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REVIEW

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Understanding and manipulating glycopeptide pathways: the example of the dalbavancin precursor A40926

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Abstract Glycopeptide antibiotics represent an important class of microbial compounds produced by several genera of actinomycetes. The emergence of resistance to glycopeptides among enterococci and staphylococci has prompted the search for second-generation drugs of this class and semi-synthetic derivatives are currently under clinical trials. Dalbavancin is obtained by chemical modification of the natural glycopeptide A40926, produced by a *Nonomuraea* sp. Recently, there has been considerable progress in the elucidation of biosynthesis of glycopeptide antibiotics; several gene clusters have been characterized, thus providing an understanding of the biosynthesis of these chemically complex molecules. Furthermore, such investigations have yielded the first glycopeptide derivatives produced by genetic or enzymatic intervention. We have isolated and characterized the *dbv* clusters, involved in the formation of the glycopeptides A40926. The development of a gene-transfer system for *Nonomuraea* sp. has allowed the manipulation of the A40926 pathway. New derivatives were obtained by inactivating selected *dbv* genes. In addition, our data suggest differences in the biosynthetic routes for heptapeptide formation between the vancomycin and the teicoplanin families of glycopeptides.

Introduction

Glycopeptides are an important class of antibiotics produced by different genera of actinomycetes. The two clinically used glycopeptides, vancomycin and teicoplanin, are particularly effective against Gram-positive bacteria, and they often represent the last line of defense against life-threatening infections due to multi-resistant pathogens. They exert their antibacterial action through the high affinity of the aglycon cavity towards the D-alanyl-D-alanine moiety of the growing bacterial peptidoglycan [15, 17, 24].

While structurally similar, vancomycin and teicoplanin actually belong to different glycopeptide families. Within their heptapeptide scaffold, they share the non-proteinogenic amino acids: β-hydroxytyrosine (Bht, at position 6), 4-hydroxyphenylglycine (Hpg, at positions 4 and 5), and 3,5-dihydroxyphenylglycine (Dpg, at position 7). Compounds in the vancomycin family present Leu, Bht, and Asn as residues 1, 2, and 3, respectively, while these positions are occupied by Hpg, Tyr, and Dpg in the teicoplanin family (Fig. 1). In addition, there are three cross-links of the aryl side chains in vancomycin and related compounds, while four cross-links exist in the teicoplanin family. Among the glycopeptides referred to here, balhimycin and chloroeremomycin belong to the vancomycin family, while A40926 and A47934 are related to teicoplanin (Fig. 1). In addition to the cross-linked heptapeptide, glycopeptides may present chlorine atoms, *N*-methyl and sulphate groups, and different sugars (attached at residues 4, 6, and 7). Teicoplanin and A40926 present also a long acyl chain linked as an amide to an amino sugar moiety attached to residue 4 (Fig. 1). This aliphatic moiety influences their pharmacokinetic behavior [1].

The increasing incidence of serious nosocomial infections due to multi-resistant Gram-positive bacteria has prompted the search for glycopeptide derivatives with improved properties. Substantial effort has been devoted to the chemically accessible portions of these complex molecules, leading to the generation of many glycopeptide derivatives, some of which are now in clinical trials [1, 11, 15, 22]. Among them, the most advanced molecule is dalbavancin (Fig. 1), a semi-synthetic derivative of A40926 [22] that shows an improved spectrum and pharmacokinetics over existing glycopeptides [9].

