Genomic-Led Discovery of a Novel Glycopeptide Antibiotic by *Nonomuraea coxensis* DSM 45129

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**ABSTRACT:** Glycopeptide antibiotics (GPAs) are last defense line drugs against multidrug-resistant Gram-positive pathogens. Natural GPAs teicoplanin and vancomycin, as well as semisynthetic ortovancin, talavancin, and dalbavancin, are currently approved for clinical use. Although these antibiotics remain efficient, emergence of novel GPA-resistant pathogens is a question of time. Therefore, it is important to investigate the natural variety of GPAs coming from so-called “rare” actinobacteria. Herein we describe a novel GPA producer—*Nonomuraea coxensis* DSM 45129. Its de novo sequenced and completely assembled genome harbors a biosynthetic gene cluster (BGC) similar to the *dbv* BGC of A40926, the natural precursor to dalbavancin. The strain produces a novel GPA, which we propose is an A40926 analogue lacking the carboxyl group on the N-acylglosamine moiety. This structural difference correlates with the absence of *dbv*29—coding for an enzyme responsible for the oxidation of the N-acylglosamine moiety. Introduction of *dbv*29 into *N. coxensis* led to A40926 production in this strain. Finally, we successfully applied *dbv*3 and *dbv*4 heterologous transcriptional regulators to trigger and improve A50926 production in *N. coxensis*, making them prospective tools for screening other *Nonomuraea* spp. for GPA production. Our work highlights genus *Nonomuraea* as a still untapped source of novel GPAs.

**1. INTRODUCTION**

*Nonomuraea* is a genus of so-called “rare” actinomycetes whose potential to produce specialized (secondary) metabolites is still rather poorly explored. Recently sequenced genomes of *Nonomuraea* species appear to be generally larger than the reference *Streptomyces* ones. The mean genome size of *Nonomuraea* (based on the three available complete assemblies) is around 12 Mbp, whereas the mean genome size of *Streptomyces* (calculated on 251 fully assembled genomes available in GenBank) equals 8.6 Mbp. The larger genomes of *Nonomuraea* spp. encode dozens of putative biosynthetic gene clusters (BGCs). *Nonomuraea* spp. were initially found to be recalcitrant to commonly used genetic engineering manipulations, but new tools are now being developed for this genus. This paves the way for unravelling the huge hidden biosynthetic potential of these organisms.

Probably the most important bioactive metabolite produced by a *Nonomuraea* species is the type IV glucose peptide antibiotic (GPA) A40926 (Figure 1) produced by *Nonomuraea gerenzanensis* ATCC 39727. Like other GPAs, A40926 acts as a selective and potent inhibitor of cell-wall biosynthesis in Gram-positive bacteria. A40926 is structurally related to the clinically relevant GPA teicoplanin (Figure 1), previously isolated from numerous *Amycolatopsis* spp. (i.e., *A. lurida* NRRL 2430, *A. japonicum* MG417-CF17, and *Amycolatopsis* sp. MJM2582). Like teicoplanin, A40926 is produced as a mixture of related compounds (major components are A40926 B and A40926 A factors), which differ in the length and branching of an aliphatic side chain (Figure 1). It was recently clarified that *N. gerenzanensis* produces the GPA in the form of O-acetyl-A40926 (with an O-acetylated mannosamine residue), but the acetyl group is lost during the alkaline extraction of the antibiotic. Since it was this deacetylated GPA that was initially named A40926, we will refer to it as A40926 hereafter.

A40926 is the precursor of the second-generation semisynthetic GPA dalbavancin (Figure 1), which is currently applied in clinics to treat severe infections caused by multidrug-resistant Gram-positive pathogens. Dalbavancin (marketed in Europe and USA under the trade names xydalba and dalvance, respectively) is the first antibiotic designated as a qualified infectious disease product by FDA because of its potency, extended dosing interval, and unique dose regimen (once-a-week), but its cost still largely exceeds that of first-generation GPAs. Therefore, improvement of A40926 production by recombinant engineering of *N. gerenzanensis*.

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has become increasingly relevant.\textsuperscript{6,16} Following the sequencing of the A40926 BGC (\textit{dbv}) almost two decades ago,\textsuperscript{18} multiple aspects of A40926 biosynthesis were investigated, including nonribosomal aglycone assembly and tailoring steps,\textsuperscript{15,19,20} self-resistance,\textsuperscript{21,22} and pathway-specific regulation of its production.\textsuperscript{6,3,24} \textit{N. gerenzanensis} was also engineered to produce A40926 derivatives that are better suited for downstream chemical modification to dalbavancin.\textsuperscript{16} Another GPA produced by a \textit{Nonomuraea} species is the type V GPA kistamicin (Figure 1) from \textit{Nonomuraea} sp. ATCC 55076, which was reported to exhibit potent antiviral activity as well as mild antibiosis against Gram-positive bacteria.\textsuperscript{2,25} Its structure contains an unusual indole−phenol cross-link which makes this GPA unique among those already known.\textsuperscript{5,26} 

