amycolatopsis* species MJM2582 produces ristocetin. To fully characterize the glycopeptide produced by MJM2582, a draft genomic sequence was produced to access the genes in the biosynthetic cluster, and culture extracts were analyzed using LC-MS. Comparative analyses using sequences from a number of previously reported glycopeptide biosynthetic clusters, including teicoplanin (32), balhimycin (33), A47934 (34), dalbavancin (35), and vancomycin (36), indicated that MJM2582 carries a novel ~79-kb gene cluster consisting of 39 ORFs (Fig. 3; see also Fig. S3 and Table S4 in the supplemental F3 material). LC-MS analysis identified a number of related glycopeptides produced by MJM2582. A single component eluting at

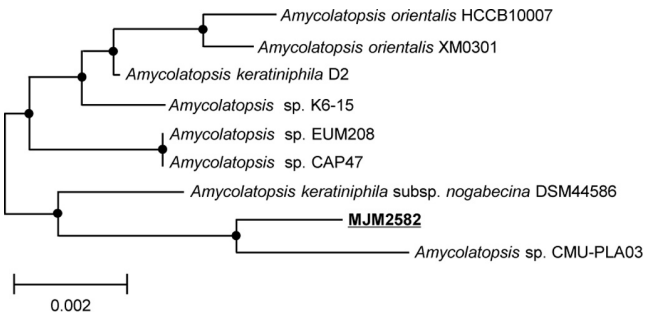
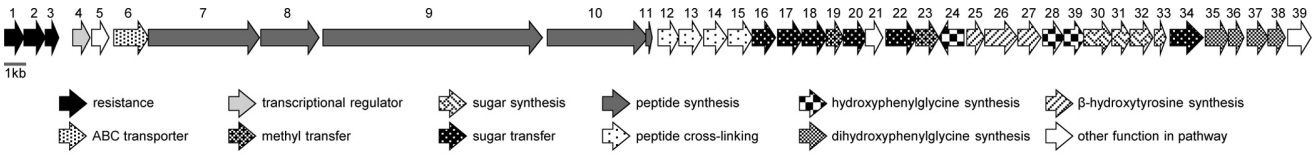


FIG 2 Phylogenetic analysis of 16S RNA sequences of MJM2582. The result indicates that MJM2582 is a previously unidentified *Amycolatopsis* species. Scale bar, distance in substitutions per nucleotide (see also Fig. S2 in the supplemental material).



1	D-lactate dehydrogenase	11	MtbH-like protein	21	inactive deacetylase	31	4-ketoreductase
2	D-alanine:D-lactate ligase	12	p450 monooxygenase	22	mannosyltransferase	32	C-3 aminotransferase
3	D-alanyl-D-alanine dipeptidase	13	p450 monooxygenase	23	methyltransferase	33	C-5 epimerase
4	StrR family transcriptional regulator	14	p450 monooxygenase	24	phenylglycine aminotransferase	34	mannosyltransferase
5	prephenate dehydrogenase	15	p450 monooxygenase	25	α/β hydrolase	35	type III polyketide synthase (DpgA)
6	ABC transporter	16	glycosyltransferase	26	NRPS AT didomain for β-hydroxytyrosine synthesis	36	dehydratase (DpgB)
7	NRPS module 1-2	17	glycosyltransferase	27	p450 monooxygenase	37	dioxygenase (DpgC)
8	NRPS module 3	18	glycosyltransferase	28	4-hydroxyphenylpyruvate dioxygenase	38	dehydratase (DpgD)
9	NRPS module 4-6	19	methyltransferase	29	phenylglycolate oxidase	39	putative aldolase
10	NRPS module 7	20	glycosyltransferase	30	NDP-hexose 2,3-dehydratase		

FIG 3 Genetic organization of the glycopeptide gene cluster identified in *Amycolatopsis* species MJM2582. Arrows, predicted ORFs, numbered consecutively. The predicted enzymatic function encoded by each ORF is listed. A detailed comparison with previously reported glycopeptide clusters can be found in Fig. S3 and Table S4 in the supplemental material. ABC, ATP-binding cassette.

9.0 min (*m/z* 1,034) is identical to an authentic standard of ristocetin A ($[M+2H]^{2+}$, *m/z* 1,034), indicating that the predicted structure of the glycopeptide from MJM2582 corresponds to ristocetin A (Fig. 4; see also Fig. S4 and S5 in the supplemental material). The other compounds are consistent with *O*-desmethyl-ristocetin A ($[M+2H]^{2+}$, *m/z* 1,027), ristocetin B ($[M+H]^{+}$, *m/z* 1,773), and *O*-desmethyl-ristocetin B ($[M+H]^{+}$, *m/z* 1,759) (Fig. 5; see also Fig. S6 in the supplemental material). This is the first report of the biosynthetic gene cluster for ristocetin and identifies a second producer strain for this glycopeptide, which had previously been isolated from *Amycolatopsis lurida* (37). Genomic sequencing of *A. lurida* showed that it carries a gene cluster with an identical organization and 91% nucleotide identity.