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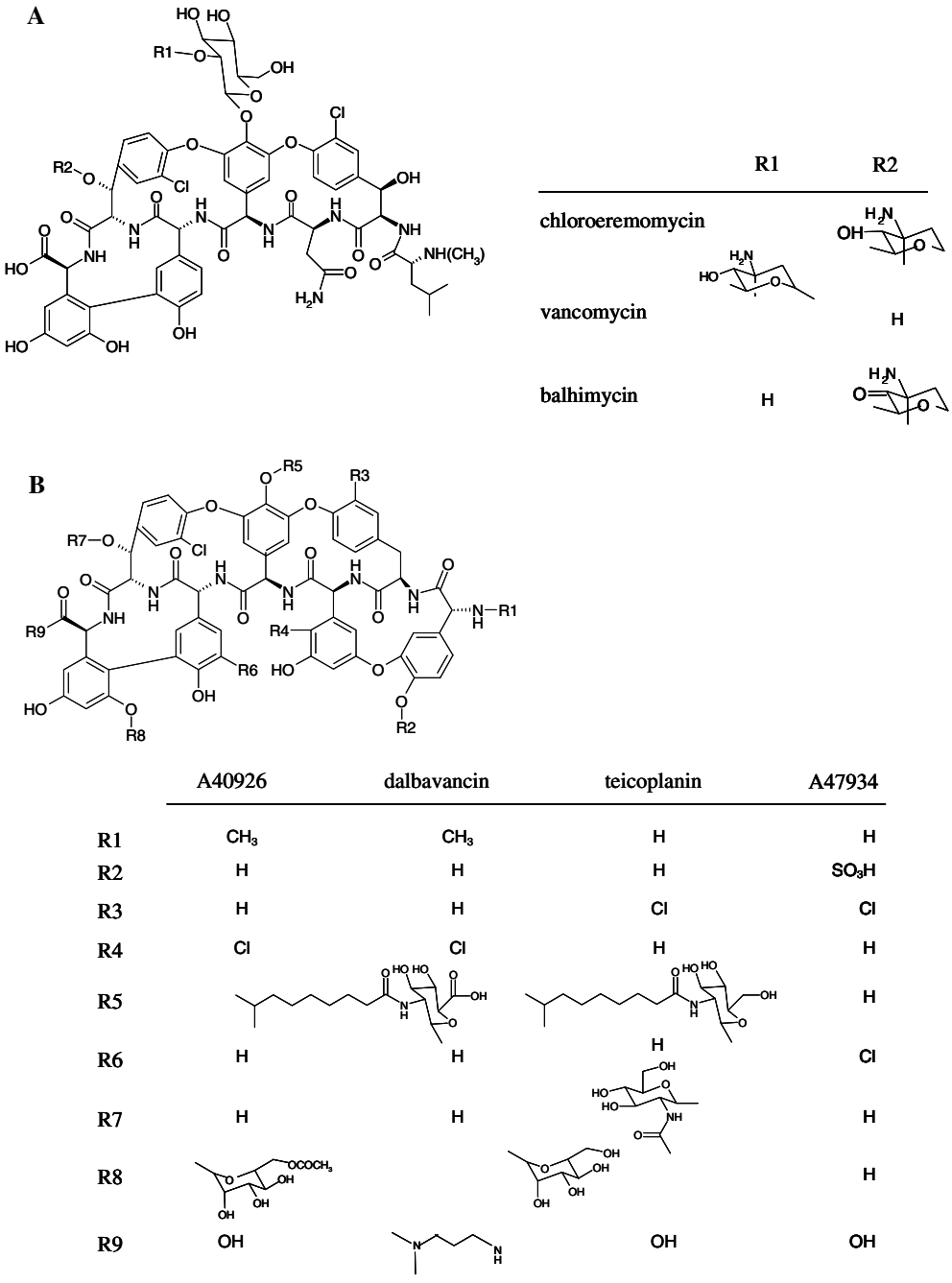

Fig. 1 Structure of vancomycin-type (**a**) and teicoplanin-type (**b**) glycopeptides

Another approach to generate novel glycopeptides is through manipulation of the biosynthetic pathway. For this reason, there has recently been considerable progress in elucidating the biosynthetic steps of glycopeptide antibiotics. Gene clusters for the biosynthesis of chloroeremomycin (*cep*), balhimycin (*bal*), A47934 (*sta*), A40926 (*dbv*), and teicoplanin (*tcp*) have been reported [18, 26, 28, 34, 35, 43]. Gene disruption experiments and chemoenzymatic studies have contributed to the elucidation of most steps involved in glycopeptide biosynthesis, while enabling also the generation of novel compounds [13, 15, 39].

Here, we provide an overview of the current understanding of glycopeptide formation, with a particular focus on the role of the *dbv* genes.

Genetic tools for *Nonomuraea* sp. ATCC 39727 and the A40926 gene cluster

The glycopeptide clusters reported to date have been derived from two *Streptomyces* spp. (*sta* and the complestatin gene cluster [7]), from two *Amycolatopsis* spp.

(*cep* and *bal*), from an *Actinoplanes* sp. (*tcp*), and from a *Nonomuraea* sp. (*dbv*). Among the producer strains, *Amycolatopsis balhimyicina* [25] and *Streptomyces toyocaensis* [23] can be genetically manipulated. To expand the possibilities of genetic manipulation within the glycopeptides, we developed a gene-cloning system for *Nonomuraea* sp. ATCC 39727 based on conjugation from *E. coli* [37]. This procedure has allowed disruption of selected *dbv* genes, while heterologous genes can be introduced at a neutral *attB* site [37].

