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The Gene Cluster for the Biosynthesis of the Glycopeptide Antibiotic A40926 by *Nonomuraea* Species

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Summary

The glycopeptide A40926 is the precursor of dalbavancin, a second-generation glycopeptide currently under clinical development. The *dbv* gene cluster, devoted to A40926 biosynthesis, was isolated and characterized from the actinomycete *Nonomuraea* species ATCC39727. From sequence analysis, 37 open reading frames (ORFs) participate in A40926 biosynthesis, regulation, resistance, and export. Of these, 27 ORFs find a match in at least one of the previously characterized glycopeptide gene clusters, while 10 ORFs are, so far, unique to the *dbv* cluster. Putative genes could be identified responsible for some of the tailoring steps (attachment of glucosamine, sugar oxidation, and mannosylation) expected during A40926 biosynthesis. After constructing a *Nonomuraea* mutant by deleting *dbv* ORFs 8 to 10, the novel compound dechloromannosyl-A40926 aglycone was isolated.

Introduction

Glycopeptides are an important class of antibiotics that interfere with bacterial cell wall synthesis, with vancomycin and teicoplanin currently used in the clinic for treating life-threatening infections caused by gram-positive bacteria [1]. The emergence of resistance to glycopeptides among enterococci and the fear that this high-level resistance may eventually become widespread in methicillin-resistant *Staphylococcus aureus* has prompted the search for second-generation drugs of this class. Promising results have been obtained with the development of semisynthetic derivatives with improved activity, expanded antibacterial spectrum, or better pharmacokinetics [2]. Chemically, glycopeptide antibiotics consist of a heptapeptide core characterized by extensive crosslinking of the aromatic side chains. Vancomycin and teicoplanin are actually representatives of two distinct families of glycopeptides that exhibit different antibacterial properties. Vancomycin is more active than teicoplanin against coagulase-negative staphylococci, while teicoplanin possesses more favorable pharmacokinetic properties than vancomycin.

Following the characterization of the first gene cluster involved in the formation of the glycopeptides chloroeremomycin [3], the genetic elements responsible for balhimycin [4], complestatin [5], and A47934 [6] formation have been described. This information has allowed the elucidation of most biosynthetic steps leading to glycopeptide formation, after establishing the biochemical properties of selected gene products overproduced in *E. coli* [7–15] or through the genetic manipulation of the balhimycin producer *Amycolatopsis mediterranei* [4, 15–19]. Interestingly, novel glycopeptide derivatives have also been generated through the manipulation of the balhimycin pathway [20] or through in vitro glycosylatyion of glycopeptide aglycones [13, 21, 22]. However, this work has been carried out mostly on the vancomycin type of glycopeptide antibiotics. Apart from the description of the gene cluster of A47934 [6], a glycopeptide devoid of any sugar, no detailed information is available on glycopeptides of the teicoplanin family.

The antibiotic A40926 [23] belongs to the teicoplanin family of glycopeptides (Figure 1). It consists of a complex of closely related molecules sharing a heptapeptide skeleton with a rigid scaffold determined by ether bonds between amino acids 1–3, 2–4, and 4–6, and a C-C bond between amino acids 5–7. In addition, two sugar residues and two chlorine atoms are present on the molecule. The components of the A40926 complex differ in the length of the acyl chain, with factor A_1 (R = *n*-decyl) as the main component (Figure 1). Besides showing an intrinsic antibacterial activity, A40926 is the precursor of the semisynthetic glycopeptide dalbavancin (formerly known as BI397 or MDL 62,397), currently under clinical development [2].

The A40926 producer strain was formerly known as *Actinomadura* species ATCC39727 [23]. Following a reclassification of the genera *Actinomadura* and *Microtetraspora* and a further reanalysis of the latter genus, the A40926 producer has been classified as a *Nonomuraea* species [24]. We describe here the characterization of the *dbv* (from *d*al*bav*ancin) cluster involved in A40926 formation in *Nonomuraea* species ATCC39727 and show that it is possible to genetically manipulate this pathway.

Results and Discussion

Identification of the *dbv* Cluster

A cosmid library of *Nonomuraea* species ATCC39727 DNA was screened with homologous, heterologous, and synthetic probes (see Experimental Procedures) followed by chromosome walking. This approach led to the identification of seven overlapping cosmids encompassing the 120 kb genomic segment reported in Figure 2. The central region of this segment was sequenced, resulting in a contiguous stretch of 89,153 nt. As described below, the *dbv* cluster consists of 37 open reading frames (ORFs), designated ORF1 through ORF37. These ORFs are illustrated in Figure 3, and their relevant features are summarized in Table 1.

The probable boundaries of the *dbv* cluster were established by comparison with other glycopeptide clusters and from the deduced functions of the *dbv* gene products. On its left end (Figure 3), the *dbv* cluster is

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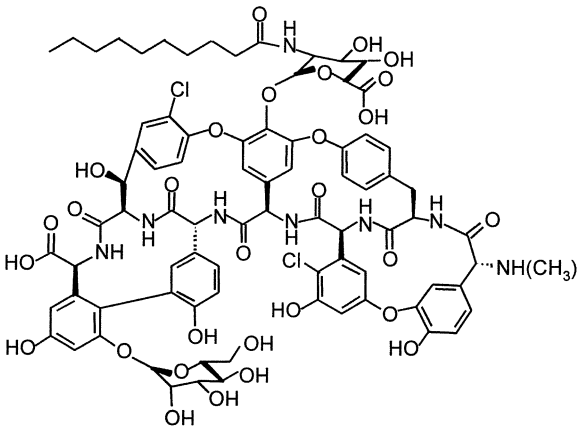