Analysis of ristocetin gene cluster. In comparison with previously characterized glycopeptide clusters, the ristocetin cluster has a number of interesting and distinctive features (see Fig. S3 and Table S4 in the supplemental material). It possesses six genes encoding glycosyltransferase (GT) enzymes, which is more than any previously reported cluster and reflects the complex pattern of glycosylation in the antibiotic produced. Phylogenetic analysis of the GTs indicates the presence of four class B and two class C enzymes, which handle the attachment of the D-arabinose, D-glucose, L-rhamnose, L-ristosamine, and two D-mannose sugars to the ristocetin aglycone (see Fig. S7 in the supplemental material). Bacterial class C GTs catalyze mannosylation using undecaprenylphosphomannose as a donor, whereas class B GTs utilize a wide variety of nucleoside diphosphate (NDP)-activated sugars (38). The sugars required for ristocetin production can all be accessed from generalized bacterial metabolism with the notable exception of L-ristosamine, a rare dideoxy sugar that requires dedicated genes in the cluster for its biosynthesis. We propose a route to TDP-activated L-ristosamine via the activity of the enzymes encoded by ORFs 30 to 33 in the cluster (Fig. 6). These are homologous to genes in the chloroeremomycin cluster that produce the related sugar epivancosamine (39), a methylated derivative of L-ristosamine that additionally requires a 3-*C*-methyltransferase (MT) that is absent from the ristocetin cluster. Another unusual feature of ristocetin is its methylation pattern, with *O*-methylation of the carboxyl terminus of the core peptide and *C*-methylation of (*S*)-3,5-dihydroxyphenylglycine (Dpg) at amino acid position 3. Accordingly, two MTs are encoded in the gene cluster (Fig. 3). ORF 19 shares significant homology with VEG18, which catalyzes the *C*-terminal *O*-methylation of a glycopeptide aglycone (40), while ORF 23 does not share homology with any previously characterized glycopeptide MTs and therefore is a candidate for the *C*-methylation of the ristocetin aglycone (see Table S4 in the supplemental material). This modification is unique among characterized glycopeptide pathways.

The four nonribosomal peptide synthetase (NRPS) proteins in the ristocetin gene cluster have a domain organization and predicted adenylation domain specificity similar to those of the teicoplanin NRPS and assemble a peptide from β-hydroxytyrosine, (*S*)-4-hydroxyphenylglycine (Hpg), and Dpg (Fig. 7; see also Table S5 in the supplemental material) (41). The rigid teicoplanin-like ristocetin aglycone is then predicted to be generated by four cytochrome P450 enzymes encoded by adjacent genes (ORFs 12 to 15), which in previously characterized pathways are known to form oxidative cross-links between the aromatic amino acid side

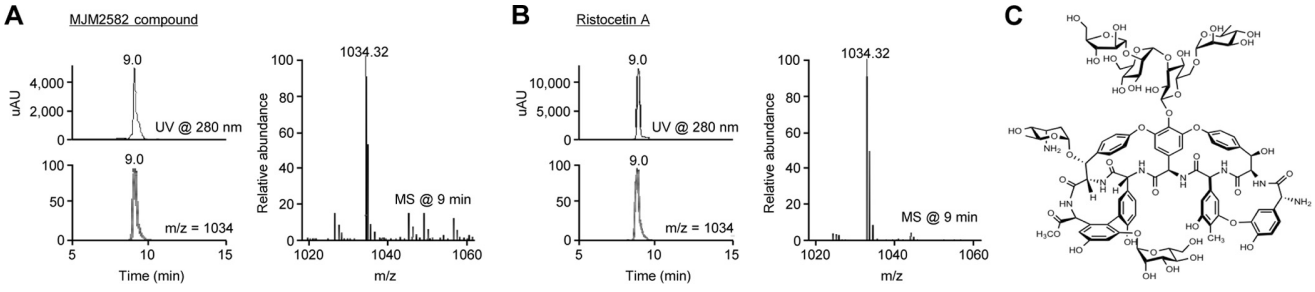


FIG 4 (A and B) LC-MS analysis of the purified glycopeptide extracted from MJM2582 (A) in comparison with authentic ristocetin A (B). (C) Predicted structure of the glycopeptide from MJM2582, corresponding to ristocetin A.