The gene cluster for A40926 biosynthesis has been cloned from *Nonomuraea*, leading to the identification, within a 77-kbp DNA segment, of 37 genes (see Fig. 2) to which putative roles in biosynthesis, export, resistance, and regulation were assigned [33]. From the deduced functions of the *dbv* gene products and by comparison with other glycopeptide clusters, the A40926 synthesis is expected to require participation of over 40 distinct enzymatic functions. These include formation of the non-proteinogenic amino acids, heptapeptide synthesis, aryl cross-links and aglycone decorations, as illustrated below.

Biosynthesis of the non-proteinogenic amino acids

Hpg, Dpg, and Bht are crucial amino acids for glycopeptide antibiotics. The enzymatic steps required for the biosynthesis of these specialized monomers have been elucidated through studies with the chloroeremomycin and balhimycin pathways [5, 14, 27, 29].

Work done with the purified *cep* DpgABCD proteins [5] and with the *bal dpgABCD* genes [27], has shown that this set of four enzymes is necessary to convert four molecules of malonyl-CoA into 3,5-dihydroxyphenylglyoxylate, consistent with feeding experiments using ^{13}C-labeled precursors in *A. orientalis* [13]. DpgA is a type-III polyketide synthase [42]. Expression of the *bal dpgABCD* genes in *Streptomyces lividans* led to accumulation of 3,5-dihydroxyphenylglyoxylate in ethyl acetate extracts. In addition, a deletion of the *hpgT* gene in *A. bahlimycina* abolished the antibiotic production and led to the accumulation of 3,5-dihydroxyphenyglyoxylate. Restoration of antibiotic production was only possible by simultaneous supplementation with Dpg and Hpg, indicating that the transaminase HpgT is essential for the formation of both amino acids [27]. Orthologous genes encoding the DpgABCD enzymes are arranged as a four gene cassette in all glycopeptide clusters, and are expected to participate in analogous reactions in A40926 biosynthesis.

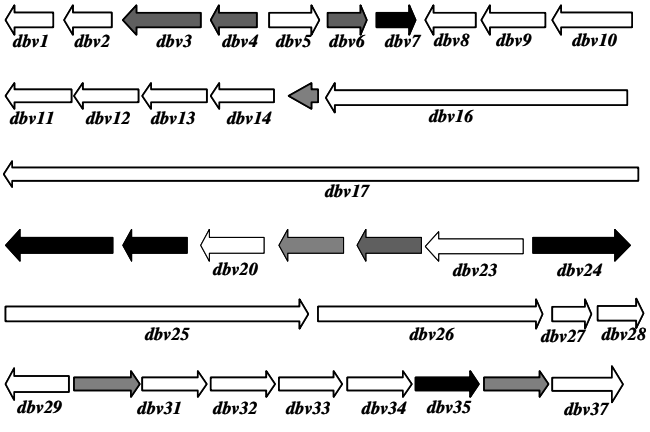

Fig. 2 Genetic organization of *dbv* cluster. Only the genes mentioned in the text are *numbered*. *Black arrows* denote genes with resistance and/or export functions, *while gray arrows* refer to regulatory genes. *Shaded arrows* indicate genes of unknown functions

Following the labeling studies suggesting Tyr as the Hpg precursor [13], new insights came from the demonstration that three *cep* enzymes, *p*-hydroxymandelate synthase (HmaS), *p*-hydroxymandelate oxidase (Hmo), and the L-*p*-hydroxyphenylglycine transaminase HpgT are involved in Hpg formation from Tyr and possibly also from prephenate, through the action of a cluster-associated prephenate dehydrogenase [14]. The three enzymes were proposed to function in a cyclic manner with Tyr acting both as the amino donor for *p*-hydroxyphenylglyoxylate and as a source of *p*-hydroxyphenylpyruvate, the substrate for HmaS [14]. Orthologues of Hmo, HmaS, and HpgT are encoded by the other glycopeptide clusters.