Figure 1. Structure of A40926

likely to be delimited by ORF1 encoding a homolog of *p*-hydroxymandelate oxidase (Hmo), one of the enzymes involved in *p*-hydroxyphenylglycine (HPG) synthesis [8, 12]. The deduced products of the next two ORFs to the left of *dbv* ORF1 (Figure 3) show significant identity to *Streptomyces coelicolor* proteins but not to sequences from other glycopeptide clusters. Thus, we assume that ORF1 delimits the left end of the *dbv* cluster. The right side of the *dbv* cluster is probably delimited by the remnant of an attachment site (*attL**, Table 1) showing 80% identity to the *attL* site of the *Streptomyces venezuelae* VWB phage, which inserts into a putative $tRNA^{Arg}$ gene [25]. Further to the right of this sequence, the next two ORFs, while similar to database sequences, find no counterparts in other glycopeptide clusters. The orientation of *dbv attL** suggests that its cognate *attR* site, if present, should be situated to its left in Figure 2. However, we were unable to locate any sequence resembling an *attR* site in the remaining 77 kb of *Nonomuraea* DNA sequence. Considering the interval comprised between ORF1 and *attL**, the *dbv* cluster spans approximately 71 kb.

Deduced Functions of the *dbv* Gene Products

Putative roles could be assigned by sequence analysis to most of the 37 *dbv* ORFs. All together, 27 of the 37 *dbv* ORFs find homologs in at least one other glycopeptide cluster (Table 1). The following sections summarize these findings, grouping ORFs governing similar portions of a hypothetical A40926 pathway.

Synthesis of Nonproteinogenic Amino Acids

Formation of the A40926 heptapeptide skeleton is expected to require the specialized synthesis of three amino acids: HPG, dihydroxyphenylglycine (DPG), and β-hydroxytyrosine (βHT). ORFs 1 and 2 (Table 1) show considerable sequence identity to Hmo and *p*-hydroxymandelate synthetase (HmaS), respectively, which are required to convert *p*-hydroxyphenylpyruvate into *p*-hydroxyphenylglyoxylate during HPG synthesis [8, 12]. ORFs 31 to 34 are closely related to DpgA through DpgD, respectively (Table 1), which are required for DPG formation [10, 16]. *dbv* ORF37 encodes a homolog of HpgT, the amino transferase required for the transamination of both *p*-hydroxyphenylglyoxylate and 3,5-dihydroxyphenylglyoxylate, to yield HPG and DPG, respectively [8, 16]. Thus, the seven genes required for HPG and DPG synthesis are also present in the *dbv* cluster. *dbv* ORF5, which encodes a homolog of prephenate dehydrogenase, may also participate in HPG synthesis. This function is likely to provide the initial *p*-hydroxyphenylpyruvate for HPG synthesis. Since HpgT uses Tyr preferentially as an amino donor, conversion of *p*-hydroxyphenylglyoxylate into HPG generates further *p*-hydroxyphenylpyruvate, which can be acted upon directly by Hmo [8].

In balhimycin and chloroeremomycin formation, conversion of Tyr into βHT requires the participation of three enzymes, one for covalent attachment of Tyr to a carrier protein, one for the actual hydroxylation, and the third (presumably) for hydrolytic release of βHT from the protein carrier [19, 26]. However, homologs of the corresponding genes are absent from the *dbv* and *sta* clusters, which instead specify polypeptides containing motifs typical of nonheme iron dioxygenases, exemplified by *dbv* ORF28 (Table 1). Homologs of these polypeptides are also encoded by the chloramphenicol [27] and ramoplanin [28] clusters. A single β-hydroxylated Phe or Asn residue, respectively, is present in the peptide skeletons of these compounds. At the moment we do not know if these apparently convergent pathways for βHT formation are interchangeable in glycopeptide formation.

Synthesis of the Heptapeptide

Synthesis of the A40926 heptapeptide precursor is catalyzed by a nonribosomal peptide synthetase (NRPS). In NRPSs, each elongating module is characterized by the presence of at least three domains: an adenylation (A) domain responsible for substrate recognition and activation, a thiolation (T) domain, which covalently binds amino acids and elongating peptides as thioesters; and a condensation (C) domain, which catalyzes peptide bond formation. The initiation module does not usually contain a C domain. In addition to these core domains, an epimerization (E) domain is usually present in those modules that convert an L amino acid into the D form. The last module is usually completed by a thioesterase (Te) domain, which hydrolyzes the thioester bond linking the completed peptide to the NRPS [29].

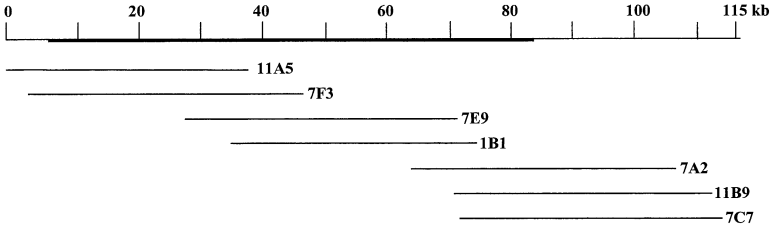

Figure 2. Cloned Portion of the *Nonomuria* Species ATCC39727 Chromosome

The thick line denotes the sequenced segment and the cosmids 11A5, 7F3, 7E9, 1B1, 7A2, 11B9, and 7C7 are indicated by the thin lines below.