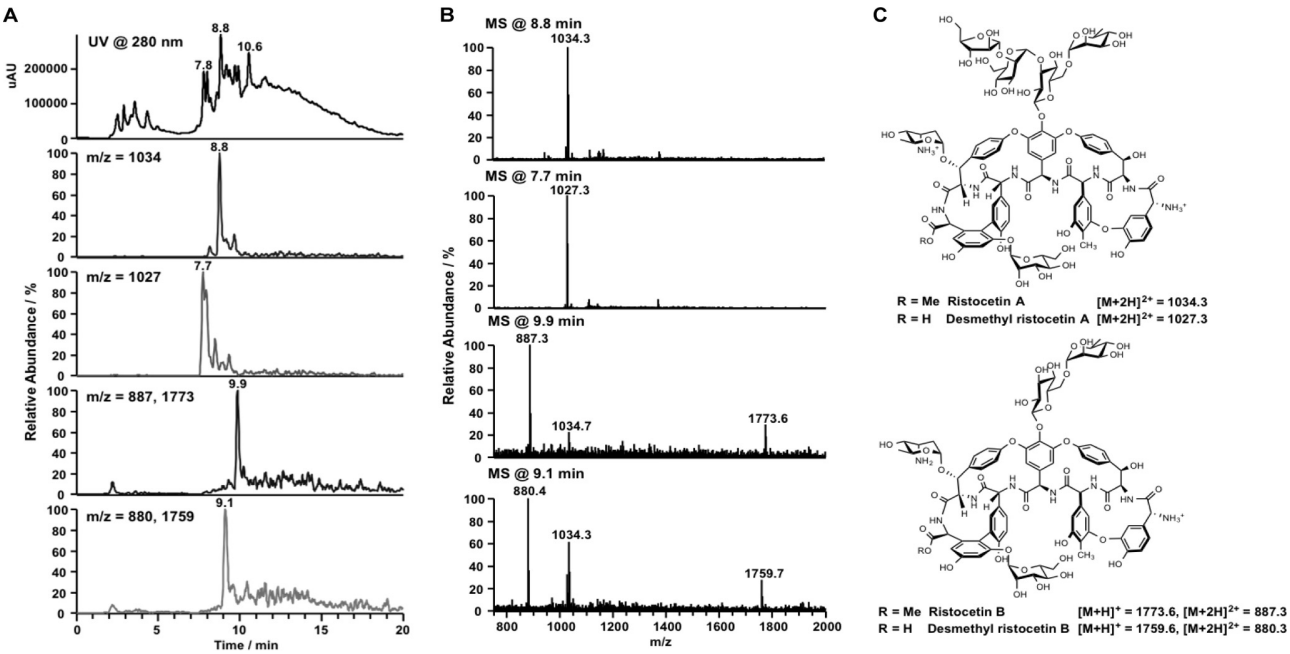


FIG 5 LC-MS analysis of the array of glycopeptides produced by MJM2582, following partial purification using HP20 resin. (A) UV chromatogram for the extract and selective ion monitoring spectra for glycopeptides. (B) Mass spectra for ristocetin derivatives. The spectrometer was tuned to the $[M+2H]^{2+}$ ion of teicoplanin, to facilitate the identification of compounds above 2,000 Da. (C) Structures and exact masses of ristocetin A and B and their corresponding desmethyl derivatives.

chains (32). Interestingly, while the organization of the NRPS into four proteins with a module distribution of 2:1:3:1 is a feature common to teicoplanin-like (type IV) glycopeptides, the organization of the tailoring enzymes is much more closely related to vancomycin-like (type I) glycopeptides. Furthermore, while the biosynthesis of Dpg and Hpg is conserved in the production of teicoplanin-like and vancomycin-like glycopeptides, distinct routes have evolved for β-hydroxytyrosine biosynthesis, with a single nonheme dioxygenase being used for the former (42) and three separate enzymes for the latter (43). Ristocetin employs the same route as vancomycin (ORFs 25 to 27), despite a teicoplanin-like aglycone. Ristocetin is the only nonchlorinated glycopeptide antibiotic reported to date and, unsurprisingly, the gene cluster does not encode a halogenase.

The 5′ end of the ristocetin gene cluster is marked by the presence of the *vanHAX* glycopeptide resistance system required for reprograming cell wall peptidoglycan biosynthesis (Fig. 3). However, no genes encoding the VanR-VanS two-component regulatory system were identified anywhere in the MJM2582 and *S. lurida* genomes, implying that *vanHAX* expression may be constitutive in this strain or otherwise controlled in a VanR/VanS-independent manner. Interestingly, alignment of the *vanHAX* promoter sequence from the ristocetin gene cluster with similar sequences from other glycopeptide gene clusters identified conserved nucleotide changes in those with no obvious VanR-VanS control system, in comparison with those known to be regulated by VanR and VanS (Fig. 8). This implies that the putatively VanR/VanS-independent promoters may share the same method of regulation.