Bht is present at positions 2 and 6 in the vancomycin family, and only at position 6 in the teicoplanin-type compounds. The synthesis of this unusual amino acid seems to follow different routes in the two glycopeptide families. In the *bal* cluster, three contiguous genes, *bhp*, encoding a hydrolase, *bpsD*, encoding adenylation (A) and thiolation domains of non-ribosomal peptide synthetases (NRPS), and *oxyD*, encoding a P450 monooxygenase, form an operon and participate in Bht synthesis [29, 30]. In-frame deletion of any of these genes in *A. bahlimyicina* resulted in mutants unable to produce balhimycin. Glycopeptide production could be restored by feeding Bht, but not 3-chloro-β-hydroxy-tyrosine, indicating that Bht is the precursor of the non-ribosomal peptide synthetase and suggesting that chlorine is added after amino acid recognition by the NRPS [29, 30]. Work performed on similar enzymes involved in nikkomycin biosynthesis unraveled the enzymatic details [6], and the likely path during balhimycin biosynthesis. In fact, tyrosine is activated by BpsD and covalently bound as a thioester to its thiolation domain, consistent with a Tyr-specific adenylation domain in BpsD [31]; subsequently, the enzyme-bound Tyr residue is hydroxylated on the benzylic carbon by OxyD, generating Bht-*S*-BpsD. This aminoacyl-*S*-protein is the likely substrate for release of free Bht by the hydrolase Bhp.

The *dbv*, *tcp*, and *sta* clusters do not specify homologues of *oxyD*, *bhp*, or *bpsD*. Instead, each encodes a putative non-heme dioxygenase, exemplified by *dbv28*, a likely candidate for Tyr hydroxylation. Generation of a *Nonomuraea* mutant with a deleted *dbv28* resulted in the formation of an A40926 derivative containing a Tyr

residue instead of Bht at position 6 [38]. Further evidence (see below) suggests that Bht may not be the substrate for the *dbv* NRPS, as proposed in Fig. 3.

Synthesis of the heptapeptide

Once the amino acid monomers become available in the producing cell, the NRPS assembly line can make the heptapeptide backbone. Synthesis of the glycopeptide skeleton is catalyzed by three distinct polypeptides for chloroeremomycin and balhimycin, and by four polypeptides in the other cases (Fig. 4). A peculiarity of the *dbv* cluster is the occurrence of two divergently transcribed gene pairs, separated by about 12 kb (Fig. 2), whereas the NRPS genes are organized as a single operon in the other four clusters. Notwithstanding the different genetic organizations, the glycopeptide NRPSs consists of seven modules, and share an almost identical domain composition (Fig. 4). The genetic organization appears co-linear with module specificity for the *bal*, *cep*, *sta*, and *tcp* NRPSs [31, 41]. Peculiarities common to all glycopeptide NRPSs are a missing epimerization domain in module 1 (expected from the stereochemistry of the first amino acid) and an extra domain of unknown function (resembling condensation and epimerization domains) flanking the terminal thioesterase domain (Fig. 4). Although it is still unresolved at which stage chlorinations and cross-linkings occur on the NRPS-assembly line, the heptapeptidyl chain is likely released by this thioesterase.

The two sets of glycopeptides, balhimycin, and chloroeremomycin on the one hand, and A40926, A47934, and teicoplanin on the other, are predicted to originate through the incorporation of identical amino acids by

