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

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ABSTRACT

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 that 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 mechanism, then subsequently specifically selects for glycopeptide antibacterials by assaying for induction of glycopeptide resistance genes. In this study, we have established a diverse natural product extract library from actinomycetes strains isolated from locations with widely varying climates and ecologies and 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 the biosynthesis of the detected glycopeptide under laboratory conditions.

INTRODUCTION

Recent years have seen ever increasing numbers of cases of infections caused by drug-resistant bacteria, creating an urgent need for new and improved antibiotics (1). Unfortunately, traditional antibiotic research and development has decreased during this period leading to a reduction in the antibiotic discovery pipeline and a developing public health crisis. Understanding how antibiotics can fail to be active is vital knowledge 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 ones. Such efforts can be assisted by recent advances in genetic engineering and synthetic biology approaches that will facilitate the targeted modification of existing antibiotic structures (2). These techniques however 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, alongside 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 by providing a physical protective barrier between the cell and its environment, and is an important mediator of the innate immune response 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 non-covalently binding to the teminus of 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). However, in common with the other antibiotics in mainstream use, resistance to glycopeptides has spread through bacterial populations, with the first clinical isolates of vancomycin-resistant Enterococci (VRE) being identified in the 1980's, and vancomycin-resistant MRSA (VRSA) emerging at the beginning of the new millennium (4). It was soon revealed that these resistant strains have responded to the challenge of vancomycin by replacement of the D-Ala-D-Ala terminus of peptidoglycan precursors with D-alanyl-D-lactate (D-Ala-D-Lac) resulting in an approximately 1000-fold lower binding affinity for vancomycin and consequently rendering the modified bacteria resistant to the antibiotic (3). This peptidoglycan remodelling requires expression of at least three genes, *vanHAX*. Transcription of *vanHAX* 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 the pathogenic bacterial strains (5-7), but the complexity of the resistance system uncovered soon led to the speculation that the resistance genes may have originated from an antibiotic producing organism where they had co-evolved for self-protection. *Streptomyces coelicolor* is a model organism for the actinomycetes, the genus responsible for the production of the two-thirds of clinically important antibiotics (8). It does not synthesize any glycopeptide antibiotics but 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 has provided a detailed understanding both of the specific mechanisms of resistance and how these are integrated with the more generalized mechanisms for bacterial cell wall homeostasis (9-17). This knowledge has allowed us to develop some simple but effective bioassay tools for identifying antimicrobial agents that are

active against cell wall biosynthesis, and here we report their use to screen a large natural product extract library 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-S3 respectively. Except where described here, media and culture conditions were as given previously (18).

Construction of the natural product extract library.

To establish the extract library, soil samples were first collected from diverse sites around south-eastern 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 medium (19) to isolate actinomycete strains (see Table S2 in the supplemental material). After incubation at 30 °C for 7 days any single colony displaying a representative actinomycete morphology was isolated by streaking on modified Bennett's (m-BN) agar medium or Gauze's No. 1 (GN1) agar medium (20) (see Table S3). This also provided the opportunity to further 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 and stored at -80 °C.

For the preparation of the crude metabolite extracts, 0.5 ml of spore (or aerial mycelium) glycerol stock solution of each isolate was inoculated in 30 ml each of three different liquid media; Bennett's (BN) medium, Glucose Soybean Starch (GSS) medium, and Dextrin Yeast Corn steep liquor (DYC) medium (Table S3). 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 vortexing vigorously. Following phase separation, the ethyl acetate layer was evaporated to dryness and re-dissolved in 3 ml methanol. The aqueous phase was mildly heated (60 °C) for 30 min to remove ethyl acetate remained in aqueous phase and freeze-dried, then re-dissolved in 3 ml water. The cell pellet was extracted with 3 ml of methanol by vigorous vortexing for 20 min then centrifuged for 5 min at 1,500 g to remove cell debris. 30 µl aliquots of each extract were added to 96 well plates, freeze dried and stored at 4 °C. Each actinomycete isolate consequently generates 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 has been give the acronym ECUM (Extract Collection of Useful Microorganisms) and is further described at www.ecum.or.kr.

Screening the 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 per 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 multi-copy, promoter probe plasmid, pIJ486, carrying *sigEp-neo* fusion DNA; see Table S1 in the supplemental material) were spread on 12 cm × 12 cm MMCGT agar plates containing 100 µg/ml of kanamycin. 6 mm sterile paper discs impregnated with the 10 µl DMSO extract sample were applied to the surface of the freshly spread plates, and activity scored after incubation at 30 °C for 2-4 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 a positive response. These positive extracts were subjected to a second-round of screening using the Δ*femX* mutant strain to identify those containing glycopeptide antibiotics capable of inducing *van* gene expression (explained in detail in the Results section). Approximately 10^7 spores of *S. coelicolor* Δ*femX* mutant strain J3130 (see Table S1) were spread on MMCGT agar medium containing no antibiotic, and 10 µl DMSO solutions of the selected inducer extracts were applied on paper discs to the plate. Plates were incubated at 30 °C for 2-4 days. Positive inducers produced a halo of glycopeptide-dependent growth of *S. coelicolor* Δ*femX* around the disc.