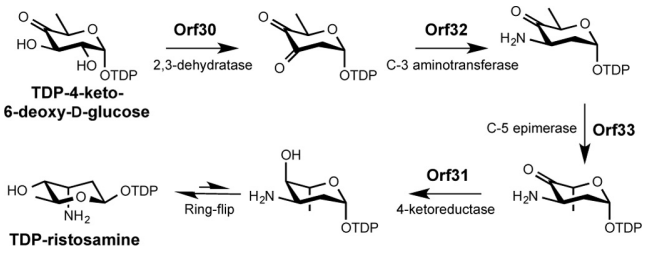


FIG 6 Predicted formation of TDP-ristosamine. The route to TDP-ristosamine was proposed based on homology of proteins encoded by ORFs 30 to 33 in the ristocetin gene cluster to the proteins previously characterized in the TDP-epivancosamine pathway (39).

DISCUSSION

We have identified the previously unknown ristocetin gene cluster, encoding interesting new enzyme activities, using a two-step screening system developed from an understanding of the detailed molecular mechanisms of glycopeptide resistance in *S. coelicolor*. Thaker et al. recently reported the discovery of the novel glycopeptide pekiskomycin using growth in the presence of vancomycin as an initial screen to enrich for glycopeptide-producing strains derived from soil samples (44). A significant proportion of the vancomycin-resistant strains (89/100 strains) isolated in this way did not contain glycopeptide biosynthetic clusters, however, and it was necessary to include an additional PCR-based screen to identify those that did. The Δ*femX* mutant bioassay employed in this work proved highly specific for compounds that induce expression of the *van* resistance genes, and a further discriminatory screen was not required. Thaker et al. elegantly exploited the results of their PCR screen to produce phylogenetic fingerprints for dereplication, discriminating against the rediscovery of known

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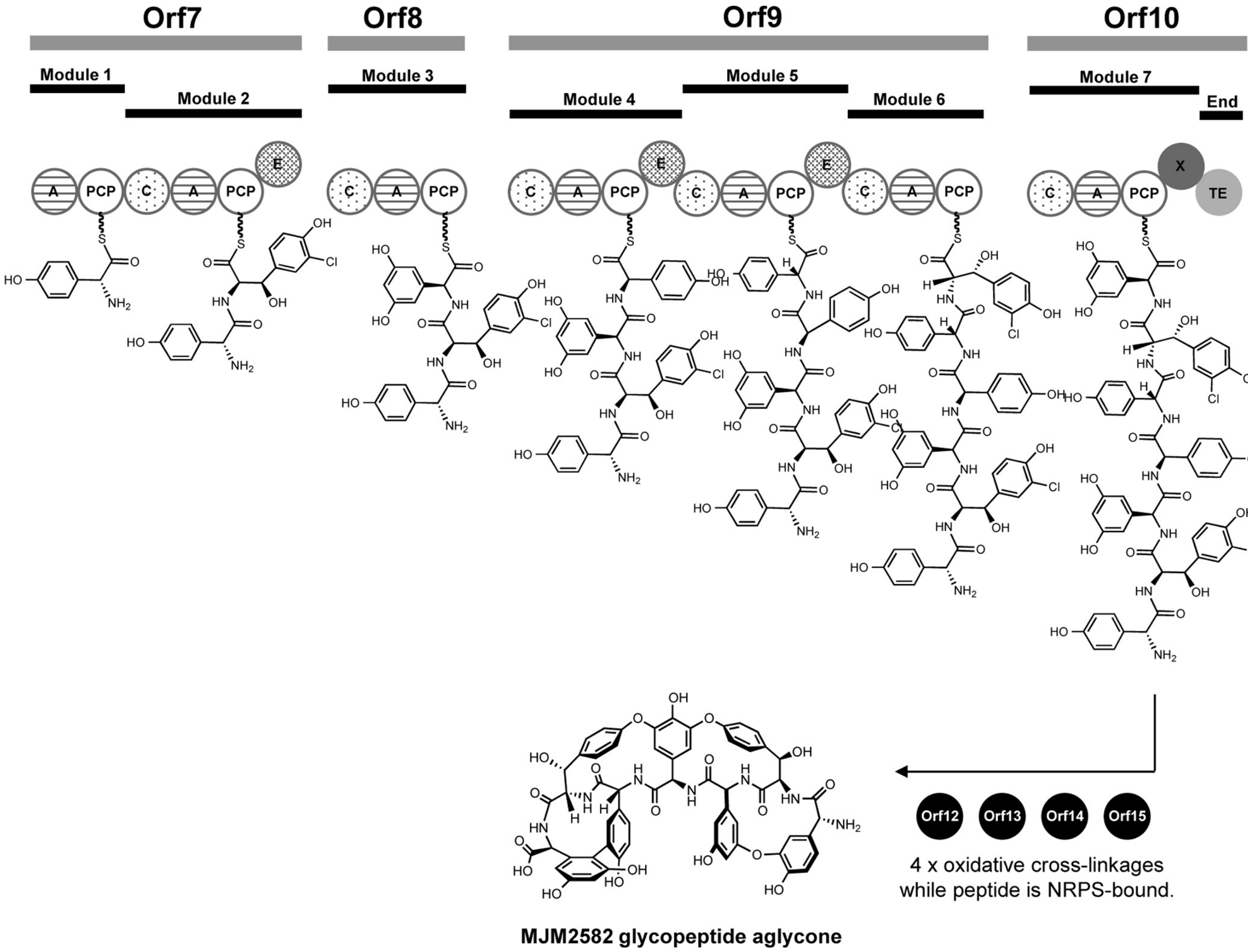