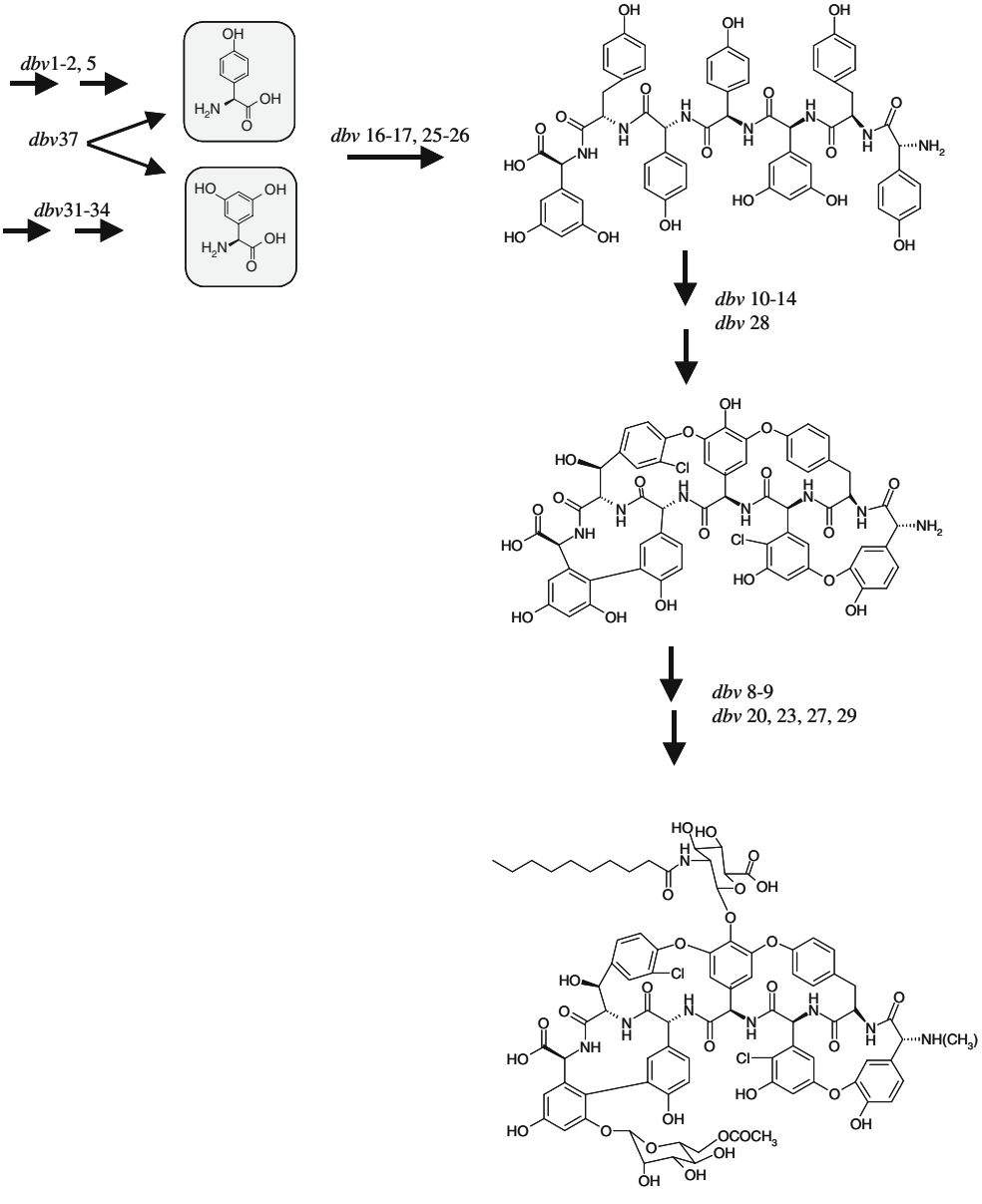

Fig. 3 Schematic illustration of the sequential enzymatic reactions in the biosynthesis of A40926, and the genes proposed to be involved

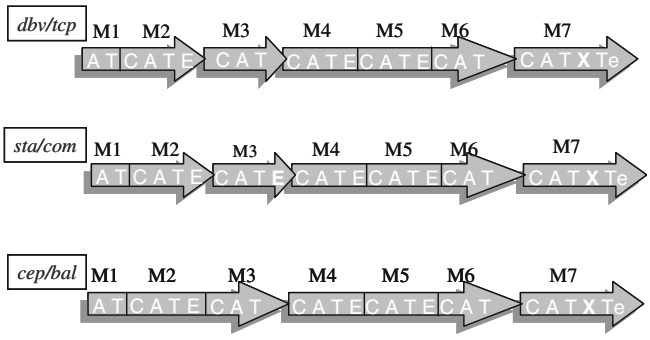

Fig. 4 Schematic representation of the module organization of the NRPS. Modules abbreviated as: *A* adenylation, *C* condensation, *E* epimerisation, *T* thiolation, *Te* thioesterase, *X* unknown function

