

Overproduction of Ristomycin A by Activation of a Silent Gene Cluster in *Amycolatopsis japonicum* MG417-CF17

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The emergence of antibiotic-resistant pathogenic bacteria within the last decades is one reason for the urgent need for new antibacterial agents. A strategy to discover new anti-infective compounds is the evaluation of the genetic capacity of secondary metabolite producers and the activation of cryptic gene clusters (genome mining). One genus known for its potential to synthesize medically important products is *Amycolatopsis*. However, *Amycolatopsis japonicum* does not produce an antibiotic under standard laboratory conditions. In contrast to most *Amycolatopsis* strains, *A. japonicum* is genetically tractable with different methods. In order to activate a possible silent glycopeptide cluster, we introduced a gene encoding the transcriptional activator of balhimycin biosynthesis, the *bbr* gene from *Amycolatopsis balhimycina* (*bbr*_{Aba}), into *A. japonicum*. This resulted in the production of an antibiologically active compound. Following whole-genome sequencing of *A. japonicum*, 29 cryptic gene clusters were identified by genome mining. One of these gene clusters is a putative glycopeptide biosynthesis gene cluster. Using bioinformatic tools, ristomycin (syn. ristocetin), a type III glycopeptide, which has antibacterial activity and which is used for the diagnosis of von Willebrand disease and Bernard-Soulier syndrome, was deduced as a possible product of the gene cluster. Chemical analyses by high-performance liquid chromatography and mass spectrometry (HPLC-MS), tandem mass spectrometry (MS/MS), and nuclear magnetic resonance (NMR) spectroscopy confirmed the *in silico* prediction that the recombinant *A. japonicum*/pRM4-*bbr*_{Aba} synthesizes ristomycin A.

The nocardioform actinomycete *Amycolatopsis japonicum* MG417-CF17 (DSM 44213) is known as the producer of the hexadentate chelating agent (S,S)-ethylenediaminedisuccinic acid [(S,S)-EDDS] (1), which is a biodegradable EDTA isomer (2) with similar properties. Though members of the genus *Amycolatopsis* are major producers of various antimicrobial compounds, such as the medically relevant rifamycin and vancomycin, no bioactive secondary metabolite has been isolated from *A. japonicum* so far.

Genome sequencing projects have revealed that the potential of actinomycetes for the production of valuable secondary metabolites is much larger than previously expected. In certain cases, more than 30 gene clusters encoding components of pathways for secondary metabolite biosynthesis have been found per actinomycete genome. For example, *Streptomyces coelicolor*, *Streptomyces avermitilis*, *Streptomyces griseus*, and *Saccharopolyspora erythraea* are each known to produce three to five secondary metabolites but actually possess more than 20 gene clusters that are predicted to encode components of biosynthetic pathways for secondary metabolites (3, 4, 5, 6). This exemplifies that a large number of these pathways are cryptic, meaning that they are expressed poorly or not at all under standardized laboratory conditions. One strategy to obtain access to this enormous genetic potential is the genome mining approach. The crucial point of genome mining is in learning how to identify, subsequently activate, and finally exploit these gene clusters, making their product accessible for evaluation as drug leads and for other biotechnological applications. The availability of hundreds of actinomycete genome sequences and the rapidly decreasing costs of genome sequencing have made genome mining the most promising tool to generate raw data for drug discovery. However, due to the biochemical heterogeneity in

secondary metabolite biosynthesis and the high number of putative gene clusters, the identification, categorization, and interpretation of the information encoded within the genomes required automation. Bioinformatic tools such as antiSMASH 2.0 (7) offer a comprehensive pipeline capable of discovering and characterizing biosynthetic loci covering the whole range of described secondary metabolite compound classes.

In order to use this information, various strategies to activate cryptic secondary metabolite gene clusters have been applied. In many cases, stress conditions or variations of culture conditions led to the production of new metabolites. More-targeted approaches are the exchange of promoters or heterologous expression of all relevant genes in a suitable host (8, 9).

Previously we elucidated the biosynthesis of the glycopeptide balhimycin in detail in *Amycolatopsis balhimycina* (10). The genus *Amycolatopsis* is known to produce various glycopeptides that are important clinical emergency antibiotics. New glycopeptides might be key compounds to treat currently spreading glycopep-

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tide-resistant pathogens, like glycopeptide-intermediate *Staphylococcus aureus* and vancomycin-resistant enterococci (VRE). A particular function is described for the highly glycosylated glycopeptide ristomycin A (also called ristocetin A), previously identified in *Amycolatopsis lurida* (11). Since ristomycin A causes thrombocytopenia and platelet agglutination, it is no longer used for the treatment of human staphylococcal infections but solely applied to assay those therapeutically unfavorable functions *in vitro* as a diagnosis compound to detect widespread hereditary genetic disorders such as von Willebrand disease and Bernard-Soulier syndrome (12).

In this study, we were able to identify and activate a new type III glycopeptide gene cluster in *A. japonicum* coding for ristomycin A production. Although ristomycin A has been in use for many years, no type III glycopeptide gene cluster has been published so far. Heterologous expression of the balhimycin pathway-specific regulator gene *bbr*_{Aba} (*bbr* gene from *A. balhimycina*) (13) in *A. japonicum* enabled us to activate the cryptic ristomycin gene cluster.

The activation of ristomycin A production in *A. japonicum* now offers the possibility of optimizing production of ristomycin A in a genetically accessible strain.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* XL1-Blue (14) was used for cloning purposes, and the methylation-deficient strain *E. coli* ET12567 (15) was used to obtain unmethylated DNA for *Amycolatopsis japonicum* transformations. *A. japonicum* MG417-CF17 (1) is the (*S,S*)-EDDS-producing wild type and was used to generate *bbr*_{Aba} and *ajrR* overexpression strains (this study). The overexpression plasmids pRM4-*bbr*_{Aba} and pRM4-*ajrR* derive from pRM4 (16), a pSET152-derived nonreplicative, Φ C31 integration vector with an integrated constitutive *ermEp** promoter, an artificial ribosomal binding site, and an apramycin resistance cassette.

Media and culture conditions. *E. coli* strains were grown in Luria broth medium (17) at 37°C and were supplemented with 100 μ g ml⁻¹ apramycin when necessary to maintain plasmids. Liquid cultures of *A. japonicum* were cultivated in 100 ml of R5 medium (18) in an orbital shaker (220 rpm) in 500-ml baffled Erlenmeyer flasks with steel springs at 27°C. Liquid/solid media were supplemented with 100 μ g ml⁻¹ apramycin to select for strains carrying integrated antibiotic resistance genes. To detect glycopeptide production, wild-type *A. japonicum* or *A. japonicum* carrying the pRM4-*bbr*_{Aba} or pRM4-*ajrR* plasmid, respectively, were incubated for 5 days in R5 medium without apramycin.

Construction of the integrative expression vector pRM4-*bbr*_{Aba}/*ajrR*. For the overexpression of *bbr*_{Aba} and *ajrR*, the *bbr*_{Aba} and *ajrR* coding regions were amplified by using the primer pair *bbr*_{Aba}-FP (FP stands for forward primer) and *bbr*_{Aba}-RP (RP stands for reverse primer) and the primer pair *ajrR*-FP and *ajrR*-RP (see Table S1 in the supplemental material), respectively. The 968-bp (*bbr*_{Aba}) and the 981-bp (*ajrR*) PCR products were integrated into pRM4 via the primer-attached NdeI and XbaI sites for *bbr*_{Aba} and NdeI and HindIII sites for *ajrR*, downstream of the *ermEp** promoter.