A single strain, designated as *Amycolatopsis* species MJM2582, was isolated from the 260 tested from the first round screen. Among the 9 different extracts from MJM2582 tested, very strong and clear induction halo were all observed from extracts prepared from aqueous and methanol extracts. In contrast, negligible halos were observed from 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 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) liquid medium at 30 °C 280 rpm for 2 days. The harvested mycelia were washed twice with an equal volume of 10.3% sucrose and then lysed in 1 ml of lysozyme solution (2 mg/ml lysozyme in 10.3% sucrose, 25 mM Tris (pH8), 25 mM EDTA, pH8) by incubating at 30 °C for 10 min. The lysate was extracted with 1 ml of 2 × Kirby mix (2 g sodium tri-isoprophylnaphthalene sulphonate, 12 g sodium 4-amino-salicylate, 5 ml 2 M tris-HCl (pH8), 6 ml

168 phenol mixture and made up to 100 ml with water) and 2 ml of phenol/chloroform solution
169 (phenol:chloroform (1:1 v/v), equilibrated with 0.1M Tris, pH8), and 1 ml of phenol/chloroform. The
170 nucleic acids in upper phase were transferred to a fresh tube and precipitated with 1/10 volume of 3 M
171 sodium acetate (pH6) and an equal volume of isopropanol at room temperature for 10 min. The pellet
172 of nucleic acids obtained by centrifugation was then resuspended in RNase solution (50 µg/ml of
173 RNaseI in 500 µl Tris-EDTA (TE) buffer) and incubated for 30 min at 37 °C to remove all RNAs. The
174 genomic DNA sample was cleaned by extraction with 200 µl of phenol/chloroform, and isopropanol
175 precipitation as described above. The DNA pellet obtained after centrifugation was washed with 70%
176 ethanol and dissolved in TE buffer solution (25 mM Tris (pH7.5), 2 ml EDTA).
177 Whole genome sequencing was performed by the DNA sequencing facility at the Department of
178 Biochemistry, University of Cambridge using a combined Illumina MiSeq and Roche 454 sequencing
179 approach. Assembly using the 454 GS De Novo Assembler (v.2.8) produced a draft sequence of 9.3
180 Mb in 149 contigs. Anti-SMASH 2.0 (http://www.secondarymetabolites.org/) (21, 22) was used to
181 identify a single glycopeptide gene cluster in each genome sequence. The DNA sequence of the cluster
182 from MJM2582 and *A. lurida* has been deposited in the GenBank database under accession number
183 KF882511 and KJ364518. Sequence comparison studies with previously reported glycopeptide
184 biosynthetic clusters i.e. teicoplanin, balhimycin, A47934, chloroeremomycin, dalbavancin and
185 vancomycin were performed using the ACT web comparison tool (23). Artemis (24) and Glimmer 3.02
186 (25) were used for prediction and annotation of open reading frames (Orfs) in the cluster sequence.
187
188 **Extraction and purification of glycopeptide antibiotics.**
189 Aerial mycelia (or spores) of each glycopeptide producer strain of interest (*Amycolatopsis* species
190 MJM2582, *Amycolatopsis lurida*, *Streptomyces toyocaensis*, *Amycolotopsis balhimycina*) (see Table S1
191 in the supplemental material) were prepared from 7 day cultures on Soya Flour Mannitol (SFM) agar

192 medium or BN agar medium at 30 °C (see Table S2). MJM2582 was tested for preparative
193 glycopeptide production in media previously reported for the production of A47934 (26), teicoplanin
194 (27), vancomycin (28) and ristocetin (29). Bioassays using *S. coelicolor* Δ*femX* and *S. coelicolor*
195 Δ*vanRS* strain showed that the only medium that reliably yielded glycopeptides was Streptomyces
196 Antibiotic Medium (SAM) used for A47934 biosynthesis (26). Seed cultures of MJM2582 were
197 prepared by transferring its mycelia (or spores) to 25 ml Glucose Yeast extract Malt extract (GYM)
198 liquid medium (20) in a 250 ml Erlenmeyer flask containing a 30 cm stainless steel spring wire and
199 incubating at 30 °C for 48 h with shaking at 250 rpm (see Table S3). 2.5 ml of dense seed culture was
200 then used to inoculate 50 ml SAM medium in 250 ml Erlenmeyer flasks containing spring wire baffles.
201 After incubation at 30 °C for 6 days with shaking at 250 rpm, the culture broth was centrifuged and the
202 cell pellet was extracted with 1% NH_4OH (1 ml per 1 g wet pellet). This mixture was centrifuged and
203 the alkaline supernatant harvested and neutralized with HCl. This pellet extract was used for initial
204 bioassay screening and a similar process was used to obtain glycopeptide extracts from *S. toyocaensis*
205 and *A. balhimycina*.