Genome mining has recently shown that other species from the genus \textit{Nonomuraea} also possess BGCs for GPAs,\textsuperscript{27} as in the cases of \textit{Nonomuraea} sp. WAC 01424 and \textit{Nonomuraea coxensis} DSM 45129. Notwithstanding the low quality of the available
draft genomic data, we recently showed that *N. coxensis* DSM 45129 carries a BGC remarkably similar to *dbv*6. We found that this BGC contains a putative regulatory gene orthologous to *dbv3*, which encodes the pathway-specific regulator of LuxR-type in *N. gerenzanensis*. The heterologous expression of this gene from *N. coxensis* (named *nocRI*) led to A40926 overproduction in *N. gerenzanensis*, indicating that it might be functional in *N. coxensis* as well. Thus, in this paper we present the fully assembled genome of *N. coxensis*, which has allowed us to properly describe the putative GPA BGC (called *noc*). Additionally, we report that *N. coxensis* produces a novel GPA complex, which we named A50926. Structural characterization of A50926 by liquid chromatography–mass spectrometry (LC-MS) and tandem MS (MS/MS) showed it has high similarity to A40926, although A50926 lacks the carbonyl group on the N-acylglicosamine (GlcN-Acyl) moiety. Consistently, the *noc* BGC lacks an orthologue of *dbv29*, which in *N. gerenzanensis* encodes the enzyme oxidizing the GlcN-Acyl moiety to an N-acylglucuronic group.19 Introduction of *dbv29* into *N. coxensis* changed the GPA production profile of this strain to A40926. Finally, we have introduced *dbv3* and *dbv4* pathway-specific regulatory genes in *N. coxensis* to trigger and overproduce A50926 by regulatory gene cross-talking. In conclusion, our results describe the biosynthesis of a novel GPA, which may have superior properties to A4092615,28 and thus may contribute to developing a platform for the combinatorial biosynthesis of third generation lipo-GPAs.

2. RESULTS AND DISCUSSION

2.1. Complete Assembly of *N. coxensis* Genome Reveals the Presence of a Novel GPA BGC. The presence of a novel GPA BGC in the genome of *N. coxensis* was recently anticipated.6,27 However, due to the poor quality of the available draft, fragments of the BGC were found on different contigs and did not cover the full expected sequence of the BGC. Therefore, we sequenced and fully assembled the genome of *N. coxensis* DSM 45129 using a combination of HiSeq Illumina and GridION ONT technologies. The circular chromosome of *N. coxensis* was found to have a smaller size in comparison to the other two previously published *Nonomuraea* genomes—only 9.07 Mbp compared to 11.85 Mbp in *N. gerenzanensis*’s and 13.05 Mbp in *Nonomuraea* sp. ATCC S5076.2 The average GC-content was 71.8%. Annotation of the *N. coxensis* genome revealed 8398 predicted protein coding sequences, five operons for 16S-23S-5S rRNA, and 73 tRNA genes. Genome analysis by antiSMASH 5.0,29 a specialized metabolite BGC identification tool, led to the discovery of 27 putative BGCs when used in the “relaxed” search mode. However, only a few BGCs showed more than 20% similarity to known BGCs (Table S1).

We thus focused our attention on the GPA-like BGC, which we denoted as *noc* (from *Nonomuraeacoxensis*). The *noc* BGC is the fourth GPA BGC described from *Nonomuraea* genus, following the *dbv* BGC from *N. gerenzanensis*,6,24 a putative GPA BGC from *Nonomuraea* sp. WAC 0142427 and the type V GPA kistamicin (*kis*) BGC from *Nonomuraea* sp. ATCC S5076.2 The average GC-content was 71.8%. Annotation of the *N. coxensis* genome revealed 8398 predicted protein coding sequences, five operons for 16S-23S-5S rRNA, and 73 tRNA genes. Genome analysis by antiSMASH 5.0,29 a specialized metabolite BGC identification tool, led to the discovery of 27 putative BGCs when used in the “relaxed” search mode. However, only a few BGCs showed more than 20% similarity to known BGCs (Table S1).

2.2. Comparative Genomics of *Nonomuraea* GPA Producers. At the time of writing, genomic information for 34 *Nonomuraea* species was available in GenBank, although there are only three complete assemblies (Table S2). Along with the four reported *Nonomuraea* GPA BGCs, we found a *kis*-like BGC in the draft genome of *Nonomuraea* sp. NN258 (Figure S1). We have then reconstructed the multilocus phylogeny (MLP) of all *Nonomuraea* species with available genomic data using conserved house-keeping proteins (Table S3). It revealed *N. coxensis* to be most closely related to *N. wenchangensis* CGMCC 4.5598, *N. polychroma* DSM 43925, and *N. turkmeniaca* DSM 43926 (Figure S2). None of these species have GPA BGCs in their genomes. *N. gerenzanensis* is most closely related to *Nonomuraea* sp. FMUSA5–5 and to the kistamicin producer *Nonomuraea* sp. ATCC S5076, whereas...
Table 1. Characterization of noc BGC genes and their comparison to the dbv and WAC 01424 GPA BGCs from Nonomuraea spp.