FIG 7 Schematic diagram showing the organization of the four NRPSs from the ristocetin gene cluster. The X domain is a nonfunctional condensation-like domain that is present in all glycopeptide NRPSs (41).

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strains and compounds, and we think that the two different screens can be highly complementary. Although more limited in scope, both also offer significant advantages over the screening of metagenomic libraries of DNA from soil for natural product biosynthetic clusters, for which the disconnection between the producing microbes and the bioactivity of the molecules that are ultimately being sought introduces a number of formidable technical challenges. Our focused approach has the advantage that any

```
                                                          -35                            -10
Sc  vanJp  ------TCGCCACATATCGTCGGCATATCGAAAACCGCATACGTGCCGGCAACACGCTGCCGCGTTCACTGGGCGT-ATGCGT
    vanKp  ------CCGGAACATATCGTCGGCGTATCGAAAACCGCATACGCGTCGGCAACGTCCCGCCGCCTTGACTGGGCGC-ATGGCC
    vanHp  ------AGGTAATATATCGTCAGGATATCGAAAAGCGCATACGGGACGGCAACACCGAGGCGCCTTGAATAGAGGC-ATGACC
St  vanHp  ------GCGGGGCGTATCGCGGGCATATCGAAAAGCACATACGTTCCGGCAACAGCACTTCCCCTTGACTGGAGGT-ATGACC
At  vanHp  GGGCCCCTCCGACGTATCGTCGGCATATCCAAAACCGCATACGTGCTCGCAACACCACCTTCCCTTGACTGGGCGC-ATGGGT
Ab  vanYp  -----------ACATATCGTCGGCATATGGAAAATCGCATACGTGCCGGCAACACATCGCCGACTTGAATGGACAC-ATGACC
Ao  vanHp  ------TGGTAACTTATTGTCGGGGCATCGAAAACCCCATAAGAAGCGGCAACAACGCGCTGCCTACAATGTCGGGCATGACC
Al  vanHp  ------TGGTAACTTATTGTCGAGGCCTCGAAAACCCCATAAGAAGCGGCAACATCGCGCTGCCTACAATGTCGGGCATGACC
MJM vanHp  ------TGGTAACTTATTGTCGGGGCATCGAAAACCCCATAAGAAGCGGCAACAACGCGCTGCCTACAATGTCGGGCATGACC
```

FIG 8 Alignment of *van* promoter sequences present in the glycopeptide antibiotic clusters from *Streptomyces coelicolor* (Sc), *Streptomyces toyocaensis* (St), *Actinoplanes teichomyceticus* (At), *Amycolatopsis balhimycina* (Ab), *Amycolatopsis orientalis* (Ao), *Amycolatopsis lurida* (Al), and *Amycolatopsis* species MJM2582 (MJM). The highly conserved sequences represent putative VanR response regulator binding sites in the −35 and −10 promoter regions (boxed). The top four strains (*S. coelicolor*, *S. toyocaensis*, *A. teichomyceticus*, and *A. balhimycina*) carry glycopeptide resistance clusters that include genes encoding a VanR-VanS two-component system, while the bottom three strains (*A. orientalis*, *A. lurida*, and *Amycolatopsis* species MJM2582) lack an obvious VanR-VanS system. Identified nucleotide sequence differences between these two groups are highlighted.

positive screen hit *a priori* comes from an actinomycete strain that is both culturable and competent for the biosynthesis of the detected antibiotic activity under laboratory conditions.