206 The MJM2582 glycopeptide was further purified by combining its pellet extract with the culture
207 supernatant, which was then applied to Diaion HP20 resin. The resin was washed with a step-wise
208 gradient of water and methanol to elute the bound glycopeptides (50-80% methanol). Fractions
209 containing glycopeptides were pooled and further purified using a D-Ala-D-Ala affinity resin. This was
210 prepared and used as previously described (30) and yielded a mixture of glycopeptides. Individual
211 glycopeptides were obtained by preparative HPLC (Agilent 1200) using a Phenomenex Luna C18(2)
212 column (250 mm × 21.2 mm, 10 μm), eluting with a linear gradient of 5 to 95% acetonitrile (+ 0.1%
213 TFA) in water (+ 0.1% TFA) over 35 min with a flow-rate of 20 ml/min. UV detection was performed
214 at 280 nm.
215

Additional antibiotic bioassays.

All other antibiotic susceptibility, resistance and induction tests using *S. coelicolor* strains were performed on Minimal Medium plus Casaminoacids Glucose Tiger milk (MMCGT) agar medium (see Table S2 in the supplemental material). Approximately 10^7 spores of *S. coelicolor* strains were spread on 12 cm × 12 cm square plates, or for 9 cm diameter round plates 10^5 spores were used. 10-30 µl of antibiotic solution (containing 30 µg antibiotic) was then soaked into sterile 6 mm paper discs and applied to the freshly spread spore lawns and incubated at 30 °C for 2-4 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 set 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 either from Sigma-Aldrich or Oxoid.

Mass spectrometry analysis of glycopeptide antibiotics.

Glycopeptide antibiotics were analyzed by liquid chromatography - mass spectrometry (LC-MS; Agilent 1100 HPLC coupled to a Thermo Scientific LCQ with an ESI source in positive-ion mode) using a Phenomenex Luna C18(2) column (250 mm × 2.0 mm, 5 µm), eluting with a linear gradient of 5 to 95% acetonitrile (+ 0.1% TFA) in water (+ 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 m/z = 1773.3, 1759.3, 1034.3 and 1027.3. High-resolution mass spectrometry was recorded on a Thermo Scientific LTQ Orbitrap and yielded the major component with an isotopic pattern of m/z = 1034.3386, 1034.8396, 1035.3406, 1035.8416, 1036.3429. This is identical to the pattern generated

from authentic ristocetin A ($[M+2H]^{2+}$; $C_{95}H_{112}N_8O_{44}^{2+}$) of m/z = 1034.3385, 1034.8395, 1035.3404, 1035.8415, 1036.3427.

RESULTS

Extensive screening of microbial extracts library using a novel two-step bioassay system identified an *Amycolatopsis* strain producing glycopeptide antibiotic.

For this study, we have initially established a geographically diverse collection of actinomycte strains. Each actinomycte strain was then cultured in three different liquid media and extracted with three different solvents consequently generating a total nine different natural product extracts. A randomly selected subset of this library covering ~5,300 actinomycete isolates was screened using a two-step procedure involving 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). *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 is therefore 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* harbouring a multi-copy *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 only exhibits kanamycin-resistant growth in the presence of such compounds (Fig. 1B). About 5% of the extracts tested strongly activated expression of the *sigEp-neo* fusion, 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 *vanHAX* 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 precursors as substrates, and under these growth conditions this function is instead performed via induction of the homologous enzyme VanK. FemX therefore becomes non-essential provided that the *van* genes are expressed (11). As a consequence, the Δ*femX* mutant strain is viable in the presence of vancomycin but non-viable in its absence, making growth of the Δ*femX* mutant itself a simple drug-dependent bioassay system for specifically screening for glycopeptide antibiotics (Fig. 1B). Of the 260 strains with extracts producing a positive response in the first round screen, extracts from a single isolate were found to strongly induce Δ*femX* 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 producer strains, and indicated a previously unidentified *Amycolatopsis* species which we designated *Amycolatopsis* sp. MJM2582 (Fig. 2; see also Fig. S2).