| noc BGC genes | homologues from dbv BGC (aa identity of protein product with noc homologue, %) | homologues from WAC 01424 GPA BGC (numbered as in Figure 2) (aa identity of protein product with noc homologue, %) | encoded protein |
|---------------|---------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|----------------|
| noc1          | dbv1 (90.6%)                                                                     | DMB42_RS42735 (31) (60%)                                                                       | hydroxymandelate oxidase (Hmo)                     |
| noc2          | dbv2 (89.3%)                                                                     | DMB42_RS42740 (30) (62%)                                                                       | hydroxymandelate synthase (HmAS)                  |
| noc3          | dbv37 (90.9%)                                                                    | DMB42_RS42745 (29) (83%)                                                                       | hydroxyphenylglycine aminotransferase (HpgT)      |
| noc4          | dbv35 (90.9%)                                                                     | DMB42_RS42730 (32) (63%)                                                                       | N\textsuperscript{a}−H\textsuperscript{a} antiporter |
| noc5          | dbv34 (93.9%)                                                                    | DMB42_RS42710 (36) (87%)                                                                       | enoyl-CoA hydratase (DpgD)                        |
| noc6          | dbv33 (89.2%)                                                                    | DMB42_RS42715 (35) (84%)                                                                       | dihydroxyphenylacetyl-CoA dioxygenase (DpgC)      |
| noc7          | dbv32 (85.1%)                                                                    | DMB42_RS42720 (34) (74%)                                                                       | enoyl-CoA hydratase (DpgB)                        |
| noc8          | dbv31 (94.3%)                                                                    | DMB42_RS42725 (33) (91%)                                                                       | type III polyketide synthase (DpgA)               |
| noc9          | dbv30 (83.5%)                                                                    | DMB42_RS42750 (28) (69%)                                                                       | 4HB-CoA thioesterase                             |
| noc10         | dbv28 (92.4%)                                                                    | DMB42_RS42760 (26) (86%)                                                                       | \(\beta\)-hydroxylase                             |
| noc11         | dbv27 (91.8%)                                                                    | DMB42_RS42755 (27) (58%)                                                                       | methyltransferase                                |
| noc12         | dbv18 (87.3%)                                                                    | a                                                                                              | ABC transporter                                  |
| noc13         | dbv19 (92.2%)                                                                    | a                                                                                              | ABC transporter                                  |
| noc14         | dbv20 (89.7%)                                                                    | a                                                                                              | mannonyltransferase                              |
| noc15         | dbv21 (86.6%)                                                                    | DMB42_RS42765 (25) (64%)                                                                       | deacetylase                                      |
| noc16         | dbv22 (92.3%)                                                                    | DMB42_RS42850 (9) (77%)                                                                        | sensory histidine kinase                          |
| noc17         | dbv23 (88.1%)                                                                    | a                                                                                              | acetyltransferase                                |
| noc18         | dbv24 (92.4%)                                                                    | DMB42_RS42845 (10) (81%)                                                                       | ABC transporter                                  |
| noc19         | dbv25 (88.7%)                                                                    | DMB42_RS42840 (11) (76%)                                                                       | NRPS modules 1–2                                 |
| noc20         | dbv26 (91%)                                                                      | DMB42_RS42835 (12) (78%)                                                                       | NRPS module 3                                    |
| noc21         | dbv27 (91.7%)                                                                    | DMB42_RS42830 (13) (77%)                                                                       | NRPS module 4–5–6                                |
| noc22         | dbv28 (91.1%)                                                                    | DMB42_RS42825 (14) (79%)                                                                       | MbtH-like protein                                |
| noc23         | dbv29 (89.8%)                                                                    | DMB42_RS42820 (15) (93%)                                                                       | cross-linking oxygenase (OxyA)                    |
| noc24         | dbv30 (89.8%)                                                                    | DMB42_RS42810 (17) (77%)                                                                       | cross-linking oxygenase (OxyC)                    |
| noc25         | dbv31 (93.5%)                                                                    | DMB42_RS42805 (18) (77%)                                                                       | cross-linking oxygenase (OxyB)                    |
| noc26         | dbv32 (91.9%)                                                                    | DMB42_RS42795 (20) (78%)                                                                       | cross-linking oxygenase (OxyE)                    |
| noc27         | dbv33 (94.1%)                                                                    | DMB42_RS42790 (21) (87%)                                                                       | halogenase                                       |
| noc28         | dbv34 (87.5%)                                                                    | DMB42_RS42780 (23) (74%)                                                                       | glycosyltransferase (GtfB)                        |
| noc29         | dbv35 (87.3%)                                                                    | DMB42_RS42775 (24) (77%)                                                                       | acyltransferase                                  |
| noc30         | dbv36 (95.9%)                                                                    | DMB42_RS42865 (6) (78%)                                                                        | VanY-carboxypeptidase                             |
| nocRI         | dbv37 (92.8%)                                                                    | DMB42_RS42860 (7) (85%)                                                                        | response regulator                                |
| nocRII        | dbv38 (94.4%)                                                                    | DMB42_RS42700 (38) (85%)                                                                       | prephenate dehydrogenase (Fdh)                   |

\textsuperscript{a}Homologue is absent.

Nonomuraea sp. WAC 01424 is distantly related to both N. coxensis and N. gerenzanensis (Figure S2). Thus, GPA-producing Nonomuraea species do not form a single phylogenetic group, which is different from what occurs in the majority of Amycolatopsis spp. producing GPAs. 30 Since N. gerenzanensis and Nonomuraea sp. ATCC 55076 are closely related and their genomes had been completely assembled, we compared their sequences using the MAUVE genome alignment tool. 31 We found that the two genomes are very similar, having few rearranged homologous segments (Figure S3A). Interestingly, the regions flanking the dbv BGC in N. gerenzanensis show synteny in Nonomuraea sp. ATCC S5076, but in this genome, they flank a miscellaneous assemblage of GPA-unrelated genes instead of the dbv genes. No dbv-like BGC is present in Nonomuraea sp. ATCC S5076. Similarly, no kis-like BGC is in the N. gerenzanensis genome, but the regions flanking the kis BGC in Nonomuraea sp. ATCC S5076 have their homologous counterparts in the N.
gerenzanensis genome (Figure S3A). Dot plots of N. gerenzanensis and Nonomuraea sp. ATCC 55076 confirm the high homology between the two strains (Figure S3B). A possible explanation is that Nonomuraea sp. ATCC 55076 and N. gerenzanensis genomes might have acquired different GPA BGCs independently through horizontal gene transfer (HGT) events from other Nonomuraea (or not) species.

Dot plots of N. coxensis and Nonomuraea sp. ATCC 55076 genomes (Figure S3C) as well as of N. coxensis and N. gerenzanensis (Figure S3D) indicate that N. coxensis is more distantly related to the other GPA producing species. Unfortunately, it was impossible to compare the genome of N. coxensis with its closest relatives N. wenchangensis CGMCC 4.5598, N. polychroma DSM 43925, and N. turkeniaca DSM 43926 (Figure S2), due to the incompleteness of their genome assemblies. Overall, it seems that the position of GPA BGCs is not conserved within Nonomuraea genomes, which contrasts to what was observed in most Amycolatopsis spp.