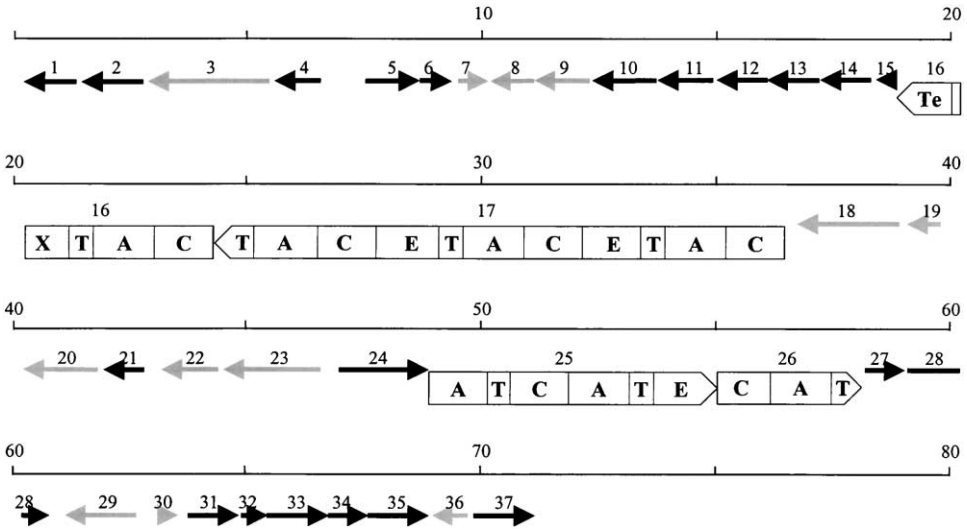

Figure 3. Genetic Organization of the *dbv* Cluster

The orientation is the same as in Figure 2. Each ORF is represented by an arrow, numbered as in Table 1, except for the NRPS genes, which are indicated by thick arrows showing the encoded domains. The ORFs unique to the *dbv* cluster are indicated in gray. Numbers on the scale bars indicate sequence coordinates (in kb).

The *dbv* ORFs 16, 17, 25, and 26 encode the 7-module NRPS required for A40926 formation. Specifically, the domain compositions of *dbv* ORF25 (A-T-C-A-T-E) and ORF26 (C-A-T) strongly indicate that the former encodes modules 1 and 2, and the latter encodes module 3. The domain composition deduced from *dbv* ORF17 (C-A-T-E-C-A-T-E-C-A-T) suggests that it encodes modules 4 through 6. *dbv* ORF16 is highly related to modules 7 from other glycopeptide NRPSs, since it specifies the domain sequence C-A-T-X*-Te (X* denotes an atypical C or E domain of unknown function). This attribution of the *dbv* ORFs to the NRPS modules is also consistent with the expected amino acid specificities of the corresponding A domains deduced from the nonribosomal code [30, 31]. Indeed, the signature amino acids from the A domains from modules 1, 4, and 5 show significant matches to HPG-specific A domains, and those from module 7 show significant matches to DPG-recognizing A domains. The matches of the signature sequences from the other A domains, while not as significant, are nonetheless consistent with module 2 and 6 recognizing a Tyr derivative and module 3 recognizing a DPG residue.

The *dbv*, *com*, and *sta* NRPS each consist of four different polypeptides, and not of three as for the *bal* and *cep* systems. In terms of domain composition, all five NRPSs present the same organization, except for the existence of an additional E domain (presumed to be inactive) in *com* and *sta* modules 3 and a putative methylation domain in *com* module 6 (proposed to *N*-methylate amino acid 6). Interestingly, all five glycopeptide NRPSs present an additional domain in module 7 (we have referred to this additional domain as X*). Its presence at the same location suggests an important role in heptapeptide synthesis. In this respect, it should be noted that the structures of the five glycopeptides imply a heptapeptide precursor of NH_2-D-D-L-D-D-L-L-COOH stereochemistry, while the number and position of E domains suggest a NH_2-L-D-L–D-D-L-L-COOH stereochemistry. The A domain from *cep* module 1 uses L-Leu preferentially over D-Leu [9]. Thus, there is one missing E domain in module 1 and an extra domain in module 7.

It should be noted that in all other glycopeptide clusters characterized thus far, there is colinearity between gene order and role of modules. The *dbv* NRPS, however, is encoded by two divergently transcribed gene pairs separated by about 12 kb (see Figure 3). Within each pair, colinearity is maintained, since the gene encoding modules 1 and 2 precedes that encoding module 3, and ORF17, encoding modules 4 to 6, precedes ORF16. The possibility that this atypical organization is due to some cloning artifact seems unlikely. First, the segments encoding the NRPS are present in more than one cosmid (Figure 2) and were thus recovered by independent cloning events. In addition, the region comprised between the two blocks of NRPS genes encodes other ORFs likely involved in A40926 biosynthesis (Table 1). At least one precedent exists for noncolinearity in actinomycete NRPS genes [32].

Two other *dbv* ORFs are likely to participate in heptapeptide synthesis. ORF15 (Table 1) encodes a short, highly conserved peptide of unknown function, specified by many actinomycete clusters containing NRPS genes. The corresponding gene is always found downstream of the Te-encoding gene. ORF36 (Table 1) encodes a type II thioesterase, a function often specified by clusters containing NRPS or polyketide synthase genes. The proposed role for these thioesterases is to hydrolyze misprimed or misacylated T domains [33, 34].

Crosslinking and Chlorination of Aromatic Residues

The A40926 aryl groups are joined by three ether links and one C-C link (Figure 1). As demonstrated for balhimycin, these crosslinking reactions are carried out by P450 monooxygenases encoded by three separate *oxy*