Direct transformation of *A. japonicum*. For transformation of *A. japonicum*, the direct transformation method of Stegmann et al. (19) was modified. Mycelia were grown in 100 ml of TSB-D (17 g Bacto tryptone, 3 g peptone 110, 5 g NaCl, 2.5 g K₂HPO₄, and 2.5 g glucose per liter Millipore H₂O) for 24 h in a 1-liter Erlenmeyer flask with 4 bottom baffles at 30°C and 220 rpm; 5-ml portions from these precultures were used to inoculate 100 ml of TSB-D, which were incubated for 36 h under the same conditions as the preculture.

Detection of ristomycin biosynthesis by HPLC-DAD. Glycopeptide production was determined by bioassays with *Bacillus subtilis* ATCC 6633 as the test organism after growth in R5 medium or by high-performance

liquid chromatography (HPLC) coupled with a diode array detector (DAD). Five microliters of each sample was analyzed by HPLC with gradient elution. For gradient elution, solvent A was 0.1% phosphoric acid and solvent B was acetonitrile, and the gradient elution was performed as follows: $t_0 = 0\%$ solvent B, $t_7 = 30\%$ solvent B, t_8 to $t_{10} = 100\%$ solvent B. The flow rate was 0.85 ml min⁻¹. Gradient elution was conducted using a Nucleosil 100-C₁₈ column (5 μ m; 125 by 3 mm) (precolumn, 20 by 3 mm) (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). Detection was carried out at 210, 230, 260, 280, 310, 360, 400, 435, and 500 nm (1260 Infinity diode array detector; Agilent Technologies, Waldbronn, Germany).

Analytical instrumentation. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 500 HD spectrometer, equipped with a BBFO cryo probe head. Spectra were referenced to residual protonated solvent signals with resonances at $\delta_{H/C}$ 2.50/39.5 (deuterated dimethyl sulfoxide [*d*₆-DMSO]). Circular dichroism (CD) spectra were measured on a Jasco J-720 spectropolarimeter. High-resolution (HR) electrospray ionization-time of flight mass spectrometry (ESI-TOF MS) data were recorded using a Bruker Daltonic maXis 4G instrument. Semipreparative HPLC was conducted using a Waters system consisting of a 600 pump, a 996 photodiode array detector, a 7725i rheodyne injector, and a PerkinElmer vacuum degasser series 200. Ristomycin monosulfate A standard was purchased from Aldrich and used without further purification for MS analyses. For NMR and CD analyses, the standard was desalted.

HPLC-ESI-MS and HPLC-MS/MS. Culture broths were prepared by centrifugation or filtration and partially purified via adsorption chromatography with Amberlite XAD16 and subsequent ethyl acetate extraction. Portions (2.5 μ l) of the different fractions were analyzed by means of HPLC-ESI-MS and HPLC-MS/MS using a Nucleosil 100-C₁₈ column (3 μ m, 100 by 2 mm) (precolumn, 10 by 2 mm) (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) coupled to an ESI mass spectrometer. LC-MS measurements were obtained from a LC/MSD Ultra Trap system XCT 6330 (Agilent Technologies, Waldbronn, Germany). Detection of *m/z* values was conducted with Agilent DataAnalysis for 6300 series Ion Trap LC/MS 6.1 version 3.4 software (Bruker-Daltonik GmbH). Analysis was carried out at a flow rate of 0.4 ml min⁻¹ with gradient elution. Solvent A was 0.1% formic acid in acetonitrile, and solvent B was 0.06% formic acid in acetonitrile. Gradient elution was performed as follows: $t_0 = 0\%$ solvent B, $t_7 = 30\%$ solvent B, t_8 to $t_{10} = 100\%$ solvent B. The flow rate was 0.4 ml min⁻¹, and the temperature was 40°C. Electrospray ionization (alternating positive and negative ionization) in Ultra Scan mode with a capillary voltage of 3.5 kV and a drying gas temperature of 350°C was used for LC-MS analysis. For tandem MS experiments, the analysis was carried out either in negative mode with an Agilent LC/MSD Ultra Trap system XCT 6330 or in positive ionization mode employing an AB SCIEX QT3200 instrument.

Scaled-up cultivation, extraction, and purification of ristomycin A. *A. japonicum*/pRM4-*bbr*_{Aba} was cultivated in a 20-liter fermentor (b20; Giovanola). The fermentor was inoculated with 2% (vol) of shaking cultures grown for 48 h in 500-ml Erlenmeyer flasks with one baffle and steel spring in tryptic soy broth (TSB). The fermentation was carried out at 27°C with an agitation rate of 1,000 rpm and an aeration rate of 0.5 vol/vol/min. After 50 h of fermentation, 1 liter of fermentation broth was taken, and bacterial cells were removed by centrifugation. Diaion HP-20 adsorbent resin (50 g liter⁻¹) was added to a portion of the supernatant (900 ml), and the mixture was agitated in a 1.5-liter Erlenmeyer flask at 120 rpm for 2 h. The HP-20 resin was then passed through a fritted funnel and washed with water, and the bound metabolites were eluted using a stepwise gradient of isopropanol-H₂O (acidified with 1% acetic acid [HOAc]) to produce five fractions (A to E). Fractions B, C, and D, eluting with 5, 10, and 15% isopropanol in acidified water, respectively, were found to potentially inhibit the growth of *B. subtilis* in an agar diffusion assay. Each bioactive fraction was rechromatographed with reversed-phase HPLC (RP-HPLC). For RP-HPLC separation, a Phenomenex Syn-

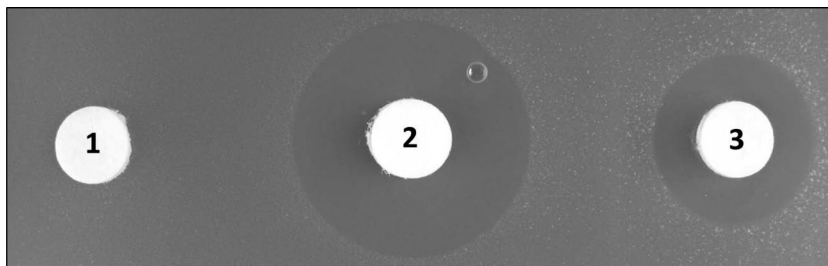


FIG 1 Bioassay after cluster activation by expression of *bbr*_{Aba} and *ajrR*, respectively. Wild-type *A. japonicum* (1), *A. japonicum*/pRM4-*bbr*_{Aba} (2), and *A. japonicum*/pRM4-*ajrR* (3) were grown for 5 days in R5 medium, and 20 μ l of culture supernatant was assayed for bioactivity against *B. subtilis*.

ergi Hydro-RP 80A column (10 by 250 mm; 4 μ m) in combination with a Phenomenex SecurityGuard AQ C₁₈ precolumn (10 by 10 mm) was used. The flow rate was 2.0 ml min⁻¹. UV monitoring at 210 and 254 nm was performed. Elution was performed as follows: (i) isocratic elution at 20:80 acetonitrile (MeCN)-H₂O (0.1% trifluoroacetic acid [TFA]) over a period of 10 min, (ii) gradient elution from 20:80 to 40:60 MeCN-H₂O (0.1% TFA) over 10 min, (iii) gradient elution from 40:60 to 100:0 MeCN-H₂O (0.1% TFA) over 20 min, and (iv) isocratic elution at 100% MeCN for an additional 10 min. This procedure yielded collectively 33 mg of ristomycin A.