2.3. Comparing noc and dbv Biosynthetic Pathways: From Genes to Products. The biosynthesis of A40926 is well understood (Figure 3). The heptapeptide core of this antibiotic is synthesized by a nonribosomal peptide synthetase (NRPS) assembly line involving Dbv25, Dbv26, Dbv17, and Dbv16 proteins. The linear peptide is cross-linked by four monoxygenases (Dbv14, Dbv12, Dbv13, and Dbv11) and halogenated by Dbv10, giving the core aglycone. This aglycone is further modified with the glycosyltransferases Dbv9 and Dbv20, which attach N-acetyl glucosamine (GlcNAc) and mannose, respectively. Then, GlcNAc is oxidized by Dbv29, deacylated by Dbv21, and acylated by Dbv8. Finally, the...
mannose moiety is acetylated by Dbv23, giving O-acetyl-A40926. Considering the A40926 pathway, it was possible to predict the biosynthetic pathway of the putative GPA from N. coxensis (Figure 3). Sets of genes required for the biosynthesis of the nonproteinogenic precursor amino acids 4-hydroxyphenylglycine (Hpg), 3,5-dihydroxyphenylglycine (Dpg), and \( \beta \)-hydroxytyrosine (further used as substrates for NRPS) are the same in noc and \( \text{dbv} \) BGCs (Table 1, Figures 2 and 3). Next, the NRPS, encoded within noc BGC, was found to have the same organization and A-domain specificities as the \( \text{dbv} \) NRPS (Figure S4, Table S4). All other genes, responsible for the cross-linking and tailoring steps, were identical in both the noc and \( \text{dbv} \) pathways (Table 1, Figures 2 and 3). However, one notable difference between \( \text{dbv} \) and noc was the absence of a \( \text{dbv} \)-29 orthologue in the latter. As mentioned above, Dbv29 is a hexose oxidase responsible for the oxidation of the GlcN-Acyl moiety of A40926.\(^{15}\) On this basis, we predicted that the noc pathway might produce an A40926 analogue lacking the carboxylic group on the GlcN-Acyl residue and therefore resembling teicoplanin in this moiety (Figures 1 and 3).

Beyond the biosynthetic genes, noc and \( \text{dbv} \) feature homologous regulatory genes. Two master regulators of A40926 biosynthesis—LuxR-like Dbv3 and StrR-like Dbv4—have orthologues coded within noc—NocRI (94% aa sequence identity) and NocRII (86% aa sequence identity), respectively.\(^{6}\) In N. gerenzanensis, both Dbv3 and Dbv4 are crucial for biosynthesis activation.\(^{23}\) Dbv4 was shown to bind the promoter regions of operons \( \text{dbv} \)-30-35 (mainly coding for Dpg biosynthesis enzymes) and \( \text{dbv} \)-14-18 (including the genes coding for cross-linking monoxygenases), and its binding sites were identified.\(^{23}\) Our in silico analysis indicates that identical binding sites are present in the promoter regions of noc20 and noc8, orthologues of \( \text{dbv} \)-14 and \( \text{dbv} \)-30, respectively (Figure S5). DNA-binding sites of Dbv3 remain uncharacterized, but its regulon was defined from gene expression analysis and includes other biosynthetic genes and Dbv4.\(^{23}\) Given all these similarities, we presume that NocRI/NocRII have functions identical to Dbv3/Dbv4 and both regulatory pairs might cross-talk between these species. Our previous results,\(^{6}\) where heterologous expression of nocRI in N. gerenzanensis improved A40926 production, support this assumption. The single GPA resistance determinant encoded within noc is Noc27, a close (87%) orthologue of Dbv7 (VanY\(_2\)), which is a D,D-carboxypeptidase involved in A40926 self-resistance.\(^{23,34}\)

Although the biosynthetic, regulatory, and resistance genes are apparently shared by the \( \text{dbv} \) and noc BGCs, their genetic organization is different. So far, almost all GPA BGCs have NRPS genes located on one strand in an order that is colinear to the order of the modules in the NRPS assembly line. The only exception is the \( \text{dbv} \) BGC, where the NRPS genes are coded on different strands and are separated by other biosynthetic genes.\(^{18}\) The noc BGC, although sharing a remarkable similarity with \( \text{dbv} \), features an organization of NRPS genes that is typical of all the other GPAs. Interestingly, only two chromosomal inversion events are needed to rearrange noc into \( \text{dbv} \) (Figure S6), indicating how a \( \text{dbv} \)-like gene arrangement might have derived from a noc-like BGC in a common ancestor of N. coxensis and N. gerenzanensis (or in an ancestral protocluster)

The putative GPA BGC in Nonomuraea sp. WAC 01424 (Figure 2) differs more substantially from both noc and \( \text{dbv} \). It lacks a noc14/\( \text{dbv} \)-20 homologue encoding for a mannosyltransferase, as well as a noc17/\( \text{dbv} \)-23 homologue encoding for a mannosyl-O-acetyltransferase (Table 1). Instead, WAC 01424 GPA BGC contains a close homologue of \( \text{stat} \) (Figure S7), which encodes for a sulfotransferase involved in the biosynthesis of A47934 from Streptomyces toyoensis NRRL 15009.\(^{35}\) Additionally, the WAC 01424 BGC-encoded halogenases seem more related to the ones from the A47934 BGC than to Noc24 and Dbv8 (Figure S7). Thus, we suggest that WAC 01424 GPA is a nonmannosylated, but sulfated, A40926 analogue, putatively with a halogenation pattern different from A40926 (Figure S8).