Amycolatopsis sp. MJM2582 produces ristocetin.

To fully characterize the glycopeptide produced by MJM2582, a draft genome sequence was produced to access the genes in the biosynthetic cluster, and culture extracts were analyzed using LC-MS. Comparative analysis using sequences from a number of previously reported glycopeptide biosynthetic clusters including teiocoplanin (32), balhimycin (33), A47934 (34), dalbavancin (35) and vancomycin (36), indicates 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 material). LC-MS analysis identified a number of related glycopeptides produced by MJM2582. A single component eluting at 9.0 min and with m/z = 1034 is identical to an authentic standard of ristocetin A ($[M+2H]^{2+}$ = 1034) indicating that the predicted structure of the glycopeptide from MJM2582 corresponds to ristocetin A (Fig. 4; see also Figs. S4 and S5 in the supplemental material). The other compounds are consistent with O-desmethyl ristocetin A ($[M+2H]^{2+}$ = 1027), ristocetin B ($[M+H]^{+}$ = 1773) and O-desmethyl ristocetin B ($[M+H]^{+}$ = 1759) (Fig. 5; see also Fig. S6). 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). Genome sequencing of *A. lurida* showed that it carries a gene cluster with an identical organization and 91% nucleotide identity.

Analysis of the ristocetin gene cluster. In comparison to 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 that 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 undecaprenyl-phospho-mannose as a donor, whereas class B GTs utilize a wide variety of 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 activity of the enzymes encoded by Orfs 30-33 in the cluster (Fig. 6). These are homologous to genes in the chloroeremomycin cluster

312 which produce the related sugar epivancosamine (39), a methylated derivative of L-ristosamine
313 additionally requiring a 3-C-methyltransferase (MT) that is absent from the ristocetin cluster. Another
314 unusual feature of ristocetin is its methylation pattern: O-methylation of the carboxyl terminus of the
315 core peptide and C-methylation of (S)-3,5-dihydroxyphenylglycine (Dpg) at amino acid position 3.
316 Accordingly, two MTs are encoded in the gene cluster (see Fig. 3). Orf19 shares significant homology
317 with VEG18 which catalyses the C-terminal O-methylation of a glycopeptide aglycone (40) while
318 Orf23 does not share homology with any previously characterized glycopeptide MTs and is therefore
319 the candidate for the C-methylation of the ristocetin aglycone (see Table S4 in the supplemental
320 material). This modification is unique amongst characterized glycopeptide pathways.

321 The four non-ribosomal peptide synthetase (NRPS) proteins in the ristocetin gene cluster have a
322 similar domain organization and predicted adenylation domain specificity to the teicoplanin NRPS and
323 assemble a peptide from β-hydroxytyrosine (βHTy), (S)-4-hydroxyphenylglycine (Hpg) and Dpg (Fig.
324 7; see also Table S5 in the supplemental material) (41). The rigid teicoplanin-like ristocetin aglycone is
325 then predicted to be generated by four cytochrome P450 enzymes encoded by adjacent genes (Orfs12-
326 15), which in previously characterized pathways are known to form oxidative cross-links between the
327 aromatic amino acid side chains (32). Interestingly, while the organization of the NRPS into four
328 proteins with a module distribution of 2-1-3-1 is a feature common to teicoplanin-like (Type IV)
329 glycopeptides, the organization of the tailoring enzymes is much more closely related to vancomycin-
330 like (Type I) glycopeptides. Furthermore, while Dpg and Hpg biosynthesis is conserved between the
331 production of teicoplanin-like and vancomycin-like glycopeptides, distinct routes have evolved for
332 βHTy biosynthesis, where a single non-heme dioxygenase is used for the former (42), and three
333 separate enzymes for the latter (43). Ristocetin employs the same route as vancomycin (Orfs 25-27),
334 despite a teicoplanin-like aglycone. Ristocetin is the only non-chlorinated glycopeptide antibiotic
335 reported to date, and the gene cluster unsurprisingly 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 (see Fig. 3). However no genes encoding the VanRS two-component regulatory system were identified anywhere in both the MJM2582 and *S. lurida* genome, implying that *vanHAX* expression may be constitutive in this strain or otherwise controlled in a VanRS-independent manner. Interestingly, alignment of the *vanHAX* promoter sequence in ristocetin gene cluster with similar sequences from other glycopeptide gene clusters identified conserved nucleotide changes in those with no obvious VanRS control system in comparison to those known to be regulated by VanRS (Fig. 8). This implies that the putatively VanRS-independent promoters may share the same method of regulation.