Platelet aggregation. For preparation of platelet-rich plasma (PRP), citrate-anticoagulated human blood was centrifuged at 200 \times g for 10 min. After centrifugation, the PRP was collected in a fresh tube, and the remaining blood was centrifuged at 2,500 \times g to obtain platelet-poor plasma (PPP). Afterwards the platelet count in the PRP was estimated with a KX21-N automatic hematology analyzer (Sysmex, Norderstedt, Germany). After adjusting the PRP to a platelet concentration of 200 \times 10⁵ μ l⁻¹ with the obtained PPP, aggregation was estimated from light transmission measurements determined with a luminoaggregometer (model 700; Chrono-Log Corp., Havertown, PA, USA). Following calibration, agonists were added at the indicated concentrations, and aggregation was measured for 10 min with a stir speed of 1,000 rpm at 37°C. The extent of aggregation was quantified as percentage of light transmission. Data analysis was performed with the aggroLink8 software (Chrono-Log). The adjusted PRP was either treated with agonists at the indicated concentrations or not treated with an agonist.

Gene expression analysis by RT-PCR. For reverse transcription-PCR (RT-PCR) experiments, the wild-type *A. japonicum* and the *bbr*_{Aba} and *ajrR* overexpression strains were grown in R5 medium. After 25 h, the cells were harvested and disrupted using glass beads and a Precellys homogenizer (Peqlab). RNA preparations were treated twice with DNase I (Fermentas). To exclude DNA contamination, negative controls were carried out by using total RNA as the template for a PCR using the primer pair sigB-RT-FP and sigB-RT-RP (see Table S1 in the supplemental material). cDNA from 3 mg RNA was generated with random hexameric primers, reverse transcriptase, and cofactors (Fermentas). PCRs were performed with the primers listed in Table S1. PCRs were carried out under the following conditions: (i) an initial denaturation step (94°C for 2 min); (ii) 27 cycles of PCR, with 1 cycle consisting of denaturation (95°C for 30 s), annealing (59°C for 30 s), and polymerization (72°C for 30 s); and (iii) an additional polymerization step (72°C for 1 min). Each PCR mixture (25 μ l) contained a 1- μ l aliquot of RT reaction product. As a positive control, cDNA was amplified from the major vegetative sigma factor (*sigB*) transcript, which is produced constitutively. The PCR products were analyzed by agarose gel electrophoresis (2.0%).

Nucleotide sequence accession number. The GenBank accession number of the genome sequence of *A. japonicum* is CP008953 (45).

RESULTS AND DISCUSSION

Activation of a cryptic glycopeptide gene cluster. Members of the genus *Amycolatopsis* are known to possess a particularly high poten-

tial for the production of secondary metabolites. They are major producers of various glycopeptides like balhimycin (*A. balhimycina*), the medically relevant vancomycin (*Amycolatopsis orientalis* NRRL 2452), and rifamycin (*Amycolatopsis mediterranei*), which is one component in the drug cocktail for the treatment of tuberculosis and inactive meningitis. However, these species are difficult to manipulate genetically because of the lack of efficient transformation systems. In contrast to these species, we were able to establish a DNA transfer protocol for *A. japonicum* (19). Although we have been working with this species in our laboratory for many years, no antibiologically active product could be identified so far. In previous work, it was shown that *A. japonicum* produces glycopeptide-resistant cell wall precursors (20) and that the genome contains an *oxyB* gene, which is involved in the production of glycopeptides (21). From these results, we assumed that *A. japonicum* may have the potential to produce a glycopeptide.

To test whether *A. japonicum* has this potential, we made use of the observation that all known glycopeptide clusters are controlled by a pathway-specific StrR-like regulator (22). Hence, we applied a new cluster activation strategy, overexpressing the gene encoding the characterized pathway-specific transcriptional regulator of the balhimycin gene cluster, *bbr*_{Aba} (13), in *A. japonicum* aiming to awake the cryptic glycopeptide gene cluster. Therefore, *bbr*_{Aba} was cloned into the integrative vector pRM4 under the control of the constitutive promoter *ermEp** and transferred into *A. japonicum* by direct transformation. The recombinant species *A. japonicum* carrying the pRM4-*bbr*_{Aba} plasmid was grown in R5 medium for 5 days, and the culture supernatant was analyzed in a growth inhibition assay. Whereas the supernatant from wild-type *A. japonicum* did not contain any biologically active compound, the culture supernatant from the recombinant *A. japonicum*-(pRM4-*bbr*_{Aba}) strongly inhibited growth of the indicator species, *B. subtilis* (Fig. 1). The metabolic profiles of the supernatants were determined by HPLC-DAD (Fig. 2). The chromatogram of the *A. japonicum*/pRM4-*bbr*_{Aba} supernatant revealed two peaks with glycopeptide-specific DAD spectra. These peaks were absent in the chromatogram of the wild-type *A. japonicum* supernatant (Fig. 2). From these results, we concluded that *A. japonicum* does indeed have the genetic potential to produce a glycopeptide antibiotic, which is synthesized only after transcriptional activation.

Genome mining in *A. japonicum*. In order to identify the gene cluster for biosynthesis of the new glycopeptide, its regulation, and the self-resistance mechanism and to evaluate the potential to synthesize additional secondary metabolites, the genome of *A. japonicum* was sequenced. The genome sequence of *A. japonicum* is approximately 8.96 Mb in size and contains 8,464 putative