2.4. Optimization of GPA-Producing Conditions for N. coxensis. N. coxensis was first described in 2007,\(^{36}\) but as far as we know, it was never tested for the production of antimicrobials. Considering the predicted similarity between the putative GPA produced by N. coxensis with A40926, we first applied to N. coxensis the cultivation and A40926 production conditions that we had previously optimized for N. gerenzanensis.\(^{22,37}\) In these conditions (namely a vegetative pre-culture in E26 medium and a GPA production step in FM2 medium using baffled flasks), N. coxensis tended to grow poorly, and no antimicrobial activity was detectable throughout the 168h cultivation from inoculum. Thus, we further screened different media and fermentation conditions previously used for growing other GPA producing strains, such as TM1 used for teicoplanin production by A. teichomyceticus\(^{38}\) and R5 adopted for balhimycin production in A. mycolatus balhimycina,\(^{39}\) as well as YM0.1 and ISP21 previously employed for the vegetative cultivation of N. coxensis\(^{6}\) (media composition detailed in the Supporting Information). The production of antimicrobial activity towards Bacillus subtilis ATCC 6633 was observed only in TM1 and ISP21 media when glass beads were added to favor dispersed growth (Figure S9). Indeed, adding glass beads to E26 medium cultures allowed us to use it for a successful vegetative pre-culture step (Figure S10A). Interestingly, routine analysis of glucose consumption in all media described above indicated that N. coxensis did not visibly consume glucose during growth (data not shown). We thus tested the glucose-lacking E26 (named E27), TM1 (TM1m), and ISP21 (ISP21m) media variants for N. coxensis growth and putative GPA production. We found that biomass accumulation was similar in E26 and E27 (Figure S10A) and that biomass and antimicrobial production were equivalent in TM1 and ISP21 as well as in their glucose lacking variants TM1m and ISP21m (Figure S10B and C). Currently, it is impossible to say why N. coxensis fails to use glucose throughout cultivation given that all necessary genes are present within its genome (Figure S10D). Thus, for all the following work with N. coxensis, E27, TM1m, and ISP21m were used.

2.5. Expression of VanY-like Activity in N. coxensis. As already mentioned, the noc BGC encodes a Dbv7 orthologue—Noc27. We therefore tested whether D,D-carboxypeptidase activity could be detected in GPA-producing cultures of N. coxensis. This was measured in membrane extracts as previously reported for N. gerenzanensis and its mutant strains.\(^{23}\) D,D-carboxypeptidase activity was measurable in N. coxensis extracts, although at an inferior level than in N. gerenzanensis (Figure S11). This indicated that Noc27 is functional and its expression correlates with the antimicrobial producing conditions. These results corroborate the hypothesis that noc genes are expressed and a novel GPA active versus B. subtilis is produced by N. coxensis. As in \( \text{dbv} \)\(^{10}\) and WAC 01424...
Figure 4. MS characterization of novel GPA complex produced by wild type N. coxensis grown in ISP2lm and TM1m media for 7 days. (a) Extracted ion chromatograms (EICs) of masses corresponding to A40926 B (left column) and the major components of the A50926 complex produced by N. coxensis WT, A50926 B (m/z 859.3, second column), and A50926 A (m/z 852.3, third column). The top row corresponds to a
BGCs, a vanY gene seems to be the only cluster-situated determinant of self-resistance in *N. coxensis*.  

2.6. Puriﬁcation and Identiﬁcation of the Novel Glycopeptide Complex Produced by *N. coxensis*.  

D-Alanine-D-Alanine (D-Ala-D-Ala) afﬁnity resin chromatography was used to capture the putative GPA from cultures of *N. coxensis* grown in ISP2lm and TM1m media. ISP2lm appeared to be the most suitable medium for GPA puriﬁcation, since the rich composition and high viscosity of TM1m interfered with afﬁnity chromatography. Analyzed by HPLC, the afﬁnity resin eluates contained two major peaks with the characteristic UV spectra of the commercially available A40926 standard, but with a different retention time (Figure S12). LC-MS analysis of these peaks revealed they corresponded to ions with *m/z* 852.3 and 859.3 ([M + 2H]2+) 28 and 14 Da smaller respectively than an A40926 standard ([M + 2H]2+ = 866.3, corresponding to A40926 B). We therefore tentatively named this new GPA complex A50926 (Figure 4a and c).

**Figure 4.** continued

![Figure 4](https://doi.org/10.1021/acschembio.1c00170)

commercial standard of A40926 and the middle and bottom rows to culture extracts from ISP2lm and TM1m, respectively. For each mass, peak heights are normalized relative to the intensity of the largest peak in the sample set, shown in brackets at the top of each column. (b) MS spectra for A40926 B, A50926 B, and A50926 A. Peak heights are normalized to the intensity of the top peak in each spectrum, shown on the top right corner of each plot. Signature in-source fragments for each of the analyzed molecules are circled in pink, blue, and green, respectively, whereas the fragment corresponding to the mannosylated aglycone common to all of them is highlighted in yellow. (c) Proposed structure for the A50926 molecules. The top schematic represents a generic proposed structure common to A40926 and A50926 while the insets below represent the differential fragments for each of the analyzed molecules, as inferred from MS and MS/MS data.