Table 1. *dbv* ORFs

	Cluster[a]				Best Match[b]			
dbv ORF	*bal*	*cep*	*com*	*sta*	Source[d]	Entry[e]	Score[f]	Proposed Function[c]
1	+	+	+	+	*sta*			Hmo
2	+	+	+	+	*sta*			HmaS
3	−	−	−	−	*S. hygroscopicus*	T03225	9e-90	positive regulator
4	+	+	+	+	*cep*			positive regulator
5	+	+	+	+	*cep*			prephenate dehydrogenase
6	−	−	−	+	*S. coelicolor*	Q03576	9e-84	response regulator
7	−	−	−	−	*Synechocystis* sp.	S77033	8e-04	carboxypeptidase
8	−	−	−	−	ND			unknown
9	+	+	−	−	*cep*			glycosyltransferase
10	+	+	+	+	*sta*			halogenase
11	+	+	-	+	*sta*			P450 monooxygenase
12	+	+	+	+	*cep*			P450 monooxygenase
13	−	−	−	+	*sta*			P450 monooxygenase
14	+	+	+	+	*bal*			P450 monooxygenase
15	+	+	+	+	*cep*			unknown
16	+	+	+	+	*cep*			NRPS, module 7
17	+	+	+	+	*sta*			NRPS, nodules 4-6
18	−	−	−	−	*S. coelicolor*	CAB89462.1	4e-58	ABC transporter
19	−	−	−	−	*S. coelicolor*	CAB89461.1	3e-67	ABC transporter
20	−	−	−	−	*S. coelicolor*	CAC32663.1	6e-59	mannosyltransferase
21	+	+	−	−	*bal*			unknown
22	−	−	+	+	*S. hygroscopicus*	T30222	2e-49	sensor protein kinase
23	−	−	−	−	*M. loti*	NP_103545.1	1e-58	acyltransferase
24	+	+	+	+	*sta*			ABC transporter
25	+	+	+	+	*sta*			NRPS, modules 1-2
26	+	+	+	+	*sta*			NRPS, module 3
27	+	+	−	−	*cep*			methyltransferase
28	−	−	−	+	*sta*			β-hydroxylase
29	−	−	−	−	*S. coelicolor*	NP_630371.1	e-126	hexose oxidase
30	−	−	−	−	*S. coelicolor*	NP_626911.1	2e-18	4HB-CoA thioesterase
31	+	+	−	+	*bal*			DpgA
32	+	+	−	+	*bal*			DpgB
33	+	+	−	+	*bal*			DpgC
34	+	+	−	+	*cep*			DpgD
35	+	+	+	+	*bal*			membrane ion antiporter
36	−	−	−	−	*A. mediterranei*	AAG52991.1	2e-25	thioesterase
37	+	+	+	+	*cep*			HpgT
attL*	−	−	−	−	*S. coelicolor*	SCO939123	4e-23	attachment site

List of *dbv* ORFs and the best matching sequences.
[a] Existence of a homolog in other glycopeptide clusters: "+" denotes its presence, and "−" denotes its absence.
[b] Best match found by Blast searches in GenBank other than glycopeptide clusters.
[c] Proposed function in A40926 biosynthesis.
[d] Organism originating the best matching sequence. When *bal*, *cep*, *com*, or *sta* are reported, it indicates that the best matching sequence derives from one of these clusters. *A.*, *Amycolatopsis*; *M.*, *Mesorhizobium*; *S.*, *Streptomyces*; ND, none detected.
[e] Accession number of the best matching sequence.
[f] Probability score of the best matching sequence.

genes [17, 18]. The *dbv* cluster contains four such genes, namely ORFs 11 through 14 (Table 1), as does the *sta* cluster, consistent with the four crosslinks present in the corresponding glycopeptides. A role for each P450 enzyme has been proposed on the basis of the partially crosslinked metabolites produced after selective inactivation of the *oxy* genes in *A. mediterranei* [17, 18]. From sequence similarities (Table 1), it is likely that *dbv* ORFs 11, 12, and 14, which are best related to OxyC, OxyB, and OxyA, respectively, are involved in crosslinking of amino acids 5–7, 4–6, and 2–4. By exclusion, ORF13 should be involved in crosslinking of amino acids 1–3.

Glycopeptides present different degrees of chlorination. In fact, one chlorine atom is present on each of the two βHT residues in balhimycin and chloroeremomycin, on one residue each of βHT and DPG in A40926 (Figure 1), and on one residue each of Tyr, HPG, and βHT in A47934. Two chlorine atoms are present on one *p*-hydroxyphenylglyoxylate and on two HPG residues in complestatin. A single halogenase gene, specified by ORF10 (Table 1), is present in the *dbv* cluster. As shown below, this ORF is very likely responsible for the introduction of both chlorine atoms in A40926, as it occurs in *A. mediterranei* [19]. Thus, there is apparently no correlation between the number of halogenase genes (one for each cluster, except two for *sta*) and the number and position of chlorine atoms in the corresponding glycopeptides. Understanding the timing and specificity of halogenation might expand the positions at which glycopeptides are chlorinated. Interestingly, the presence of chlorine atoms appears to enhance antibacterial activity in some glycopeptides [35].

Addition and Modification of Sugars, and N-Methylation

The following modifications are expected to occur on the newly formed A40926 aglycone: *N*-methylation of the terminal HPG residue, addition of a glucosamine moiety to the hydroxyl group of amino acid 4, *N*-acylation of the glucosamine residue, oxidation of the primary alcohol of the glucosamine moiety to yield a carboxyl group, and addition of a mannose residue to the hydroxyl group of amino acid 7 (Figure 1). Five proteins, encoded by *dbv* ORFs 9, 20, 23, 27, and 29, are likely to be involved in these tailoring steps. Only ORFs 9 and 27 find counterparts in other glycopeptide clusters (Table 1).

The deduced sequence of ORF9 is highly related to the GtfB glycosyltransferase encoded by the *bal* and *cep* clusters (Table 1), which glycosylate amino acid 4 [13, 21]. Thus, most likely ORF9 modifies this same amino acid, using an activated amino sugar instead of UDP-glucose. *dbv* ORF27 is likely to catalyze *N*-methylation of the terminal HPG residue, since it is highly related to the *cep* methyltransferase (Table 1), which has been shown to methylate in vitro advanced glycopeptide intermediates [14].

The other three tailoring steps are unique to A40926 biosynthesis. Glycopeptides of the teicoplanin family are *N*-acylated at the glucosamine moiety with an acyl chain derived from fatty acid degradation [36]. *dbv* ORF23 encodes a protein highly related to members of the acyltransferase 3 family, so it might participate in *N*-acylation. Comparison of the *dbv* genes with those directing the synthesis of teicoplanin [37] might help identify candidate genes for this reaction.