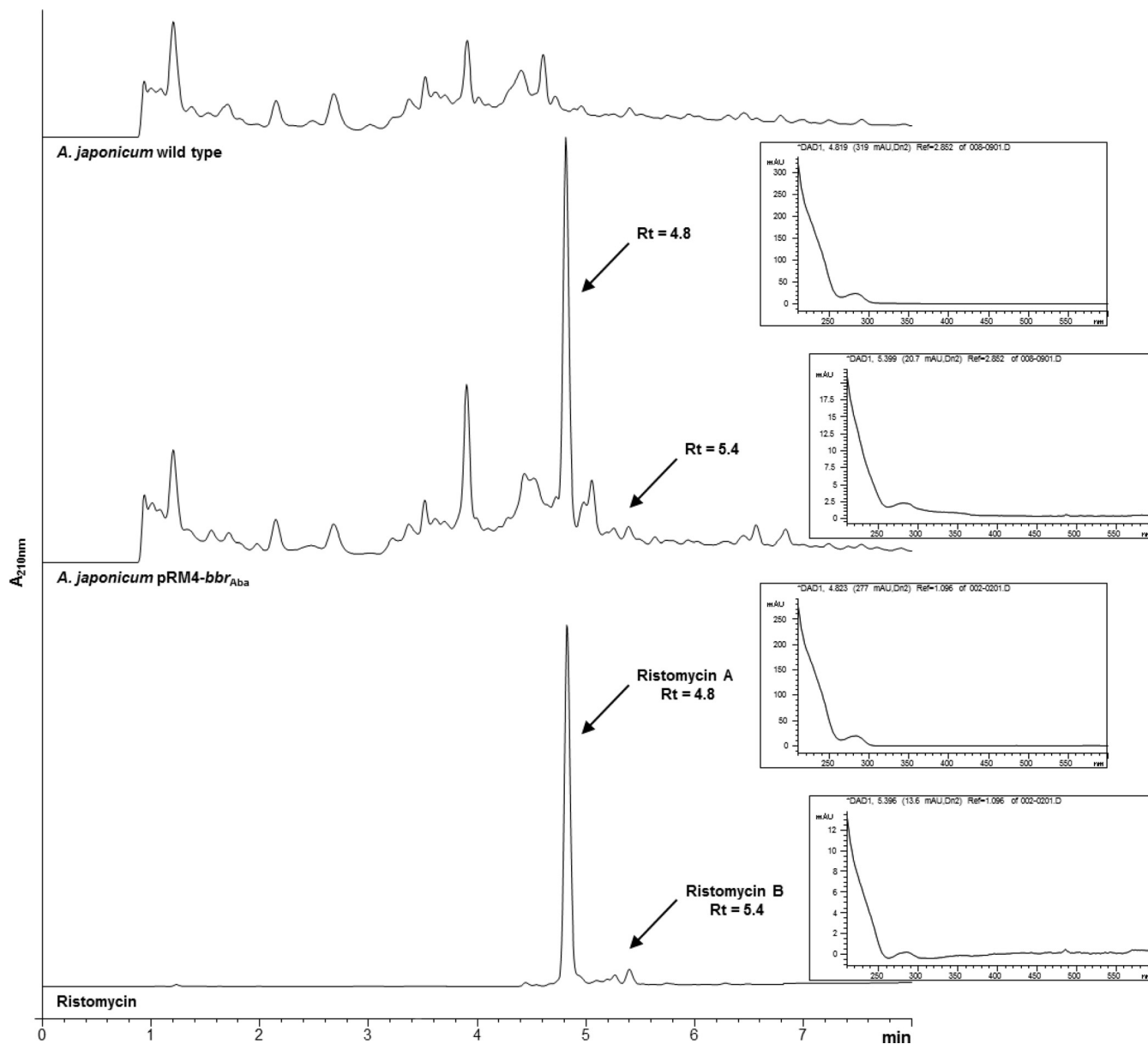


FIG 2 HPLC chromatograms of wild-type *A. japonicum* and *A. japonicum/pRM4-bbr_{Aba}* after growth for 5 days in R5 medium and ristomycin standard. The boxed regions show the corresponding DAD spectra. Rt, retention time; mAU, milliabsorbance units.

open reading frames (ORFs) (45). Extensive genome analysis using antiSMASH 2.0 (7) revealed the presence of 29 putative secondary metabolite gene clusters (see Table S2 in the supplemental material). One of the gene clusters (cluster 4) encodes the synthesis of the polyketide synthase (PKS) compound ECO-0501, which was already identified in the vancomycin producer *Amycolatopsis orientalis* ATCC 43491 by a genome-scanning technology (23). ECO-0501 defines a new structural class of the polyketide antibiotic octanoic acid glucuronide and possesses activity against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (24). However, since this cluster and the corresponding product were already known, we focused our attention on the type III PKS/nonribosomal peptide synthetase (NRPS) hybrid gene cluster (cluster 24)

(Table S2) which showed high similarity to described glycopeptide gene clusters, such as the balhimycin (25), teicoplanin (26), and A47934 (27) gene clusters. Cluster 24 consists of 39 distinct ORFs (AJAP_31985 to AJAP_32175) (Fig. 3) with a total size of almost 69 kb. The putative functions of all 39 gene products were deduced by comparative amino acid sequence analysis with homologues from known glycopeptide clusters (Table 1). Cluster 24 is predicted to encode all the enzymes required for biosynthesis of a glycopeptide, comprising enzymes responsible for assembly and export of the glycopeptide, self-resistance, and gene regulation. By considering these deduced functions, the boundaries of the glycopeptide gene cluster 24 were predicted. The left border is probably delimited by *orf1*, a *dahp* homologue, involved in tyrosine precursor supply for the synthesis of the aprotogenic amino acids (28), and the right border is most

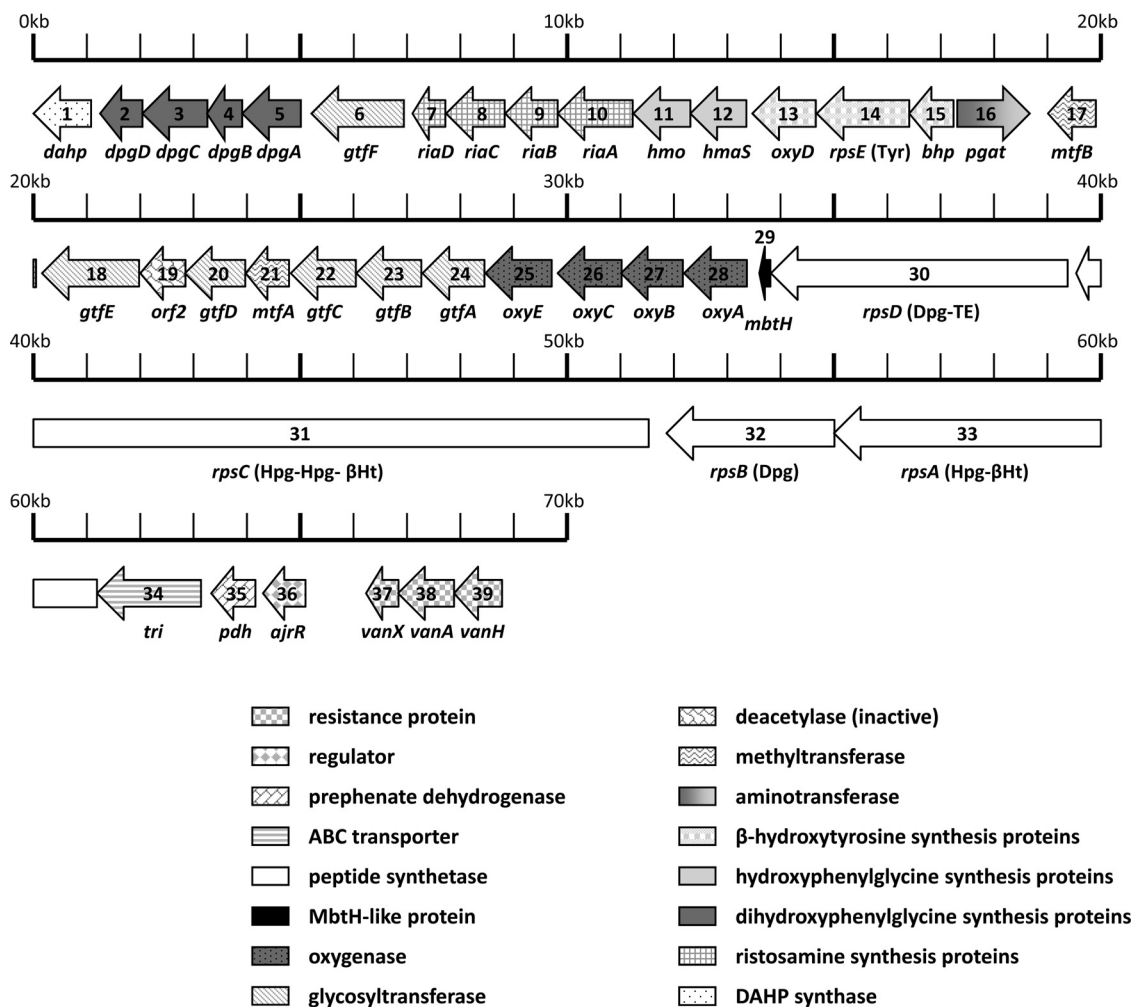


FIG 3 Genetic organization of the ristomycin A (*ris*) cluster identified in *A. japonicum*. Predicted ORFs are represented by an arrow drawn to scale and are numbered as in Table 1. Gene names are indicated underneath the corresponding ORFs. Predicted functions of genes are listed. TE, thioesterase.

likely delimited by *orf39*, a *vanH* homologue, involved in self-resistance (20) (Table 1).