**Figure 5.** Production of A40926 in *N. coxensis pSad29* grown in ISP2lm for 7 days. (a) EICs for masses corresponding to A40926 B (red trace), A50926 B (purple), and A50926 A (green) in puriﬁed extracts of *N. coxensis pSad29* (top chromatogram) and *N. coxensis WT* (middle) in comparison to an A40926 commercial standard. The intensity for the top peak in each chromatogram is shown in brackets under the sample name. (b) MS spectrum of A40926 B from *N. coxensis pSad29* cultures. Monoisotopic masses corresponding to [M + 2H]2+ and [M + H]+ adducts are highlighted in red, and the deviation between the observed accurate mass and the predicted mass for A40926 is represented in parts per million. (c) MS/MS spectra of A40926 B produced by *N. coxensis pSad29* and an A40926 B commercial standard.
All three molecules showed similar MS spectra with single, double, and triple charge proton adducts as well as in-source fragments corresponding to the aglycone carrying the mannose moiety and the GlcN-Acyl moiety (Figure 4b,c). The mannosylated aglycone fragment (m/z 1374.3) was common to all three peaks (Figures 4b and S13), indicating that they share the same aglycone structure and mannose decoration. In contrast, the in-source fragment corresponding to the acylated sugar carried the signature mass difference for each molecule (Figures 4b, S14, and S15): the main A50926 peak ([M + H]⁺ = 1717.5361) had a fragment with m/z 344.2, whereas the A40926 standard had a fragment with m/z 358.22 (Figures 4b, S14, and S15). Further MS and MS/MS analyses of these fragments (Figures S14 and S16) allowed us to assign this 14 Da mass difference to the glucosamine moiety. The masses are consistent with this sugar featuring a regular 6-hydroxyl group in A50926 versus being carboxylated in A40926 (Figures 1, 3, 4c, S14, S15, and S16). This correlates with the lack of a homologue of dbv29 in the noc BGC, as it encodes the enzyme responsible for the oxidation of the C-6 hydroxyl group of GlcN-Acyl into a carboxylic acid in A40926. The second A50926 peak ([M + H]⁺ = 1703.5172) had a further 14 Da...
mass difference in the GlcN-AcyI moiety (Figures 4b and S14), but in this case MS/MS showed this difference to be in the acyl chain (Figure S16), which is consistent with an A50926 congener with a C11 acyl chain instead of a C12 acyl chain. This is equivalent to the A and B series of congeners in the A40926 complex.41 Based on this analysis and accurate mass data (Figure 4b), we named the compound with [M + H]⁺ = 1717.54 A50926 B (Figure 4c) and the compound with [M + H]⁺ = 1703.52 A50926 A (Figure 4c).

2.7. Single Gene Expression Leads to A40926 Production in N. coxensis. To support our MS-based characterization of A50926, we hypothesized that we could convert N. coxensis into an A40926 producer by overexpression of the dbv29 gene from N. gerenzanensis, which encodes the hexose oxidase required for oxidation of the C-6 hydroxyl group of GlcN-AcyI into the corresponding carboxylic acid. To achieve this, we used the pSET152A expression platform, which has proven to be very effective for gene overexpression in both N. coxensis and N. gerenzanensis.42 dbv29 was cloned into pSET152A to generate pSAD29, which was then introduced into N. coxensis by conjugation from Escherichia coli. N. coxensis pSAD29⁺ was grown in ISP21m medium for 168 h, and the resulting GPA complex was purified using D- Alpha-A-A affinity resin. LC-MS analysis determined that N. coxensis pSAD29⁺ was able to produce a molecule with an identical retention time and MS spectrum to that of A40926 (observed m/z 1731.5181, calculated A40926 [M + H]⁺ 1731.5107, 4.27 ppm difference) (Figure 5a and b).

MS/MS analysis of the molecule showed it also had an identical fragmentation pattern to the A40926 standard, including the in-source fragment with m/z 358.22 characteristic of the carboxylated GlcN-AcyI moiety (Figures 5c and S17). Traces of A50926 could also be detected in the extract of the complemented strain, indicating that while complementation was very efficient, conversion from A50926 to A40926 was not complete (Figure 5a). Alongside the BGC homology (Figure 2), this provides strong evidence that A50926 is chemically identical to A40926 with the exception of the carboxylated GlcN-AcyI. However, we cannot completely rule out small differences, such as acyl chain branching.

2.8. Heterologous Expression of Transcriptional Regulators dbv3 and dbv4 to Enhance the Production of A50926 in N. coxensis. In previous work, we overexpressed the two dbv BGC situated master regulators in N. gerenzanensis (dbv4 and dbv3) to successfully improve A40926 production.6 Therefore, hereby, we used the previously constructed expression vectors pSAD4 and pSAD3 carrying dbv4 and dbv3, respectively, in N. coxensis to trigger and improve A50926 production. First, we observed that N. coxensis pSAD3⁺ and pSAD4⁺ recombinant strains grown in the E27 and VSP vegetative media produced an antimicrobial activity against B. subtilis (Figure S18A), whereas their parental wild type strain did not exhibit any antimicrobial activity in these media. Overexpression of dbv3 also triggered antimicrobial activity on VM0.1 and ISP2 solid media, whereas the wild type was not active (Figure S18B). Consistently, in both ISP21m and TM1m production media N. coxensis pSAD3⁺ and pSAD4⁺ produced more antibiotic than the wild type (Figures S18C and 6a and b). In ISP21m (Figure 6a), at 192 h N. coxensis pSAD3⁺ reached the maximum production of approximately 45 μg mL⁻¹, exceeding both wild type (approximately 20 μg mL⁻¹) and N. coxensis pSAD4⁺ (approximately 30 μg mL⁻¹) productivities. In TM1m medium (Figure 6b), N. coxensis pSAD3⁺ produced approximately 50 μg mL⁻¹ after 192 h of cultivation. At the same time point in TM1m the wild type and N. coxensis pSAD4⁺ produced approximately 16 and 22 μg mL⁻¹ of antibiotic, respectively. The control strain carrying the “empty” pSET152A vector performed exactly as the wild type (data not shown). No significant differences between biomass accumulation or pH were observed among the recombinant strains, or in comparison with the parental N. coxensis wild type strain. Thus, overexpression of dbv3 and dbv4 regulatory genes triggered or improved the production of A50926 in N. coxensis under different cultivation conditions.

3. CONCLUSIONS

A novel GPA, A50926, was identified from N. coxensis DSM 45129. Detailed MS and MS/MS analysis indicates that A50926 differs from the previously characterized A40926 GPA by lacking the carboxyl group on the GlcN-AcyI moiety attached to Hpg4 of the GPA glycore, resembling teicoplanin in this part of the molecule. A compound with the same chemical structure was described 25 years ago as a chemically prepared derivative of A40926 (named RA⁳⁶). Extensive study of antibacterial activities of RA in vitro⁴⁶ indicated that RA has slightly better antimicrobial activity than A40926: minimal inhibitory concentrations (MICs) of RA were 2–4 times lower against different staphylococcal and enterococcal strains when compared to A40926. The difference of chemical structure between the newly described A50926 and A40926 correlates with the absence of dbv29 orthologue in the A50926 BGC (noc). Consistently, when dbv29 was introduced into N. coxensis, we obtained A40926 production in the recombinant strain. Otherwise, both noc and dbv BGCs share all biosynthetic genes, which are closely related. Heterologous expression of A40926 regulatory genes dbv3 and dbv4 in N. coxensis improved A50926 production.