Homologs of *dbv* ORF20 are present in *S. coelicolor* (Table 1) in the ramoplanin [28] and teicoplanin (M.S. and S.D., unpublished results) gene clusters. Ramoplanin and teicoplanin also contain a mannosyl residue attached to an HPG residue of the peptide core. Interestingly, all three polypeptides contain motifs typical of the protein mannosyltransferases family (Figure 4). These data suggest a role for ORF20 in A40926 mannosylation. It is interesting to note that the protein mannosyltransferase family (pfam02366 from the CDD database) does not show any obvious resemblance to glycosyltransferases that use NDP-activated sugars. This might be related to the use of other sugar-activating moieties. In addition, while NDP-dependent glycosyltransferases are located in the cytoplasm, mannosylation might take place on the outer side of the cytoplasmic membrane, as suggested by the ease with which some glycopeptide producers can mannosylate exogenously added demannosylated glycopeptides [38]. Since glycopeptides do not cross the cytoplasmic membrane, mannosylation is likely to occur outside of the cell.

dbv ORF29 contains motifs typical of FAD binding and shows considerable matches to other proteins described as hexose oxidases (Table 1). This gene is likely to govern oxidation of the glucosamine moiety to yield the corresponding acid. In addition, the presence of a putative signal sequence at the N terminus suggests that the product of ORF29 is secreted, and hence that oxidation occurs outside the cytoplasm.

Resistance, Export, Regulation, and Unknown Functions

The deduced product of ORF7 contains motifs typical of the VanY-family of carboxypeptidases (Table 1). These enzymes are involved in the extracytoplasmic removal of the terminal alanine residue from nascent peptidoglycan, reducing the extent of glycopeptide binding to its molecular target [39]. *dbv* ORF7 may thus be involved in conferring some level of A40926 resistance to the producing strain. Like its homolog from the *bal* cluster (W. Wohlleben, personal communication), it resembles the VanY-family of carboxypeptidases, which in vancomycin-resistant enterococci represent ancillary resistance determinants. It thus appears that different resistance mechanisms are operating in glycopeptide clusters, with *vanHAX*-like genes present in the *sta* [6] and *tcp* [38] clusters, *vanY*-like genes in the *bal* and *dbv* clusters, and no obvious resistance determinant in the *cep* cluster.

Four *dbv* ORFs are likely to encode export functions. Homologs of ORFs 24 and 35 are present in other glycopeptide clusters, and they encode predicted ABC- and ion-dependent transmembrane transporters, respectively (Table 1). *dbv* ORFs 18 and 19 (Table 1) appear to encode an additional ABC-type transporter, with ORF18 being the transmembrane partner. The best matching sequences to these two ORFs are also adjacent in the *S. coelicolor* genome.

Among the four putative regulatory genes present in the *dbv* cluster, homologs of the putative positive regulator *dbv* ORF4 are present in all glycopeptide clusters (Table 1). An additional putative positive regulator is encoded by *dbv* ORF3 (Table 1). The two members of a putative two-component signal transduction system may also be specified by the *dbv* cluster, although the corresponding genes, ORF6 and ORF22, are not physically linked (Table 1).

Three additional genes are present in the *dbv* cluster. ORF30, located just 5′ to the *dpgAD* homologs, encodes a polypeptide highly similar to hypothetical proteins of unknown function identified from bacterial genome sequences (Table 1), which all display the conserved motifs typical of 4-hydroxybenzoyl-CoA thioesterases [40]. Thus, the product of ORF30 may be a thioesterase, possibly involved in facilitating the release of a DPG precursor during synthesis of this polyketide-derived amino acid. No significant matches to proteins of known function could be found for ORFs 8 and 21. Homologs of the latter are also present in the *bal* and *cep* clusters, while ORF8 is so far unique to the *dbv* cluster.