Proposed glycopeptide biosynthesis in *A. japonicum*. In the last two decades, considerable progress has been made in understanding the genetics and biochemistry of glycopeptide biosynthesis (10, 22, 29). The profound knowledge of the individual enzymes participating in glycopeptide biosynthesis makes it possible to almost completely predict the structure of the final product.

(i) **Synthesis of the nonproteinogenic amino acids.** Glycopeptides consist of a heptapeptide backbone constituted mainly by aprotogenic aromatic amino acids such as 3,5-dihydroxyphenylglycine (Dpg), *p*-hydroxyphenylglycine (Hpg), and β-hydroxytyrosine (β-Ht). Since detailed information on their biosynthesis is available (30, 31, 32, 33, 34), it is possible to conclude that most likely, *orf5* to *orf2* and *orf16* are responsible for the synthesis of Dpg, *orf11*, *orf12*, and *orf16* are responsible for Hpg synthesis, and *orf15* to *orf13* are responsible for synthesis of βHt (Fig. 3 and Table 1).

(ii) **Synthesis of the aglycon. (a) Synthesis of the linear backbone.** The amino acids are assembled by nonribosomal peptide synthetases to form a heptapeptide (35). The *A. japonicum* glyco-

peptide gene cluster contains four NRPS genes (*orf33* to *orf30*) that are predicted to encode the enzymes catalyzing the assembly of the heptapeptide backbone. The genetic organization and domain composition of these NRPS genes and the predicted specificity of the A domains indicate that the ORF33 protein, including module 1 and module 2, incorporates Hpg and β-Ht; ORF32, including module 3, incorporates Dpg; ORF31, including modules 4, 5, and 6, incorporates Hpg, Hpg, and β-Ht; and ORF30, including module 7, incorporates Dpg. This amino acid composition was confirmed by all three amino acid prediction tools used by antiSMASH 2.0 (7, 36). Hence, the assembled heptapeptide has the predicted amino acid sequence Hpg¹-β-Ht²-Dpg³-Hpg⁴-Hpg⁵-β-Ht⁶-Dpg⁷. The organization of the epimerization domains within the modules predicts a stereochemistry of L-D-L-D-D-L-L, which is consistent with the stereochemistry of teicoplanin (26), but inconsistent with L-D-D-D-D-L-D of A47934 (27) and D-D-L-D-D-L-L of balhimycin (35), respectively. *orf29*, located downstream of the NRPS genes encodes a 69-amino-acid small Mbth-like polypeptide. Mbth-like peptides are common features of NRPS biosynthesis gene clusters acting as facilitators of the peptide-assembling machineries by stimulating adenylation reac-

TABLE 1 Comparison of the *ris* cluster ORFs with ORFs from published glycopeptide clusters^a

ORF no.	Locus tag	Gene	Glycopeptide cluster ^b				Amino acid similarity (%)	Proposed function ^d	Biosynthetic role ^e
			No. of amino acids	BAL	STA	TEI			
1	AJAP_31985	<i>dhlp</i>	358	Dahp*	[-]	[-]	87	3-Deoxy-7-phosphoheptulonate synthase	Precursor supply
2	AJAP_31990	<i>dpdD</i>	263	DpgD*	DpgD	DpgD	90	Enoyl-CoA hydratase/enhances DpgA activity	Dpg formation
3	AJAP_31995	<i>dpnC</i>	404	DpgC*	DpgC	DpgC	90	3,5-Dihydroxyphenylacetyl-CoA oxidase	
4	AJAP_32000	<i>dpnB</i>	220	DpgB*	DpgB	DpgB	83	Enoyl-CoA hydratase/enhances DpgA activity	
5	AJAP_32005	<i>dpnA</i>	366	DpgA*	DpgA	DpgA	97	3,5-Dihydroxyphenylacetyl-CoA synthase (type III PKS)	
6	AJAP_32010	<i>gtfF</i>	575	[-]	[-]	[-]		Mannosyltransferase	Mannosyl transfer
7	AJAP_32015	<i>riaD</i>	205	DvaD*	[-]	[-]	91	dTDP-4-keto-6-deoxy-glucose-5-epimerase	Sugar/ristosamine formation
8	AJAP_32020	<i>riaC</i>	369	DvaB*	[-]	[-]	94	C-3 aminotransferase	
9	AJAP_32025	<i>riaB</i>	325	DvaE*	[-]	[-]	80	NAD-dependent epimerase/dehydratase	
10	AJAP_32030	<i>riaA</i>	469	DvaA*	[-]	[-]	92	NDP-hexose 2,3-dehydratase	
11	AJAP_32035	<i>hmo</i>	356	Hmo*	Hmo	Hmo	91	<i>p</i> -Hydroxymandelate oxidase	Hpg formation
12	AJAP_32040	<i>hmaS</i>	350	HmaS*	HmaS	HmaS	81	<i>p</i> -Hydroxymandelate synthase	
13	AJAP_32045	<i>oxyD</i>	396	OxyD*	[-]	[-]	92	P450 monooxygenase	β -Ht formation
14	AJAP_32050	<i>rpsE</i>	577	BpsB*	[-]	[-]	88	Nonribosomal peptide synthetase/adenylation domain Hydrolase	
15	AJAP_32055	<i>rhp</i>	274	Bhp*	[-]	[-]	91		
16	AJAP_32060	<i>pgat</i>	453	PgaT*	HpgT	HpgT	94	<i>p</i> -Hydroxy- and 3,5-dihydroxyphenylglycine aminotransferase	Dpg/Hpg formation
17	AJAP_32065	<i>mfbB</i>	346	[-]	[-]	[-]		Methyltransferase	Methylation
18	AJAP_32070	<i>gtfE</i>	605	[-]	[-]	[-]	79	Mannosyltransferase	Mannosyl transfer
19	AJAP_32075	<i>orf2</i>	281	Bal2	[-]	Orf2/Dbv21*	78	Inactive <i>N</i> -acetylglucosamine deacetylase	Sugar addition/deacetylation
20	AJAP_32080	<i>gtfD</i>	374	[-]	[-]	GtfA*	73	UDP- <i>N</i> -acetylglucosamine transferase	Peptide methylation
21	AJAP_32085	<i>mfa</i>	270	[-]	[-]	[-]		Methyltransferase	Sugar addition
22	AJAP_32090	<i>gtfC</i>	404	BgtfC*	[-]	[-]	70	UDP- <i>N</i> -acetylglucosamine transferase	
23	AJAP_32095	<i>gtfB</i>	405	BgtfB*	[-]	GtfB	81	UDP- <i>N</i> -acetylglucosamine transferase	
24	AJAP_32100	<i>gtfA</i>	385	BgtfA*	[-]	[-]	73	UDP- <i>N</i> -acetylglucosamine transferase	
25	AJAP_32105	<i>oxyC</i>	413	OxyC	StaJ	OxyC*	88	P450 monooxygenase	Cross-linking (aa 5 to 7)
26	AJAP_32110	<i>oxyB</i>	398	OxyB*	StaH	OxyB	90	P450 monooxygenase	Cross-linking (aa 4 to 6)
27	AJAP_32115	<i>oxyE</i>	385	[-]	StaG*	OxyE	85	P450 monooxygenase	Cross-linking (aa 1 to 3)
28	AJAP_32120	<i>oxyA</i>	391	OxyA	StaF	OxyA*	86	P450 monooxygenase	Cross-linking (aa 2 to 4)
29	AJAP_32125	<i>mbtH</i>	69	MbtH*	StaE	MbtH	94	Adenylation, loading of NRPS	Peptide synthesis/ β -Ht formation
30	AJAP_32130	<i>rpsD</i> (Dpg-TE)	1853	BspC (Dpg-TE)	StaD (Dpg-TE)	TeiD (Dpg-TE)		Nonribosomal peptide synthetase	Peptide synthesis
31	AJAP_32135	<i>rpsC</i> (Hpg-Hpg- β -Ht)	3994	BspC (Hpg-Hpg- β -Ht)	StaC (Hpg-Hpg- β -Ht)	TeiC (Hpg-Hpg- β -Ht)		Nonribosomal peptide synthetase	
32	AJAP_32140	<i>rpsB</i> (Dpg)	1050	BspB (Hpg-Hpg- β -Ht)	StaB (Dpg)	TeiB (Dpg)		Nonribosomal peptide synthetase	