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 mechanism 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 (89/100) of the vancomycin-resistant strains isolated in this way did not however contain glycopeptide biosynthetic clusters and it was necessary to include an additional PCR based screen to identify those that do. The Δ*femX* mutant bioassay employed in this work proved highly specific for compounds which induce expression of the van resistance genes, and a further discriminatory screen was not required. Nevertheless, Thaker et al. elegantly exploited the results of their PCR screen to produce phylogenetic fingerprints for dereplication, discriminating against the rediscovery of known strains and

compounds, and we believe 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 products biosynthetic clusters where the disconnection between the producing microbe 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 positive screen hit apriori comes from an actinomycete strain which is both culturable and competent for the biosynthesis of the detected antibiotic activity under laboratory conditions.

We envisage that the direct relationship between producing organism, genome 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 identified as active in the first round of *sigEp-neo* screening but inactive in the subsequent glycopeptide screen, and 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 the extracts that produced a positive response in the *sigEp-neo* screen would need to be reanalyzed in the improved hybrid screens.

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FIGURE LEGENDS

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 nine extracts per strain. Extracts from approximately 5,300 different isolates were tested in the first round *sigEp-neo* fusion reporter system, and 260 different isolates gave extracts which 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 designated MJM2582. (B) Plates demonstrating the response of the *sigEp-neo*, Δ*femX* reporter systems to antibiotics (numbered list shown). Antibiotics that target DNA gyrase (novobiocin), the ribosome (thiostrepton) or early peptidoglycan biosynthesis (tunicamycin) do not induce the *sigEp-neo* system, while those targeting late peptidoglycan biosynthesis (bacitracin, moenomycin A, ramoplanin, vancomycin, teicoplanin, ristocetin) do. The amount of novobiocin, thiostrepton and tunicamycin used in the bioassays (30 μg each) is sufficient to produce a halo of growth inhibition when assayed against *S. coelicolor*. Extracts obtained from known glycopeptide producer strains (*A. balhimycina*, *S. toyocaensis*) and from MJM2582 also induced the *sigEp-neo* system. Only glycopeptide antibiotics (but not teicoplanin) or extracts containing glycopeptides, including MJM2582, acted as inducers in the Δ*femX* bioassay. (C) Bioassay showing the spectrum of activity of the MJM2582 extract against

525 glycopeptide sensitive (Δ*vanRS*), constitutively resistant (Δ*vanS*) and inducibly resistant (M600) strains
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527
528 **FIG 2** Phylogenetic analysis of MJM2582 16S RNA sequences. The result indicates that MJM2582 is
529 a previously unidentified *Amycolatopsis* species. Scale bar indicates the distance in substitutions per
530 nucleotide. See also Fig.S2 in the supplemental material.
531
532 **FIG 3** Genetic organization of the glycopeptide gene cluster identified in *Amycolatopsis* sp. MJM2582.
533 Predicted Orfs are represented by arrows, and numbered consecutively. The predicted enzymatic
534 function encoded by each Orf is listed. A detailed comparison to the previously reported glycopeptide
535 clusters can be found in Fig. S3 and Table S4 in the supplemental material.
536
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539 to ristocetin A.
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543 spectra for glycopeptides. (B) Mass spectra for each ristocetin derivative. The spectrometer was tuned
544 to the $[M+2H]^{2+}$ ion of teicoplanin to facilitate the identification of compounds above 2000 Da. (C)
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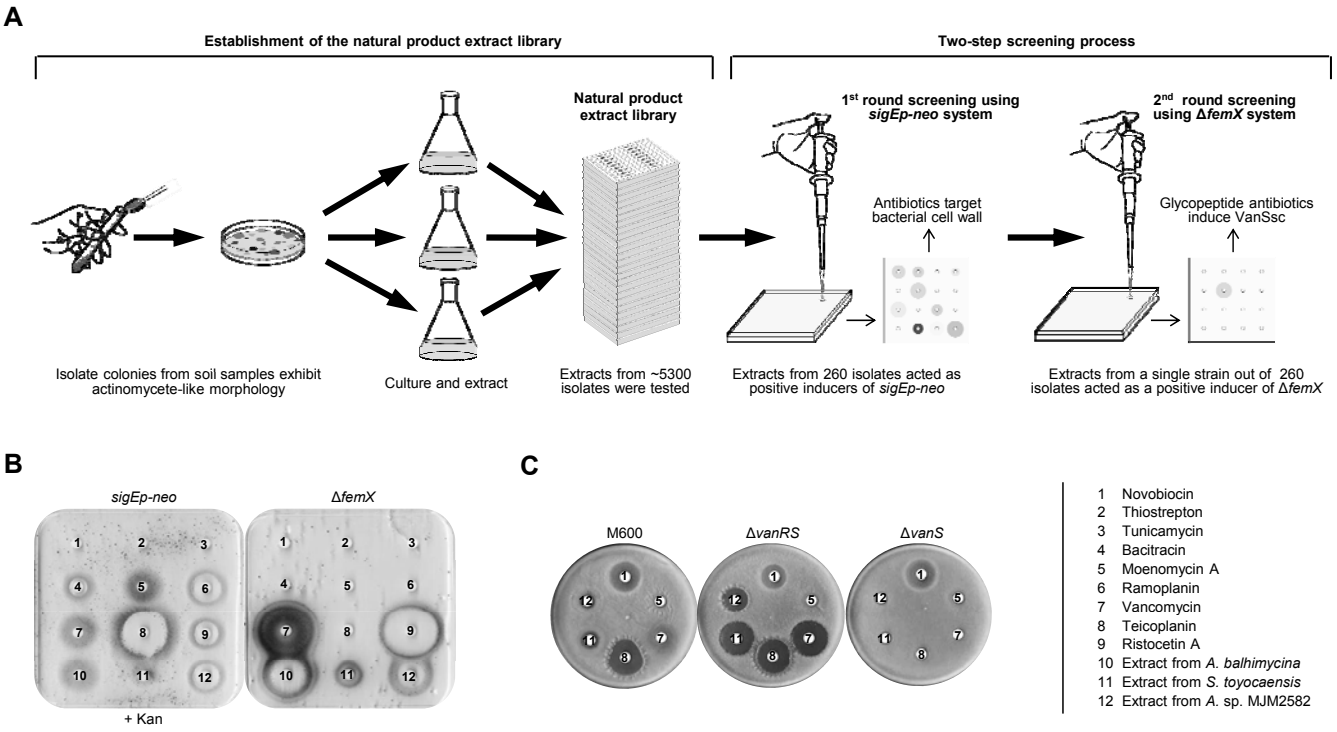
546