We envision that the direct relationships between producing organism, genomic sequence, and antibiotic activity afforded by our screening procedure will facilitate both the isolation of new glycopeptide antibiotics and the provision of novel verified enzyme activities for use in synthetic biology approaches to explore the production and design of modified antibiotic structures. In addition, the extracts that were identified as active in the first round of *sigEp-neo* screening but were inactive in the subsequent glycopeptide screen, which were not considered further in this work, provide an enriched library from which to seek other classes of natural products active against the bacterial cell wall. The native *S. coelicolor* VanS sensor is not induced by teicoplanin-like compounds, and we are currently developing hybrid sensors to broaden the range of glycopeptide structures that can be detected. Only extracts that produced positive responses in the *sigEp-neo* screen would need to be reanalyzed in the improved hybrid screens.

ACKNOWLEDGMENTS

This work was supported by funding from the Royal Society (United Kingdom) (grant 516002.K5877/ROG), the Medical Research Council (United Kingdom) (grant G0700141), and St. John's College, University of Cambridge.

We thank Andy Hesketh (University of Cambridge, Systems Biology Centre) for helpful discussions and comments on the manuscript.

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REFERENCES

1. **Levy S, Marshall B.** 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nat. Med. **10**(Suppl):S122–S129. http://dx.doi.org/10.1038/nm1145.
2. **Wohlleben W, Mast Y, Muth G, Röttgen M, Stegmann E, Weber T.** 2012. Synthetic biology of secondary metabolite biosynthesis in actinomycetes: engineering precursor supply as a way to optimize antibiotic production. FEBS Lett. **586:**2171–2176. http://dx.doi.org/10.1016/j.febslet.2012.04.025.
3. **Leclercq R, Derlot E, Duval J, Courvalin P.** 1988. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. N. Engl. J. Med. **319:**157–161. http://dx.doi.org/10.1056/NEJM198807213190307.
4. **Pearson H.** 2002. 'Superbug' hurdles key drug barrier. Nature **418:**469–470. http://dx.doi.org/10.1038/418469b.
5. **Billot-Klein D, Gutmann L, Bryant D, Bell D, Van Heijenoort J, Grewal J, Shlaes DM.** 1996. Peptidoglycan synthesis and structure in *Staphylococcus haemolyticus* expressing increasing levels of resistance to glycopeptide antibiotics. J. Bacteriol. **178:**4696–4703.
6. **Arthur M, Reynolds P, Courvalin P.** 1996. Glycopeptide resistance in enterococci. Trends Microbiol. **4:**401–407. http://dx.doi.org/10.1016/0966-842X(96)10063-9.
7. **Courvalin P.** 2005. Genetics of glycopeptide resistance in Gram-positive pathogens. Int. J. Med. Microbiol. **294:**479–486. http://dx.doi.org/10.1016/j.ijmm.2004.10.002.
8. **Bentley SD, Chater KF, Cerdeño-Tárraga AM, Challis GL, Thomson NR, James KD, Harris DE, Quail MA, Kieser H, Harper D, Bateman A, Brown S, Chandra G, Chen CW, Collins M, Cronin A, Fraser A, Goble A, Hidalgo J, Hornsby T, Howarth S, Huang CH, Kieser T, Larke L, Murphy L, Oliver K, O'Neil S, Rabbinowitsch E, Rajandream MA, Rutherford K, Rutter S, Seeger K, Saunders D, Sharp S, Squares R, Squares S, Taylor K, Warren T, Wietzorrek A, Woodward J, Barrell BG, Parkhill J, Hopwood DA.** 2002. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature **417:**141–147. http://dx.doi.org/10.1038/417141a.
9. **Hong H-J, Hutchings MI, Neu JM, Wright GD, Paget MS, Buttner MJ.** 2004. Characterisation of an inducible vancomycin resistance system in *Streptomyces coelicolor* reveals a novel gene (*vanK*) required for drug resistance. Mol. Microbiol. **52:**1107–1121. http://dx.doi.org/10.1111/j.1365-2958.2004.04032.x.
10. **Hong H-J, Paget MSB, Buttner MJ.** 2002. A signal transduction system in *Streptomyces coelicolor* that activates the expression of a putative cell wall glycan operon in response to vancomycin and other cell wall-specific antibiotics. Mol. Microbiol. **44:**1199–1211. http://dx.doi.org/10.1046/j.1365-2958.2002.02960.x.