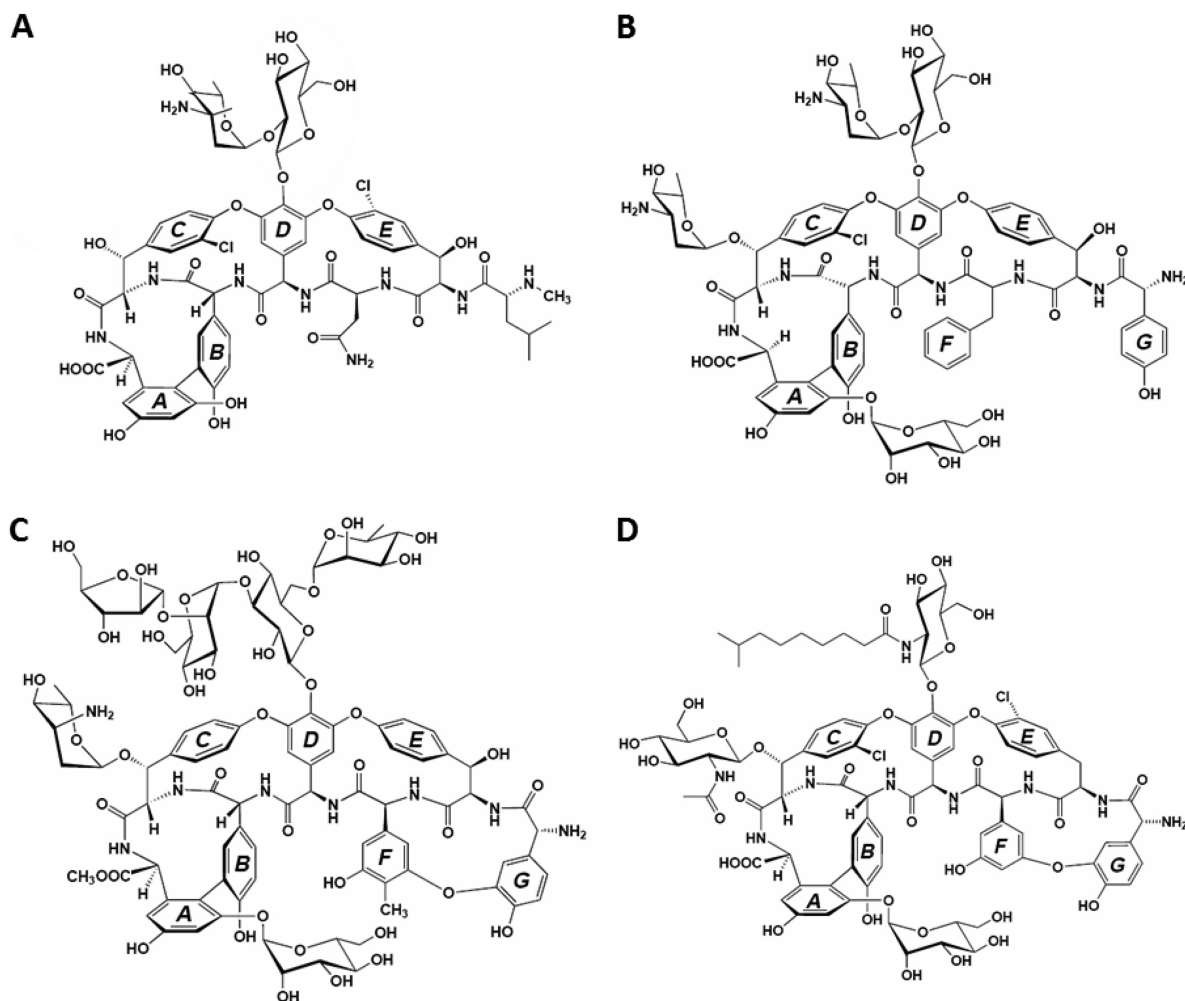


FIG 4 Classification of the glycopeptides. (A) Type I glycopeptides exemplified by vancomycin. These glycopeptides contain aliphatic chains in amino acids 1 and 3. (B) Type II glycopeptides exemplified by actinoidin A. These glycopeptides contain aromatic aliphatic chains in amino acids 1 and 3. (C) Type III glycopeptides exemplified by ristomycin A. These glycopeptides are like type II glycopeptides, and they contain an extra F-O-G ring system. Ring abbreviations: A, Me-L-Dpg; B, D-Hpg; C, L- β -Ht; D, D-Hpg; E, D- β -Ht; F, Me-L-Dpg; G, L-Hpg. (D) Type IV glycopeptides exemplified by teicoplanin. These glycopeptides are like type III glycopeptides plus they have aliphatic side chains on sugar.

are not directly involved in the synthesis and tailoring of the glycopeptide, are located at the left (*orf1*) and right (*orf34* to *orf39*) borders (Fig. 3). *orf39* to *orf37* encode the VanHAX-like glycopeptide resistance cassette. However, neither a *vanRS* (encoding the two-component system) nor a *vanY* (D,D-carboxypeptidase) homologue are located within the *A. japonicum* glycopeptide gene cluster. Previous cell wall precursor analyses (20) demonstrated that *A. japonicum* synthesized a glycopeptide-resistant cell wall, although no glycopeptide production could be observed, suggesting constitutive expression of the resistance genes.

The *orf34*-encoded protein is likely to specify export functions, since it is predicted to be an ABC-type transporter exhibiting high similarity to the balhimycin exporter Tba (15) (Table 1).

The *orf36* encodes a StrR-like transcriptional activator with high similarity to the balhimycin pathway-specific transcriptional activator Bbr_{Aba} (13) (see below).

Two additional genes, presumably encoding a 3-deoxy-7-phosphoheptulon synthase (DAHP synthase) homologue (*orf1*) and a prephenate dehydrogenase homologue (*orf35*) are predicted to opti-

mize the precursor supply of the aromatic amino acids (28). While prephenate dehydrogenase genes are encoded in all described clusters, the DAHP synthase-encoding gene is lacking in some gene clusters, like the teicoplanin and A47934 gene clusters (22).