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

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

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 s*p. MJM2582 (MJM). The highly conserved sequences represent a putative VanR response regulator binding site in the -35 and -10 promoter region (boxed). The top four strains (Sc, St, At, Ab) carry glycopeptide resistance clusters which include genes encoding a VanRS two-component system, while the bottom three strains (Ao, Al and MJM) lack an obvious VanRS system. Nucleotide sequence differences identified between these two groups are highlighted.



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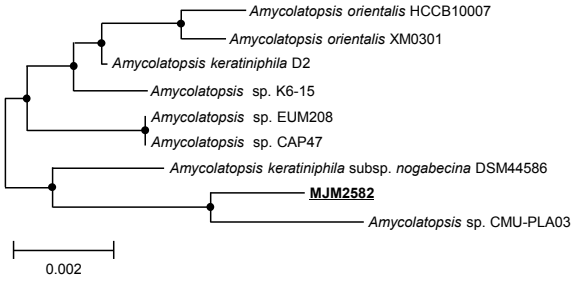

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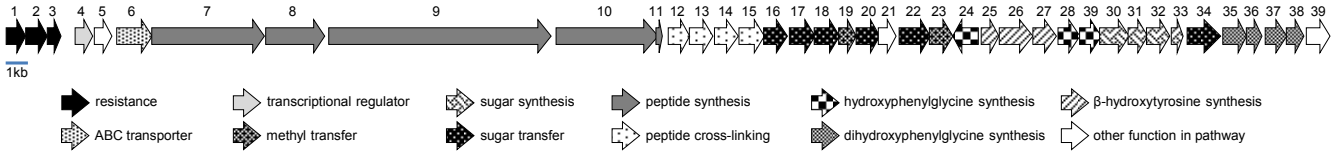

1	D-lactate dehydrogenase	11	MtbH-like protein	21	inactive deacetylase	31	4-ketoreductase
2	D-alanine:D-lactate ligase	12	p450 monooxygenase	22	mannosyltransferase	32	C-3 aminotransferase
3	D-alanyl-D-alanine dipeptidase	13	p450 monooxygenase	23	methyltransferase	33	C-5 epimerase
4	StrR family transcriptional 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		