Manipulation of the A40926 pathway

In order to confirm the involvement of the *dbv* cluster in A40926 biosynthesis, we employed a gene transfer system recently developed for *Nonomuraea* species ATCC39727 (S. Stinchi et al., submitted) to construct a gene replacement mutant. A DNA segment from the *dbv* cluster, in which the 5′ end of ORF 8, most of ORF10, and the entire ORF9 was replaced by the *tsr* marker (see Figure 5A), was cloned into an *int*-deleted version of pSET152. The resulting plasmid pVHom1 was introduced into the *Nonomuraea* chromosome by homolo-

```
          1                                                  50                                                 100
 ramOrf29 ~~~~~~~~~~ ~~~~~~~~~~ ~VDVPRVRPP GA....APAP RRRRWRFWQS PDGQPAWARP ALLGIAALAA VLYTANLARS GYP.MYYAVA VKSMSVSWPA
 mtaOrf18 ~~~~~~~~~~ ~~~~~~~~~~ ~MSHITMTPP SACRDPAPAG RFPRWAVWRS PPGQPWWARP ALLCIAATAA VLYAWNLPLV DYA.PRYSDA VKSMSENWKA
np_628205 MTTHYDPTTH ADGPPTPVGE DGGPPSAAP. .AHRQPRPAR RPRLGRVWRG RPEDPRWVRP AFLGLLLATA LLYLYNLSAS GYANSFYSAA VQAGSQSWKA
consensus                       -------P- -A-----PA- R------W-- ----P-W-RP A-L------A -LY--NL--- -Y----Y--A V---S--W-A

          101                                                150                                                200
 ramOrf29 FWTGAFDPAA SITIDKLAGA FVPQALSARV FGFHQWSLAL PQAVEGVIAV LVLYRAVRRW HGPGAGLAAA GLFATTPIVS SMFGHSMEDG ALTLCLVLAA
 mtaOrf18 FLYGTVDVQA TYTLDKLAGA FVPQAISVKI FGFHAWALAL PQVIEGVISV LVMYRIVRRW AGVVPGLLAA AVFTITPVAA SMFGHSMADG ALVMCLVLAV
np_628205 FFFGSLDAGN AITVDKPPAA LWPMALAVRI FGLSSWSILV PEVLMGVGTV AVVYAAVRRR FSPAAGLIAG AVLALTPVAA LMFRFNNPDA LLALLMSVAC
consensus F--G--D--- --T-DK---A --P-A----- FG---W---- P----GV--V -V-Y--VRR- -----GL-A- -----TP--- -MF-----D- -L------A-

          201                                                250                                                300
 ramOrf29 DAFGAAVTRG SPARLALAGA WIGLGFQAKM MQAWLVLPAL VVTYLAGAPV RARARVVHVA AAVAATLAVS LLWVLALTLL PGSHRPWADG TTSGNAFAMV
 mtaOrf18 DSYQRAVLEG RLRSLVWAGV WVGLGFQAKM LQAWMILPAL AIGYLLSAPI GLRRRLQHLG IAGVVTLVVS LSWITLYHVT PAADRPYISG TTNSSAAAMV
np_628205 YLVVRALEDG RSKWLVWAGV AIGFAFLAKT LQAFLILPAL ALVYGVCAPV RLRKRLGQLA LATAAIVVSG GWWVAVVELW PASSRPYIGG SQNNSFLELT
consensus -----A---G ----L--AG- --G--F-AK- -QA---LPAL ---Y---AP- --R-R----- -A-------- --W------- P---RP---G ----------

          301                                                350                                                400
 ramOrf29 FGYNGFDRAG IHVPGALTTG FTDG...... .......... .........G AAAGGSWTAL AADRLATQIG WWYPLALTGL LLGLARWR.. ....TARAGL
 mtaOrf18 FGYNGLGRLG INLPGALPPN YMGSVIGPAP PKRSTQLPRP RPGMVIPEIG IEHGGGWGKL FGGRLGVASG WLYPLALMAL LCGLWWWRRA ERTDPARGGM
np_628205 FGYNGLGRLN GEETGSVGGG ..GGGMGGGG GGQWGET... .......... .....GWDRM FNSEIGGQIS WLLPAALILL VAGLVATRRA ARTDGARASF
consensus FGYNG--R-- ----G----- ---------- ---------- ---------- ------W--- ---------- W--P-AL--L --GL---R-- -----AR---

          401                                                450                                                500
 ramOrf29 LFWGLWLLTA AVVLSRI.TI QHNAYLAVLA PPLAALAAAG AVQLWRTHRD .GTAPWLLPA VVVVQAGWTL WLATRYPSFL AGLTWTAPIA AVLAVVVLA.
 mtaOrf18 VMWGVWLLTF ALPYSAVFVI PHSAYVAVLA PPVAALSGIG IVMFWRAYRS GGRMAWIFPL AIVAELAWAV WLWSFYPTFL PWAMWGAVAL GVVAVVALAL
np_628205 LVWGGSLLMT AAVFSYMAGI FHQYYTVALA PYLAAVTGMG AATLWE.RRE RPWASIALAA AVTASAAWGY VLLNRTPDYL PWLKVPVLVG GLVAALGLVF
consensus --WG--LL-- A---S----I -H--Y---LA P--AA----G ----W---R- ---------- -------W-- -L----P--L ---------- ---A---L--

          501                                                550
 ramOrf29 AR.PTARRPA VV.....VVV AGLLAVPVAW GASVLNPRYA GTSFEAG.AG PS.G..PVGV
 mtaOrf18 ARLVRPRRSS LVSAGLTIGV AAMLAAPATW SASVLDPRYG GSSFDAN.AG PA.ARTPGG*
np_628205 AGRL.GRRTA LAAAGL..GL VASLAAPTAY TLSTVREGHS GSIVTAGPAG ASMGGGPGGG
consensus A-----RR-- ---------- ---LA-P--- --S------- G----A--AG ------P-G-
          601                                                650
```

Figure 4. Alignment of Putative Mannosyltransferases

The sequences are abbreviated as follows: dbv, *dbv* ORF20; ram, ORF19 from the ramoplanin gene cluster [28]; Sc, *S. coelicolor* CAC32663.1 [45]. Residues indicated in red in the consensus line are present in the protein mannosyltransferase family (http://www.ncbi.nlm.nih.gov/Structure/cdd).

gous recombination (see Experimental Procedures). In this way, a first strain was isolated, designated Non1, and carrying the construct of Figure 5A, integrated by a single crossover event. Serial passages of Non1 eventually yielded strain Non1Δ8-10, which showed, upon PCR analysis, the 2.7 and 1.2 kb products expected from replacement of the wild-type segment with the in vitro mutated copy (Figure 5A). (The *tsr* marker turned out to be unsuitable for recognizing gene replacement events due to high background growth of the wild-type.) This interpretation was confirmed by Southern hybridization, which showed that the 4.0 and 2.7 kb bands in