11. **Hong H-J, Hutchings MI, Hill L, Buttner MJ.** 2005. The role of the novel Fem protein VanK in vancomycin resistance in *Streptomyces coelicolor*. J. Biol. Chem. **280:**13055–13061. http://dx.doi.org/10.1074/jbc.M413801200.
12. **Hutchings MI, Hong H-J, Buttner MJ.** 2006. The vancomycin resistance VanRS signal transduction system of *Streptomyces coelicolor*. Mol. Microbiol. **59:**923–935. http://dx.doi.org/10.1111/j.1365-2958.2005.04953.x.
13. **Hutchings MI, Hong H-J, Leibovitz E, Sutcliffe IC, Buttner MJ.** 2006. The σ^E cell envelope stress signal of *Streptomyces coelicolor* is influenced by a novel lipoprotein, CseA. J. Bacteriol. **188:**7222–7229. http://dx.doi.org/10.1128/JB.00818-06.
14. **Koteva K, Hong H-J, Wang XD, Nazi I, Hughes D, Naldrett MJ, Buttner MJ, Wright GD.** 2010. A vancomycin photoprobe identifies the histidine kinase VanSsc as a vancomycin receptor. Nat. Chem. Biol. **6:**327–329. http://dx.doi.org/10.1038/nchembio.350.
15. **Hesketh A, Hill C, Mokhtar J, Novotna G, Tran N, Bibb M, Hong H-J.** 2011. Genome-wide dynamics of a bacterial response to antibiotics that target the cell envelope. BMC Genomics **12:**226. http://dx.doi.org/10.1186/1471-2164-12-226.
16. **Novotna G, Hill C, Vicent K, Liu C, Hong H-J.** 2012. A novel membrane protein, VanJ, conferring resistance to teicoplanin. Antimicrob. Agents Chemother. **56:**1784–1796. http://dx.doi.org/10.1128/AAC.05869-11.
17. **Kwun MJ, Novotna G, Hesketh AR, Hill L, Hong H-J.** 2013. In vivo studies suggest that induction of VanS-dependent vancomycin resistance requires binding of the drug to D-Ala-D-Ala termini in the peptidoglycan cell wall. Antimicrob. Agents Chemother. **57:**4470–4480. http://dx.doi.org/10.1128/AAC.00523-13.
18. **Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA.** 2000. Practical *Streptomyces* genetics. The John Innes Foundation, Norwich, United Kingdom.
19. **Masayuki H, Hideo N.** 1987. Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. J. Ferment. Technol. **65:**501–509. http://dx.doi.org/10.1016/0385-6380(87)90108-7.
20. **Atlas RM.** 2004. Handbook of microbiological media. CRC Press, Boca Raton, FL.
21. **Medema MH, Blin K, Cimermancic P, de Jager V, Zakrzewski P, Fischbach MA, Weber T, Takano E, Breitling R.** 2011. antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. Nucleic Acids Res. **39:**W339–W346. http://dx.doi.org/10.1093/nar/gkr466.
22. **Blin K, Medema MH, Kazempour D, Fischbach MA, Breitling R, Takano E, Weber T.** 2013. antiSMASH 2.0: a versatile platform for genome mining of secondary metabolite producers. Nucleic Acids Res. **41:**W204–W212. http://dx.doi.org/10.1093/nar/gkt449.
23. **Carver T, Berriman M, Tivey A, Patel C, Böhme U, Barrell BG, Parkhill J, Rajandream MA.** 2008. Artemis and ACT: viewing, annotating and comparing sequences stored in a relational database. Bioinformatics **24:**2672–2676. http://dx.doi.org/10.1093/bioinformatics/btn529.
24. **Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA, Barrell B.** 2000. Artemis: sequence visualization and annotation. Bioinformatics **16:**944–945. http://dx.doi.org/10.1093/bioinformatics/16.10.944.
25. **Delcher AL, Bratke KA, Powers EC, Salzberg SL.** 2007. Identifying bacterial genes and endosymbiont DNA with Glimmer. Bioinformatics **23:**673–679. http://dx.doi.org/10.1093/bioinformatics/btm009.
26. **Neu JM, Wright GD.** 2001. Inhibition of sporulation, glycopeptide antibiotic production and resistance in *Streptomyces toyocaensis* NRRL 15009 by protein kinase inhibitors. FEMS Microbiol. Lett. **199:**15–20. http://dx.doi.org/10.1111/j.1574-6968.2001.tb10644.x.
27. **Lee JC, Park HR, Park DJ, Son KH, Yoon KH, Kim YB, Kim CJ.** 2003. Production of teicoplanin by a mutant of *Actinoplanes teicomyceticus*. Biotechnol. Lett. **25:**537–540. http://dx.doi.org/10.1023/A:1022842203917.
28. **McIntyre JJ, Bull AT, Bunch AW.** 1996. Vancomycin production in batch and continuous culture. Biotechnol. Bioeng. **49:**412–420.
29. **Philip JE, Forest L, Schenck JR.** 1961. Preparation of ristocetin A salts. US patent 2990329A.