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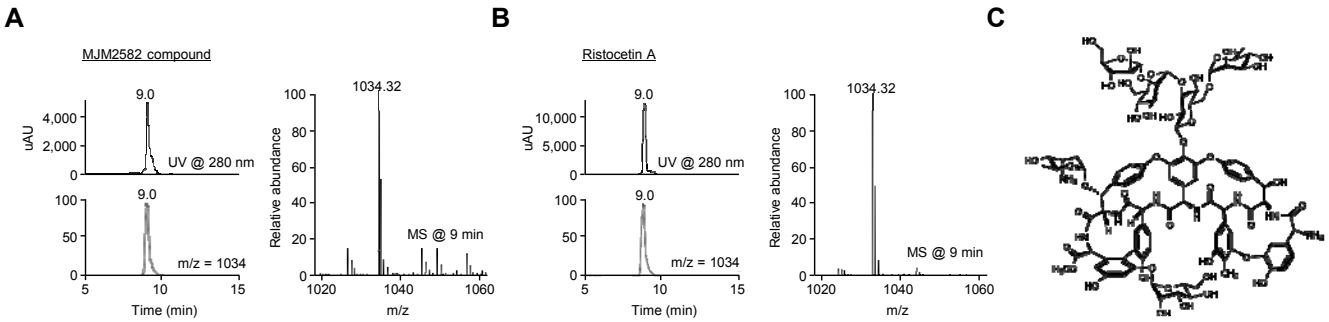

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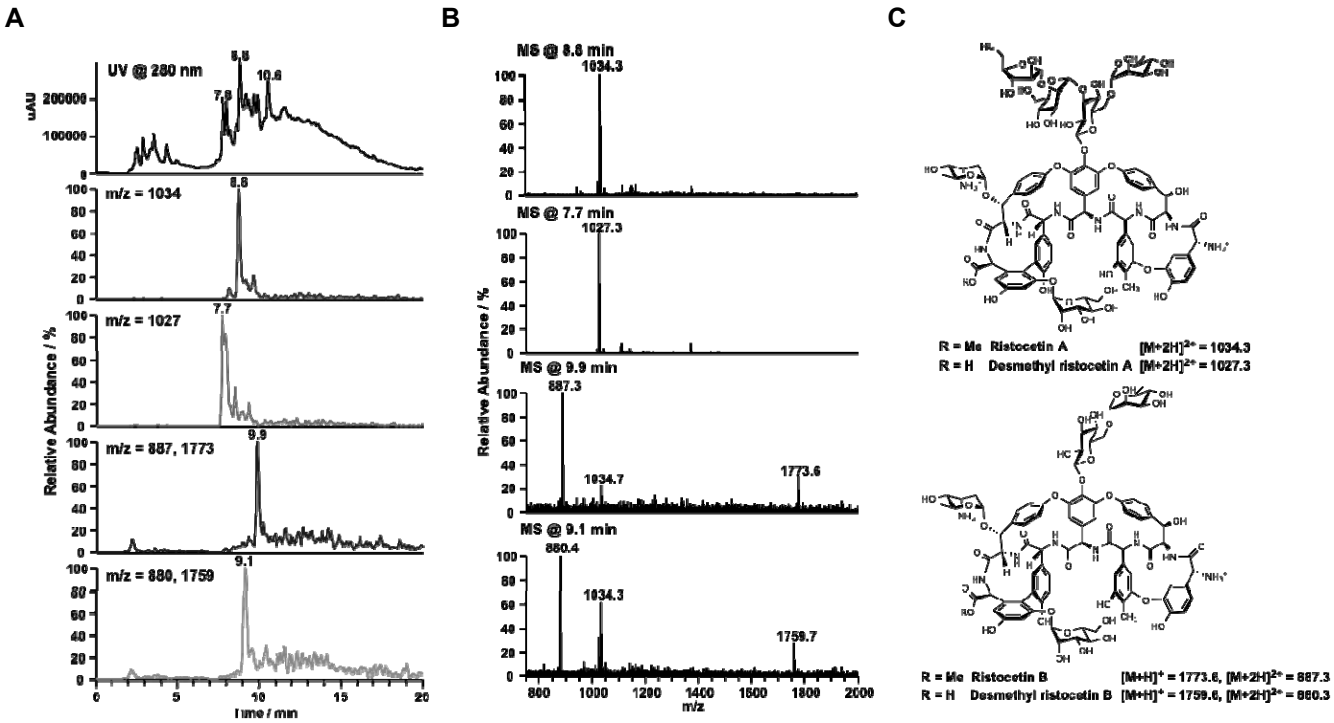

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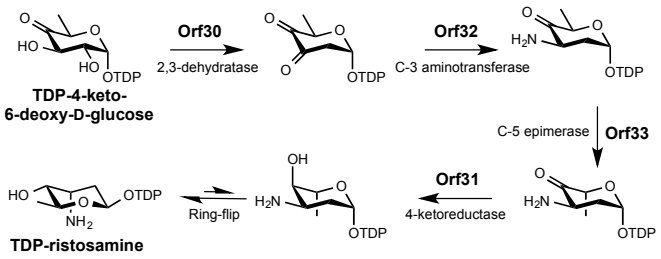