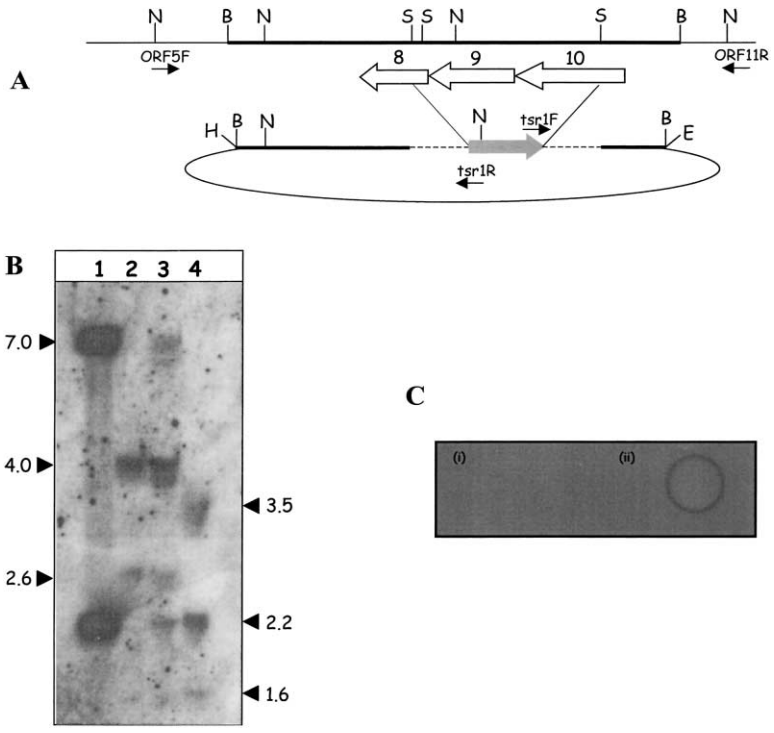

Figure 5. Gene Replacement in *Nonomuraea*

(A) Scheme of gene replacement. The upper part shows the relevant portion of the *dbv* cluster, while the lower portion is a schematic of plasmid pVHom1. The thick line denotes the DNA fragment cloned in pWHom1, while the thin line represents the flanking segments in the *dbv* cluster. The white arrows indicate the affected ORFs, while the gray arrow represents the *tsr* gene. Small arrows indicate PCR primers, and the dashed lines indicate the segments deleted in strain Non1Δ8-10. Relevant restriction sites are abbreviated as follows: B, BamHI; E, EcoRI; H, HindIII; N, NcoI; S, SacI.

(B) Southern hybridization. Genomic DNA from *Nonomuraea* wild-type (lane 2), strain Non1 (lane 3), strain Non1Δ8-10 (lane 4), and control pVHom1 (lane 1) were digested with NcoI and hybridized to the 6.3 kb BamHI fragment (A). Fragment sizes (in kb) are indicated.

(C) Bioassay on *B. subtilis* BAU102: i, strain Non1Δ8-10; ii, wild-type.

the wild-type DNA were replaced by bands of 3.5 and 2.2 kb (Figure 5B), consistent with the predictions from Figure 5A. On the basis of the extent of the deletion introduced, strain Non1Δ8-10 is expected to be devoid of functional products of ORF8 through ORF10. Strain Non1Δ8-10 produced a significantly lower amount of antimicrobial activity than the parent strain (Figure 5C).

The metabolites produced by strain Non1Δ8-10 were analyzed by LC-MS and compared with those of the wild-type and with an A40926 standard. The wild-type produced a major peak with a 13.9 min retention time (Figure 6A), which showed a molecular ion $[MH]^+$ of 1731 in the corresponding mass spectrum, as did the A40926 standard. In the case of strain Non1Δ8-10, the 13.9 min peak was absent, and a major peak was observed with a 6.7 min retention time and molecular weight of 1306 (Figure 6B). Furthermore, this peak shows a UV spectrum (not shown) indistinguishable from that of A40926, with a maximum at 278 nm. (The HPLC profile of Figure 6B shows also another major peak at 13.1 min, with a molecular weight of 833 and a UV spectrum unrelated to that of A40926.) The 1306 molecular weight is compatible with the A40926 mannosyl aglycone devoid of chlorine atoms. The isotopic profile of the 6.7 min peak demonstrates the absence of chlorine atoms, and MS-MS fragmentation yielded a first fragment at 1144 mass units, consistent with loss of the mannose unit from the molecular ion. These results strongly indicate that the main metabolite produced by strain Non1Δ8-10 is the mannosyl-deschloroaglycone of A40926 and confirm the hypothesized role for the product of ORFs 9 and 10 as a glycosyltransferase and a halogenase, respectively. Since the deletion strain Non1Δ8-10 carries also a defect in ORF8, this latter gene could either play no essential role in A40926 formation or be required for modification of the glucosamine moiety. The construction of mutant strains lacking only ORF8 should distinguish between these two possibilities.

Significance

The glycopeptide A40926 is the precursor of the semisynthetic glycopeptide dalbavancin, currently under clinical development. The *dbv* cluster, required for A40926 formation, represents the first example of a gene cluster from a *Nonomuraea* species and the first description of the genes required for formation of a teicoplanin-like glycopeptide. The cluster consists of 37 ORFs and presents some unique features absent from other glycopeptide clusters, including divergently transcribed genes encoding the A40926 NRPS. Some *dbv* genes could be assigned to particular tailoring steps: ORF9 is responsible for glucosamine attachment, while ORFs 20 and 29 are likely to encode a mannosyltransferase and a hexose oxidase. These and other *dbv* genes that specify tailoring steps unique to A40926 formation can represent additional tools for eventually generating glycopeptide analogs. A new glycopeptide derivative was obtained after deletion of three *dbv* genes. The ability to genetically manipulate the A40926 producer should help elucidate the role of *dbv* genes and should allow the production of new glycopeptides of the teicoplanin family that are currently hard to make by synthetic or semisynthetic approaches.

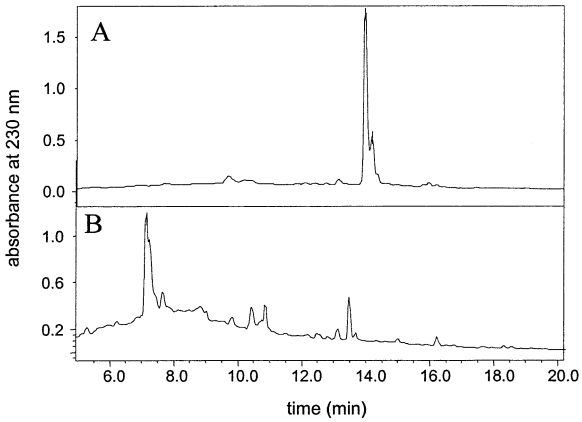

Figure 6. Metabolite Analysis

HPLC analysis of culture media of (A) wild-type and (B) strain Non1Δ8-10.