Categorization of the *A. japonicum* glycopeptide by considering the genetic features. The *in silico* data strongly suggested that the final product of the identified gene cluster is a 6-fold-glycosylated, twice methylated, and fully cross-bridged glycopeptide. The genetic organization and domain composition of the NRPS specifies an amino acid sequence L-Hpg¹-D- β -Ht²-L-Dpg³-D-Hpg⁴-D-Hpg⁵-L- β -Ht⁶-L-Dpg⁷. The incorporation of aromatic amino acids at positions 1 and 3 together with the predicted complete cross-linked heptapeptide by the four oxygenases and the absence of any acyltransferase or halogenase imply that the *A. japonicum* gene cluster has the biosynthetic potential to produce a type III glycopeptide (Fig. 4). Until now, only the structure of the type III glycopeptides, exemplified by ristomycin A (produced by *A. lurida*) was known; no biosynthesis gene cluster was known. Therefore, the *A. japonicum* gene cluster identified

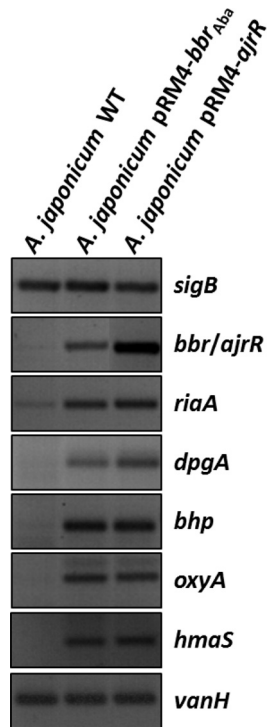


FIG 5 Transcriptional pattern of representative ristomycin biosynthesis genes. The gene names are indicated to the right of the gel. Cultures were grown in R5 medium for 25 h. *sigB* is the major sigma factor of *A. japonicum* and was used as a housekeeping gene to normalize the RNA. WT, wild type.

here is the first type III glycopeptide gene cluster described so far (Fig. 3). The amino acid sequence and stereochemistry of the glycopeptide encoded by the *A. japonicum* gene cluster are consistent with the sequence and stereochemistry of ristomycin A. Furthermore, the number and predicted function of the tailoring enzymes (six glycosyltransferases and two methyltransferases) are in agreement with the ristomycin A decorations (Fig. 4). Considering all these congruences of the described ristomycin A structure and the genetic content within the identified *A. japonicum* gene cluster, we propose that this gene cluster encodes a glycopeptide which is either ristomycin A itself or a highly similar derivative.

Expression of the *A. japonicum* StrR-like regulator AjrR. The two closely related species *A. japonicum* and *A. balhimycina* each harbor a glycopeptide gene cluster encoding the regulators AjrR and Bbr_{Aba}, respectively. These StrR-like regulators exhibit 84 and 91% amino acid identity and similarity, respectively. However, Bbr_{Aba} initiates the transcription of the glycopeptide gene cluster in *A. balhimycina*, while the *A. japonicum* gene cluster is of cryptic nature under the identical laboratory conditions.

One reason why the glycopeptide cluster in *A. japonicum* is not expressed under standard conditions could be that the regulator AjrR is not functional. To evaluate its functionality, we overexpressed *ajrR* under the control of the constitutive *ermEp** in *A. japonicum*. The supernatant of the recombinant *A. japonicum*/pRM4-*ajrR* inhibited growth of *B. subtilis*, while the supernatant of wild-type *A. japonicum* did not (Fig. 1). HPLC-DAD analyses confirmed the biosynthesis of the glycopeptide (data not shown). These results demonstrated the *in vivo* functionality of AjrR and

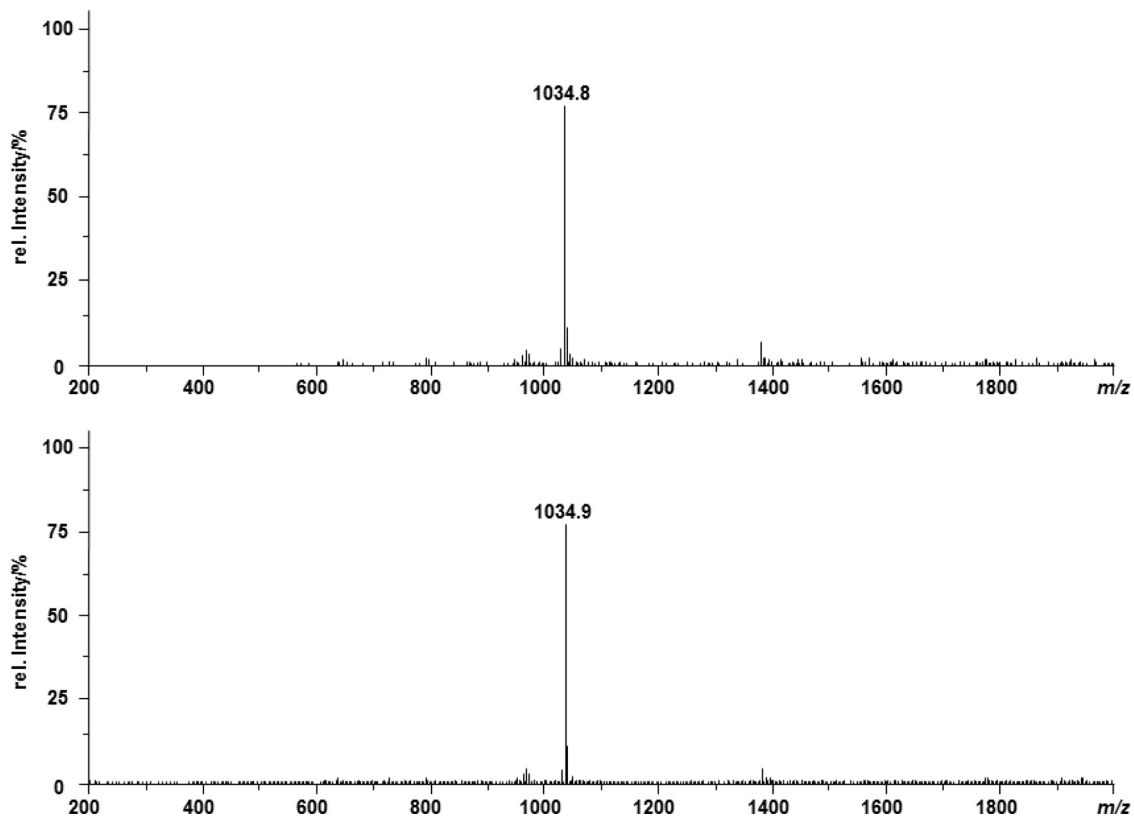


FIG 6 HPLC-ESI-MS analysis (positive mode) of *A. japonicum* glycopeptide and ristomycin A. (Top) Ristomycin A standard (0.1 mg ml^{-1}). (Bottom) *A. japonicum*/pRM4-*bbr*_{Aba} glycopeptide. $[\text{M} + 2\text{H}]^{2+}$ was observed at m/z 1,034.8 and 1,034.9. The relative intensity is shown on the y axes.