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30. **Holding AN, Spencer JB.** 2008. Investigation into the mechanism of phenolic couplings during the biosynthesis of glycopeptide antibiotics. ChemBioChem **9:**2209–2214. http://dx.doi.org/10.1002/cbic.200800303.
31. **Paget MSB, Emmanuelle L, Buttner MJ.** 1999. A putative two-component signal transduction system regulates σ^E, a sigma factor required for normal cell wall integrity in *Streptomyces coelicolor* A3(2). Mol. Microbiol. **33:**97–107. http://dx.doi.org/10.1046/j.1365-2958.1999.01452.x.
32. **Li TL, Huang F, Haydock SF, Mironenko T, Leadlay PF, Spencer JB.** 2004. Biosynthetic gene cluster of the glycopeptide antibiotic teicoplanin: characterization of two glycosyltransferases and the key acyltransferase. Chem. Biol. **11:**107–119. http://dx.doi.org/10.1016/j.chembiol.2004.01.001.
33. **Shawky RM, Puk O, Wietzorrek A, Pelzer S, Takano E, Wohlleben W, Stegmann E.** 2007. The border sequence of the balhimycin biosynthesis gene cluster from *Amycolatopsis balhimycina* contains *bbr*, encoding a StrR-like pathway-specific regulator. J. Mol. Microbiol. Biotechnol. **13:**76–88. http://dx.doi.org/10.1159/000103599.
34. **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. http://dx.doi.org/10.1073/pnas.102285099.
35. **Sosio M, Stinchi S, Beltrametti F, Lazzarini A, Donadio S.** 2003. The gene cluster for the biosynthesis of the glycopeptide antibiotic A40926 by *Nonomuraea* species. Chem. Biol. **10:**541–549. http://dx.doi.org/10.1016/S1074-5521(03)00120-0.
36. **Jeong H, Sim YM, Kim HJ, Lee DW, Lim SK, Lee SJ.** 2013. Genome sequence of the vancomycin-producing *Amycolatopsis orientalis* subsp. *orientalis* strain KCTC 9412T. Genome Announc. **1:**e00408–e00413. http://dx.doi.org/10.1128/genomeA.00408-13.
37. **Grundy WE, Sinclair AC, Theriault RJ, Goldstein AW, Rickher CJ, Warren HB, Jr., Oliver TJ, Sylvester JC.** 1956–1957. Ristocetin, microbiologic properties. Antibiot. Annu. 687–692.
38. **Coutinho PM, Deleury E, Davies GJ, Henrissat B.** 2003. An evolving hierarchical family classification for glycosyltransferases. J. Mol. Biol. **328:**307–317. http://dx.doi.org/10.1016/S0022-2836(03)00307-3.
39. **Chen H, Thomas MG, Hubbard BK, Losey HC, Walsh CT, Burkart MD.** 2000. Deoxysugars in glycopeptide antibiotics: enzymatic synthesis of TDP-L-epivancosamine in chloroeremomycin biosynthesis. Proc. Natl. Acad. Sci. U. S. A. **97:**11942–11947. http://dx.doi.org/10.1073/pnas.210395097.
40. **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. http://dx.doi.org/10.1073/pnas.0807564105.
41. **Donadio S, Sosio M, Stegmann E, Weber T, Wohlleben W.** 2005. Comparative analysis and insights into the evolution of gene clusters for glycopeptide antibiotic biosynthesis. Mol. Genet. Genomics **274:**40–50. http://dx.doi.org/10.1007/s00438-005-1156-3.
42. **Stinchi S, Carrano L, Lazzarini A, Feroggio M, Grigoletto A, Sosio M, Donadio S.** 2006. A derivative of the glycopeptide A40926 produced by inactivation of the β-hydroxylase gene in *Nonomuraea* sp. ATCC39727. FEMS Microbiol. Lett. **256:**229–235. http://dx.doi.org/10.1111/j.1574-6968.2006.00120.x.
43. **Mulyani S, Egel E, Kittel C, Turkanovic S, Wohlleben W, Süssmuth RD, van Pée KH.** 2010. The thioesterase Bhp is involved in the formation of β-hydroxytyrosine during balhimycin biosynthesis in *Amycolatopsis balhimycina*. ChemBioChem **11:**266–271. http://dx.doi.org/10.1002/cbic.200900600.
44. **Thaker MN, Wang W, Spanogiannopoulos P, Waglechner N, King AM, Medina R, Wright GD.** 2013. Identifying producers of antibacterial compounds by screening for antibiotic resistance. Nat. Biotechnol. **31:**922–927. http://dx.doi.org/10.1038/nbt.2685.