Although the majority of noc and dbv genes are orthologous, the dbv BGC is significantly rearranged in comparison to the noc BGC, as well as all other characterized GPA BGCs. We have proposed a series of genetic inversions that could have occurred in a common Nonomuraea ancestor to explain these different genetic architectures. Both BGCs are quite similar to the putative GPA BGC from Nonomuraea sp. WAC 01424. The latter lacks genes required for the addition of mannose, but possesses a gene encoding a sulfotransferase and an additional gene encoding a halogenase. Thus, the putative nonmannosylated GPA from Nonomuraea sp. WAC 01424 might be sulfated and have a different chlorination pattern than A40926/A50926. Consequently, Nonomuraea sp. WAC 01424 GPA BGC seems an attractive source for new tailoring genes to obtain A40926 derivatives with altered pharmacological properties. Notwithstanding the GPA BGC similarity, multilocus phylogeny of Nonomuraea spp. shows that GPA producers are not clustered together: GPA producers are found in distinct clades within the genus. Our analysis indicates that type IV and V GPA BGCs are common in Nonomuraea spp., which is in contrast to how rare these BGCs were believed to be. This is comparable to studies that show that BGCs for types I–III–IV GPA are common in Amycolatopsis, and type V GPAs in Streptomyces.⁴⁷,⁴²,⁴³ This highlights how rare actinomycete genera, such as Nonomuraea, may represent a rich untapped source of novel GPAs, as well as GPA tailoring enzymes for the diversification of existing GPA scaffolds.
4. METHODS

4.1. Bacterial Strains and Cultivation Conditions. Bacterial strains and plasmids used in this work are summarized in Table S6. Compositions of all the media used for cultivation and GPA production are also given in the Supporting Information. All media components and antibiotics were supplied by Sigma-Aldrich, unless otherwise stated. For routine maintenance, N. gerenzanensis and N. cossensis strains were cultivated on ISP3 agar medium supplemented with 50 µg mL⁻¹ apramycin-sulfate when appropriate. For genomic DNA isolation, N. gerenzanensis and N. cossensis strains were cultivated in liquid VSP medium on an orbital shaker at 220 rpm and at 30 °C. The working cell banks (WCBs) for N. gerenzanensis and N. cossensis strains were prepared as described previously. E. coli DH5α was used as a routine cloning host, and E. coli ET15576 pUZ8002 was used as a donor for intergeneric conjugations. E. coli strains were cultivated at 37 °C in LB liquid or agar media supplemented with 100 µg mL⁻¹ of apramycin-sulfate, 50 µg mL⁻¹ of kanamycin-sulfate, and 25 µg mL⁻¹ of chloramphenicol when appropriate.

4.2. Plasmid Construction and Generation of Recombinant N. cossensis Strains. To construct the pSAD29 expression vector, the coding sequence of dbv29 (1601 bp) was amplified from the genomic DNA of N. cossensis using dbv29_F/R primer pair (Table S7) and Q5 high-fidelity DNA polymerase (New England Biolabs). The resulting amplicon was digested with EcoRI and EcoRV restriction endonucleases and cloned into pSET152A and Q5 high-fidelity DNA polymerase (New England Biolabs). The resulting plasmid was digested with endonuclease HpaI, which cleaves downstream of the polylinker sequence (Table S7) and Q5 high-fidelity DNA polymerase (New England Biolabs). The resulting plasmid was verified by endonuclease restriction mapping and sequencing at BMG Genomics.

pSAD29, as well as pSAD3, pSAD4, and pSET152A, were transferred to N. cossensis conjunctively, as described previously. Transconjugants were selected as resistant to 50 µg mL⁻¹ of apramycin-sulfate. Obtained strains were verified by PCR. To verify the integration of pSAD29, a ∼1.1 kbp fragment of pSAD29 was amplified using the dbv29_seq_int/PAM_seq_R (Table S7) primer pair, in which dbv29_seq_int anneals within dbv29 and PAM_seq_R anneals upstream the EcoRV cleavage site of pSET152A. To verify the integrations of pSAD4 and pSAD3, ∼1 kbp and ∼2 kbp fragments were amplified respectively using PAM_seq_F/dbv4_R and PAM_seq_F/dbv3_seq_R primer pairs (Table S7). Finally, the integration of pSET152A was verified by amplifying aaC(3)IV with the aaC(3)JV_R primer pair (Table S7). In all cases, genomic DNA was isolated using the Kirby procedure.

4.3. N. cossensis Cultivation for A50926 Production. To initialize the cultivation of N. cossensis one WCB vial was inoculated into a 250 mL Erlenmeyer flask with 50 mL of VSP reactivation medium containing 6 glass beads (ø5 mm). After 72 h of incubation on a rotary shaker at 220 rpm, 30 °C the culture was used to inoculate (10% v/v) 500 mL Erlenmeyer flask containing 100 mL of E27 vegetative medium and 12 glass beads (ø 5 mm). Following 72 h of incubation on a rotary shaker at 220 rpm, 30 °C this culture was used to inoculate (10% v/v) 500 mL Erlenmeyer flask containing 100 mL of ISP21m or TM1m production media containing 12 glass beads (ø 5 mm). A50926 production cultures were then incubated up to 240 h on a rotary shaker at 220 rpm, 30 °C. Samples were collected at regular time points to estimate biomass accumulation (dry weight), pH, and A50926 production.