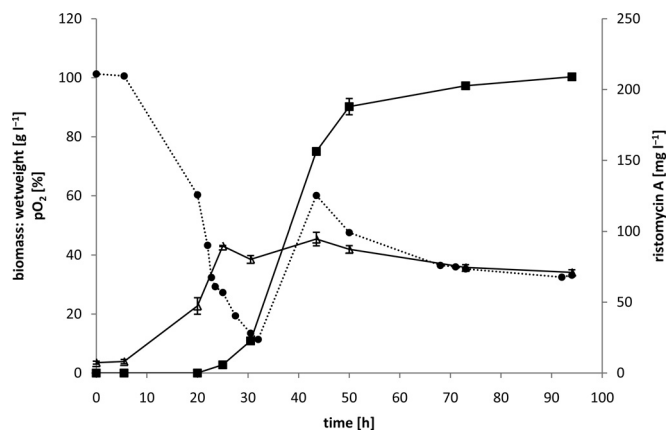


FIG 7 Batch fermentation of *A. japonicum*/pRM4-*bbr*_{Aba} in R5 medium. Biomass (Δ), ristomycin A (\blacksquare), and partial O_2 pressure (pO_2) (\bullet) are depicted. *A. japonicum*/pRM4-*bbr*_{Aba} reached its maximal biomass value after 44 h of incubation, decreasing afterwards slowly during 2 days of further fermentation. Metabolizing of different sugars in R5 medium (sucrose [α -1,2-glycosidic linked glucose and fructose] and glucose) is reflected by a diauxic shift (between 24 and 44 h) during cultivation, apparent in growth retardation and pO_2 .

suggest that *ajrR* is not transcribed in the wild-type species. Therefore, transcriptional analyses of *ajrR* and representative biosynthetic genes (*riaA*, *dpgA*, *bhp*, *oxyA*, *hmaS*, and *vanH*) were performed after growth of wild-type *A. japonicum*, *A. japonicum*/pRM4-*bbr*_{Aba} and *A. japonicum*/pRM4-*ajrR* in production medium R5 (Fig. 5). In wild-type *A. japonicum*, no *ajrR* transcription was detected, and it is therefore not surprising that no other investigated biosynthetic gene was transcribed. However, in *A. japonicum*/pRM4-*ajrR* and *A. japonicum*/pRM4-*bbr*_{Aba}, transcription of all the investigated genes could be observed. Coincident to the observations of Schäberle et al. (20) that *A. japonicum* produces a glycopeptide-resistant cell wall, whereas no glycopeptide production could be detected, we could observe transcription of the *vanH* resistance gene in the wild type. It will be interesting to ascertain why *bbr*_{Aba} is transcribed under standard conditions whereas *ajrR* is not.

Isolation and spectroscopic characterization of the *A. japonicum* glycopeptide. In order to confirm the assumption de-

duced from the genome sequence, the chemical structure of the glycopeptide was analyzed. Initial experiments with *A. japonicum*/pRM4-*bbr*_{Aba} growing in shake flasks revealed a glycopeptide production with quantities up to 50 mg liter⁻¹. The crude extract was fractionated and analyzed for the presence of the glycopeptide (Fig. 2). A major compound with a quasimolecular ion $[M + 2H]^{2+} = m/z$ 1,034.8 (Fig. 6) and a minor compound with $[M + 2H]^{2+} = m/z$ 887.7 (data not shown) were detected. Since the masses of the major and minor components were in agreement with the masses of ristomycin A and B, the isolated glycopeptides were compared with a commercially available ristomycin standard. HPLC-DAD and HPLC-ESI-MS analyses showed that both the glycopeptides synthesized by *A. japonicum*/pRM4-*bbr*_{Aba} and ristomycins A and B eluted at the same time, possessed the same UV profile (Fig. 2), and showed in ESI-MS/MS analyses (positive and negative mode) an identical fragmentation pattern (see Fig. S1 to Fig. S3 in the supplemental material).

To corroborate the findings and to obtain additional information on the nature of the sugar units, the major glycopeptide synthesized by *A. japonicum*/pRM4-*bbr*_{Aba} was isolated in a pure form and compared with a ristomycin A standard employing NMR, CD, and high-resolution mass spectrometry. For this purpose, the initial shake flask cultivation was scaled up to a 20-liter volume using a Giovanola b20 fermentor. Glycopeptide production was detected after 24 h and reached a maximum amount up to 200 mg liter⁻¹ after 50 h and stayed almost constant during 2 more days of fermentation (Fig. 7). For the isolation of the glycopeptide, 1 liter of fermentation broth was taken after 50 h of fermentation and separated by centrifugation into supernatant and mycelium. Subsequent workup of the extract led to the isolation of the major glycopeptide in a highly pure form at a yield of 37 mg l⁻¹. HR-MS analysis confirmed that the isolated glycopeptide showed the same exact mass as that of the ristomycin A standard and therefore possessed the same molecular formula of C₉₅H₁₁₀N₈O₄₄ (see Fig. S4 and S5 in the supplemental material). ¹H and the ¹³C NMR spectra of the isolated glycopeptide and the ristomycin A standard were absolutely superimposable, with the exception of additional peaks in the commercially available ristomycin standard which can be attributed to the presence of up to 10% ristomycin derivatives in the sample (Fig. S6 to S14). Further analysis of the one-dimensional (1D) and two-dimensional (2D) NMR data (Fig. S15

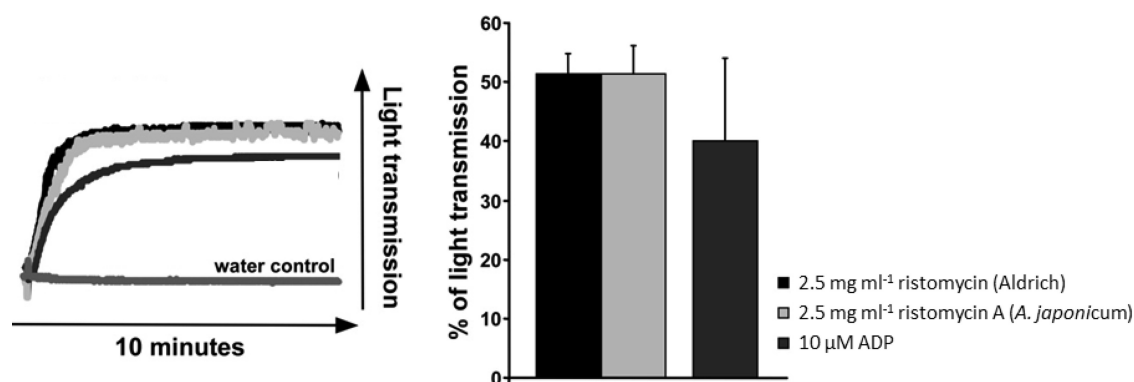


FIG 8 Ristomycin-dependent platelet aggregation. (Left) Representative tracings of aggregometry after stimulation of human platelets with 2.5 mg ml⁻¹ commercial (black line) or *A. japonicum* ristomycin A (light gray line) as well as 10 μ M ADP (dark gray line) and water control. (Right) Results of aggregometry after stimulation of human platelets with 2.5 mg ml⁻¹ commercial or *A. japonicum* ristomycin A as well as 10 μ M ADP. The values are arithmetic means plus standard errors of the means (SEM) (error bars) for four experiments.

to Fig. S20) allowed the complete assignment of all hydrogen and carbon atoms (Table S3), which supported the hypothesis that the isolated compound possessed the same planar structure and the same relative configuration as that of ristomycin A. Since the CD spectra of the isolated glycopeptide and a commercially available ristomycin A standard were consistent (Fig. S21), it was deduced that the isolated glycopeptide also possessed the same absolute configuration as that of ristomycin A. From these data, we concluded that the identified cryptic gene cluster is responsible for the production of ristomycin A.

Ristomycin A-dependent platelet aggregation. Measuring von Willebrand factor (vWF) activity is essential for the diagnosis of von Willebrand disease (vWD). A common test method is the ristomycin platelet-induced agglutination method, which allows discrimination among specific subtypes of the vWD. In a platelet aggregation assay, we could demonstrate that ristomycin A isolated from *A. japonicum* has the same *in vitro* function as the reference ristomycin (Fig. 8).

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