the A domains from modules 4, 5, and 7 (A-4, A-5, and A-7, respectively), while different amino acids must be recognized by A-1 and A-3. Since A40926, A47934, and teicoplanin contain an unmodified Tyr residue at position 2, it seems reasonable to assume that Tyr is the substrate for this module, while the *bal* and *cep* systems incorporate Bht. A non-ribosomal code has been proposed for the A domains on the basis of the amino acid residues present in the substrate-binding pocket [4, 36]. A compilation of the amino acids proposed as signature for the non-ribosomal code is reported in Table 1. While the two sets present significant differences in the signature residues for modules 1 and 3, they are virtually identical for the remaining modules, including module 2. This suggests that other A domain residues, in addition to those already identified [4, 36], contribute to specificity of substrate amino acid recognition. Since the glycopeptide NRPSs have a common origin [8], similarity levels among the A domains might be correlated with substrate specificity. A phylogenetic analysis of the A domains is illustrated in Fig. 5. It can be seen that the branching patterns are coherent with the corresponding modules, except for the repartition of the A-3 domains into two distant subfamilies, *bal–cep* versus *dbv–sta–tcp*. For the homospecific modules 4, 5, and 7, the branching pattern appears relatively random, except for a higher relatedness between the *bal* and *cep* sequences, and for a tendency of the *dbv* domains to cluster with *bal–cep*, as seen with many *dbv* genes [8]. The A-1, A-2, and the A-6 domains, instead, branch into distinct subfamilies, with *bal–cep* on one side and *dbv–sta–tcp* on the other (Fig. 5). This is consistent with the different specificities expected for modules 1 and 2 in the two NRPS sets, and suggests that module 6 might also recognize different amino acids in the two sets. Since Bht is the substrate for the *bal* module 6 (and presumably for *cep* too), the corresponding *dbv* module (and presumably the *sta* and *tcp* modules too) may recognize Tyr instead, consistent with the results from deletion of *dbv28* [38]. If this is the case, then hydroxylation must occur after amino acid recognition by the NRPS.

Cross-linking and halogenation

Cross-linking of the aromatic side chains occur in order to rigidify the heptapeptide and create the high-affinity site for the cell-wall target. As demonstrated for balhimycin, these reactions are carried out by P450 monooxygenases encoded by three contiguous *oxy* genes. Likewise, in the *dbv* cluster, four *oxy* genes are present, consistent with the additional cross-link between the aryl side chain at amino acid residues 1 and 3 in A40926 (Fig. 1). Inactivation of the *oxy* genes in *A. balhimycina* and analysis of the accumulated intermediates has established the role for each P450 enzyme and a proposed order for the oxidation steps: the first OxyB catalyzes oxidative formation of the ether link between amino acid residues 4 and 6 (4–6 link), then OxyA carries out the 2–4 ether coupling, and the last OxyC forms the 5–7 C–C bond [2, 3]. Recent work has indicated that OxyB actually acts on a thioester-bound hexapeptide intermediate, raising the intriguing possibility that formation of the oxidative cross-links requires an interaction between the NRPS and the P450 enzymes [47].

Many glycopeptides are characterized by the presence of chlorine atoms on the aryl moieties: one each on residues 2 and 6 in balhimycin, chloroeremomycin, and teicoplanin; one each on residues 3 and 6 in A40926; and one each on residues 3, 5, and 6 in A47934 (Fig. 1). A single halogenase gene is present in the *bal*, *cep*, *dbv*,

Table 1 Amino acid residues defining the A domain specificity pocket

	D-235		A-236		W-239		T-278		I-299		A-301		A-322		I-330		K-517	
	bc	dst	bc	dst	bc	dst	bc	dst	bc	dst	bc	dst	bc	dst	bc	dst	bc	dst
A-1	D	D	A	A	F	F	Y	H	L	L	G	G	M	L	M	L	K	K
A-2	D	D	T	A	S	S	H	H	V	V	A	A	A	A	I	V	K	K
A-3	D	D	L	A	T	Y	H	N	L	L/A^{d}	G	G	E	T	V	L	K	K
A-4	D	D	I	I	F	F	H	H	L	L	G	G	L	L	L	L	K	K
A-5	D	D	A	A	V	L/F^{s}	H	H	L	L	G	G	L	L	L	L	K	K
A-6	D	D	A	A	S	S	H	H	L	V/I^{t}	G	A	A	G/A^{t}	I	V	K	K
A-7	D	D	P	P/A^{s}	Y	Y	H	H	G	G	G	G	T	T	L	L	K	K

The top row shows the residues and their positions in the GrsA sequence [4, 36]. Clusters are abbreviated as *b*, *c*, *d*, *s*, and *t*, for *bal*, *cep*, *dbv*, *sta*, and *tcp*, respectively. When two different residues are present at a given position in the *dbv–sta–tcp* set, both are reported and a superscripts, abbreviated as before, indicates the A domain showing a different amino acid