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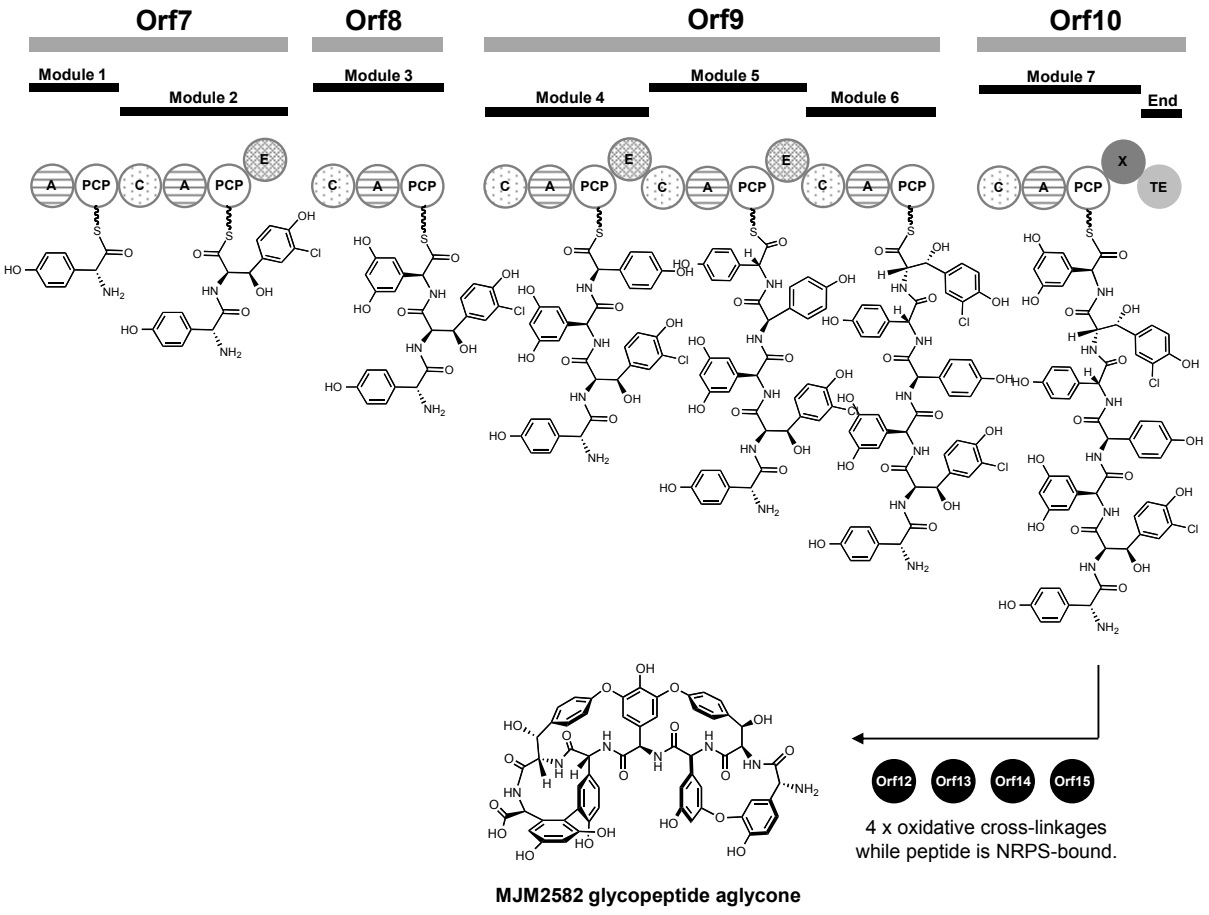

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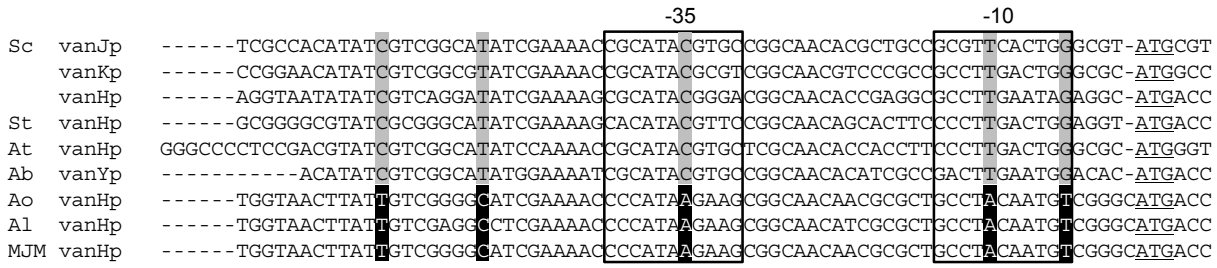

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