Experimental Procedures

Isolation of the *dbv* Cluster

A cosmid library of *Nonomuraea* ATCC39727 DNA in the vector Supercos was prepared by the Stratagene customer library service (Stratagene, La Jolla, CA). An enriched library of *Nonomuraea* DNA made with 2–3 kb BamHI fragments in pUC19 was screened by hybridization to the *bal dpgA* gene [15]. The insert from one positive clone was then used to screen the cosmid library, together with the oligo probes Pep6 and Pep8 [41] and the *bal gtfB* gene [4]. After restriction mapping and chromosome walking, seven cosmids were identified, leading to the set of overlapping inserts shown in Figure 2. Cosmids 1B1, 11A5, and a 20 kb ClaI deletion of cosmid 7C7 were sequenced by custom sequencing (SeqLab, Gottingen, Germany). The *dbv* sequence was analyzed with programs from the Wisconsin Package (version 10, Accelrys). Prediction of amino acid specificity of the A domains was performed as described [30, 31] using the search available at http://raynam.chm.jhu.edu/~nrps/index2.html.

Gene Replacement

A 6.3 kb BamHI fragment spanning from ORF5 to ORF11 was cloned into pUC18. Two internal SacI fragments, for a total of 2.8 kb, were then replaced with the *tsr* gene, PCR amplified from pIJ39 [42]. Then, the resulting 5.4 kb insert was excised as an EcoRI-HindIII fragment and cloned into the corresponding sites of pSET152 [43], generating plasmid pVHom1. (This cloning strategy removes 1.2 kb from the 3′ end of the *int* gene in pSET152.) Conjugation between *E. coli* ET12567 (pUB307) harboring pVHom1 and *Nonomuraea* ATCC39727 was performed as described [41]. Briefly, an overnight culture of the donor strain and an exponential, sonicated culture of the recipient strain were plated on BTT agar (10 g/l glucose, 1 g/l yeast extract, 1 g/l beef extract, 2 g/l casitone, 20 g/l agar). Plates were incubated for 24 hr at 28°C before overlaying them with 3 ml soft agar containing 280 and 200 μg apramycin and nalidixic acid, respectively. After a further 3 week incubation, one apramycin-resistant *Nonmuraea* exconjugant was isolated and designated strain Non1. This apramycin-resistant strain was grown under nonselective conditions in RARE3 medium (10 g/l glucose, 4 g/l yeast extract, 10 g/l malt extract, 2 g/l Bacto peptone, 2 g/l $MgCl_2 \cdot H_2O$, 5 g/l glycerol) for 4 days before plating on BTT agar. The resulting colonies were screened for plasmid loss by PCR using the primer pairs ORF5F (5′-GTGGCCGGGGTGCTGGAGCGGATCG-3′) and tsr1R (5′-GCG ATGCCGATGGGGGTGTCGTGGC-3′) or tsr1F (5′-CCGAGAC CGTGAGCCGGCGCAAGCC-3′) and ORF11R (5′-TGGTCGGTCAGC TGTCGGTCTATGATCCG-3′), as shown in Figure 5A. For PCR analy-

sis, a loopful of mycelium from an agar plate was resuspended in 100 μl of distilled water, incubated at 95°C for 10 min, and centrifuged at 13,200 × g for 5 min. Then, 14 μl of the supernatant was used for PCR amplification. Southern hybridizations were performed using the nonradioactive DIG DNA labeling and detection kit (Roche).

Metabolite Identification

Nonomuraea was grown in RARE3 medium for 7 days at 28°C. After centrifugation, 90 ml of broth was mixed with 1 volume of 0.1 M sodium borate (pH 12) and kept for 1 hr at 50°C. After filtering through a 0.45 μm filter, the filtrate was brought to pH 7.5 with HCl, and antibiotic activity was evaluated by agar diffusion against *Bacillus subtilis* BAU-102 [44]. For HPLC and MS analysis, the extracts were adsorbed onto 7.5 ml of an HP20 resin prewashed with CH_3OH and equilibrated with 20 mM ammonium formate (pH 6.6) (buffer A). After a 12 hr incubation, the resin was recovered by filtration, washed with 15 ml buffer A, and eluted with 20 ml of 1:1 1.5% NH_4OH: CH_3COCH_3. The resulting fraction was dried and dissolved at 10 mg/ml in 70:30 CH_3CN: buffer A. Metabolites (1 mg) were injected onto a 5μ-Symmetry C18 column (4.6 mm × 25 cm) using a Waters system (Waters Chromatography, Milford, MA). Metabolites were resolved employing a binary solvent system composed of phase A (5:95 20 mM ammonium formate [pH 4.5]: CH_3CN) and phase B (100% CH_3CN), using a linear gradient from 30% to 70% phase B in 30 min at 1.0 ml/min. A zero-dead-volume tee (Valco, Houston, TX) was used to produce a 5:95 post-column effluent split, diverting the majority of the effluent to a Waters 996 Photodiode Array Detector and the remainder to the ESI interface of a Finnigan LCQ Duo mass spectrometer. Full scan mass spectra were acquired in positive (profile or centroid) mode from 600 to 1900 amu, with a data-dependent zoomscan and MS2 programmed in the selected mass range. MS2 spectra were obtained with an isolation width of 3 amu and normalized collision energy of 28%.

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Accession Numbers

The complete DNA sequence of the A40926 gene cluster has been deposited in the GenBank database with the accession number AJ561198.