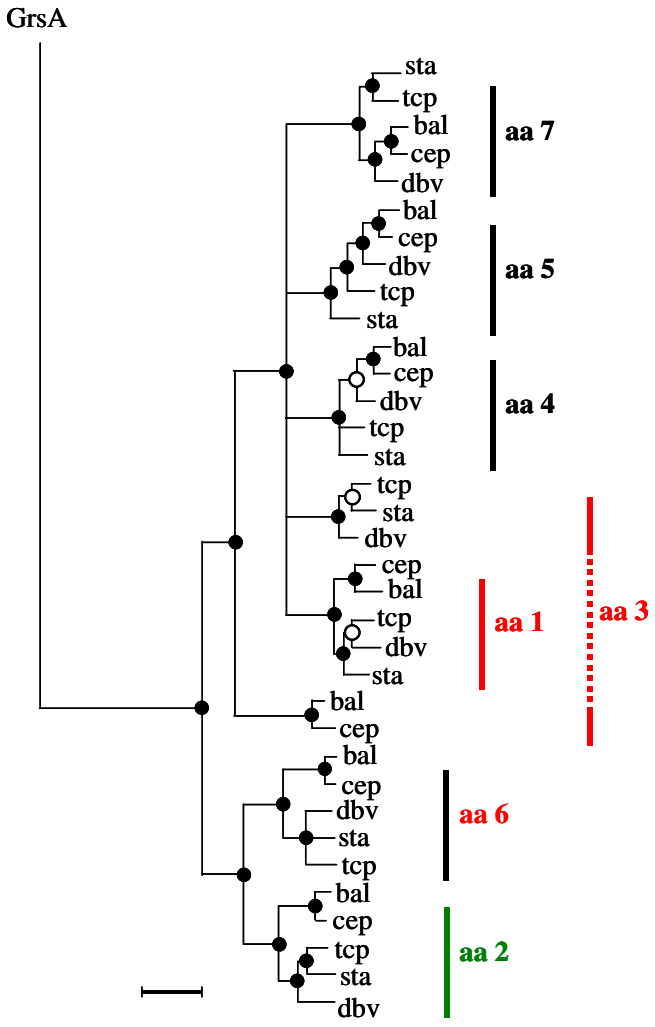

Fig. 5 Phylogenetic analysis of adenylation domains. The figure reports a tree of the seven A domains, using GrsA as out-group. The *bar* represents inferred 100 nucleotide substitutions per 100 residues. Branch points supported by bootstrap resampling are denoted by *solid* ($\geq$ 80) or *open circles* ($<$ 80). The *thick vertical lines* delimit the corresponding NRPS modules

and *tcp* clusters, while the *sta* clusters contain two such genes. Deletion of the bahlimycin halogenase gene *bhaA* yields dechloro-balhimycin [39]. Deletion of *dbv10* also abolished halogenation, indicating that a single gene is able to modify two chemically distinct moieties during A40926 biosynthesis [34]. The actual substrates for halogenation have not been identified yet. Mutants blocked in the cross-linking reactions accumulate chlorinated linear peptides [26, 29]. It is also possible that halogenation, as the cross-linking reaction, requires a direct interaction of the halogenase with NRPS-bound peptides. If tyrosine β-hydroxylation does occur on an NRPS-bound intermediate during A40926 biosynthesis, then a third type of enzyme would act during heptapeptide synthesis. Whatever the order and timing of events, the fully cross-linked and halogenated heptapeptide is the aglycone intermediate for subsequent tailoring steps.

Aglycone-decorating steps

The major source of diversity in glycopeptides originates from the tailoring steps occurring on the aglycone. In the case of A40926, the final molecule presents an *N*-methyl on residue 1, an *N*-acyl-aminoglucuronic acid moiety on residue 4, and an acetyl-mannosyl group on residue 7 (Fig. 1). Consistently, the *dbv* cluster contains genes encoding a methyltransferase (*dbv27*), a glycosyltransferase (*dbv9*), a hexose oxidase (*dbv29*), an acyl transferase (*dbv8*), a mannosyltransferase (*dbv20*), and an acetyltransferase (*dbv23*). The exact order of these steps is not precisely known. Deletion of *dbv8–10* results in the formation of dechloromannosyl-A40926, indicating that *N*-methylation and mannosylation can occur in the absence of the glycosidic moiety at position 4 [34]. Deletion of *dbv23* results in the production of desacetyl-A40926 (M. Sosio et al., unpublished data). We hypothesize that attachment of glucosamine and *N*-methylation occur in the cytoplasm, and are followed by sugar *N*-acylation. Sugar oxidation, mannosylation, and mannose acetylation may occur once the glycopeptide has been exported from the cell. It should be noted that the glycosyltransferases attaching sugars to amino acids 4 and 6 utilize NDP-activated sugars as substrates. In contrast, the mannosyltransferases belong to the protein mannosyltransferase family and are likely to utilize a different activated sugar.

Novel glycopeptide derivatives

As understanding of glycopeptide biosynthesis was increasing, novel derivatives were obtained by a combination of mutasynthesis, heterologous gene expression, and chemoenzymatic approaches.

Novel amino acids were incorporated by the NRPS using mutasynthesis. The observation that feeding Bht to a Δ*bhp* strain restored balhimycin production prompted the generation of fluorobalhimycins upon feeding 3-fluoro-β-hydroxytyrosine [45]. Similarly, feeding a *dpgA* mutant with Dpg analogues and their putative precursors resulted in completed cyclized aglycone containing methoxy groups at amino acid residues 7 [46].

The glycosyltransferases have been the object of intense investigations leading to novel glycopeptides [12, 15, 18–21, 33, 35]. These enzymes attach neutral or amino sugars at positions 4 and 6, and can further glycosylate the sugar moiety present at position 4. They appear to be relatively tolerant of aglycone structures, and the *cep* enzyme GtfB can attach glucose to the teicoplanin aglycones, while the teicoplanin glycosyltransferase can attach glucosamine to a vancomycin aglycon [12, 15, 19–21, 33]. In a combinatorial fashion, the sugars from chloroeremomycin and teicoplanin can be added to any glycopeptide aglycone. Chemical diversity can be further expanded by using the *N*-acyltransferase (exemplified

by *dbv8* and its *tcp* orthologue), which can be used to add an acyl chain to glycopeptides aglycones previously decorated with different sugars [16, 18].

Export, resistance, and regulation

The A40926 biosynthetic scheme (Fig. 3) accounts for 24 *dbv* genes. On the basis of sequence similarities, eight genes are predicted to encode the elements involved in export, resistance, or regulation. However, limited information is available on these topics in glycopeptide producers.

In the *dbv* cluster, four genes are likely to play a regulatory function [34]: *dbv6* and *dbv22* are predicted to encode the response regulator and the sensor kinase of a two-component signal transduction system; *dbv3* and *dbv4* encode putative positive regulators. Although their roles have not been determined yet, it is worth noting that *dbv4* orthologues are present in all glycopeptide clusters [8]. Their gene products belong to StrR-family of positive regulator, which are known to bind to specific DNA motifs in the promoter regions of selected genes [10, 40]. This suggests that common regulatory mechanisms control some aspects of glycopeptide formation.

Glycopeptide resistance has been intensively studied in pathogenic enterococci [15, 44]. In the glycopeptide producers, *vanHAX* genes are associated with the *sta* and *tcp* clusters, and they have been shown to confer glycopeptide resistance to the producer strain [28] or to a heterologous host [32]. In the *bal* and *dbv* clusters, resistance might be conferred by a VanY-homologue (encoded by *dbv7*), while no resistance determinants are associated with the *cep* cluster. It should be noted that glycopeptide producers face a significant challenge for glycopeptide resistance in comparison with pathogenic bacteria. Indeed, while non-producing strains need to protect their extracytoplasmic targets only, glycopeptide producers have to cope also with the possible binding of these antibiotics to their D-alanyl-D-alanine-ending cytoplasmic targets. Interestingly, all glycopeptide clusters contain orthologues specifying an ABC transporter and a membrane ion antiporter [8], exemplified by *dbv24* and *dbv35*, respectively. It is conceivable that one or both genes may be involved in protecting intracellular targets from inhibition by glycopeptides.

Conclusions and future perspectives

Molecular analysis of glycopeptide biosynthesis has provided unexpected enzyme chemistry for the synthesis of these complex compounds, as well as different logics for the synthesis of similar intermediates. In addition, the use of different approaches has allowed the generation of several glycopeptide derivatives that would have been inaccessible by chemical means. As our understanding of glycopeptide pathways increases, including insight into resistance and regulation, so will our ability to manipulate these complex pathways for increased yields and novel compounds.

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