4.4. VanY-Related Activity Measurement. d,L-carboxypeptidase activity in Nonomuraea spp. was measured in FM2 production medium (N. gerenzanensis) and ISP21m (N. cossensis) at 24, 48, 72, 96, 120, and 144 h time points. Mycelial lysates were prepared as described previously. The enzyme activity releasing d-Ala from the tripeptide N-Acetyl-L-Lys-d-Ala-d-Ala (10 mM) was followed spectrophotometrically by a d-amino acid oxidase/peroxidase coupled reaction that oxidizes the colorimetric substrate 4-aminoantipyrine to chinoxime. d,L-carboxypeptidase activity was normalized to dry biomass weight, as previously reported. One unit is defined as the amount of enzyme that is able to convert 1 µmol of substrate in 1 min.

4.5. HPLC and LC-MS Analysis of GPAs. For quantitative measurement, A40926 and A50926 were extracted from N. cossensis cultures with equal volumes of borate buffer composed of 100 mM H₂BO₃ (Sigma-Aldrich) and 100 mM NaOH (Sigma-Aldrich), pH 12. During this extraction the O-acetylated forms were converted in the corresponding decacetylated GPAs A40926 and A50926. A40926 and A50926 were analyzed using HPLC as previously reported. In all cases the injection volumes of studied samples and standards were the same (50 µL). Concentration of A50926 was estimated as follows:

\[ \text{A50926 concentration} (\text{mg L}^{-1}) = C(A40926 \text{ std}) \times A50926(A40926 \text{ std}) \times 2 \]

Where, C(A40926 std) is the concentration of the commercial A40926 sample; A(A50926 std) is the area sum of the peaks corresponding to A50926 B; A(A40926 std) is the area of the peak corresponding to the standard A40926 factor B0 and 2 is the dilution factor.

High resolution liquid chromatography–mass spectrometry (LC-MS) and fragmentation (MS/MS) analysis of A40926 and A50926 was carried out on a SYNAPT G2-Si mass spectrometer equipped with an Acquity UPLC (Waters). Samples were injected onto a Waters Acquity UPLC BEH 1.7 µm, 1 × 100 mm C18 column, and eluted with a gradient of (A) acetonitrile/0.1% formic acid in (A) water/0.1% formic acid with a flow rate of 0.08 mL min⁻¹ at 45 °C. The concentration of B was kept at 1% for 2 min followed by a gradient up to 40% B over 9 min, ramping to 99% B in 1 min, kept at 99% B for 2 min and re-equilibrated at 1% B for 4 min. MS data were collected in positive mode with the following parameters: resolution, scan time 0.5 s, mass range m/z 50–2000 calibrated with sodium iodide, capillary voltage = 2.5 kV; cone voltage = 40 V; source temperature = 120 °C; desolvation temperature = 350 °C. Leunkephalin peptide was used to generate a mass calibration with m/z 556.2766 for positive mode, measured every 90 s during the run. For MS/MS fragmentation, a data directed analysis (DDA) method was used with the following parameters: precursor selected from the 4 most intense ions; MS/MS threshold 5000; scan time 2 s; no dynamic exclusion. Collision energy (CE) was ramped between 8 and 35 at low mass (m/z 50) and 10–70 at high mass (m/z 1200).

4.6. Purification of GPAs Using d-Ala-d-Ala-Based Affinity Resin. GPAs were purified by affinity chromatography with a d-Alanine-d-Alanine (d-Ala-d-Ala) based resin. Activation of 5 mL HitTrap NHS-activated HP affinity columns (GE Healthcare) and ligand binding was conducted as described before with modifications. Briefly, the resin was activated with 30 mL of 1 mM HCl, followed by injection of 200 mM d-Ala-d-Ala dipeptide, dissolved into 5 mL of coupling buffer (0.2 M NaHCO₃, pH 7.0). After 30 min incubation, the resin was washed with three cycles of 0.5 M ethanolamine hydrochloride, 0.5 M NaCl (pH 4.0, 30 mL), followed by 0.1 M sodium acetate, 0.5 mM NaCl (pH 4.0, 30 mL), alternately. Finally, the resin was wash with 50 mL coupling buffer and left to equilibrate for at least 1 h before use.

N. cossensis cultures were extracted in borate buffer as reported above, the pH in the obtained extracts was adjusted to 7.5 with HCl, and they were applied to the affinity chromatography system. Thus, extracts in borate buffer, coming from N. cossensis strains cultivated in TM1m or ISP21m media, were filtered with 0.45 µm cutoff and loaded onto a d-Ala-d-Ala column at a flow rate of 0.5 mL min⁻¹. After extensive washing with coupling buffer, the bound GPA was eluted with 0.1 M NaOH and the eluate was lyophilized.

4.7. Bioassays for the Detection of A50926. Agar plug or Whatman paper disc (GE Healthcare) antibiotic diffusion assays were used to determine antimicrobial activities. An overnight B. subtilis ATCC 6033 culture in Mueller-Hinton broth II (cation adjusted, Sigma-Aldrich) was used to inoculate (1% v/v) a fresh culture, which was grown to OD₆₀₀ = 0.6. A 200 µL portion of this culture was then added to 25 mL of 0.7% (w/v) Mueller-Hinton agar (Condalab) and plated. After solidification of the media, agar plugs cut from the plates with N. cossensis lawns, or Whatman paper discs containing GPAs,
were placed on the agar surface. Bioassay plates were incubated for 16 h at 37 °C before examination.

4.8. Sequencing and Annotation of the N. coensis Genome. The genome of N. coensis was sequenced using a combination of HiSeq Illumina and GridION ONT technologies. The Illumina data was obtained from SRA (PRJNA165411), while for the ONT data, a sequencing library (SQK-LSK109) was prepared using the Ligation Sequencing Kit (Oxford Nanopore Technologies) according to the manufacturer’s instructions and run on a GridION sequencer in an R9.4.1 flowcell (both Oxford Nanopore Technologies). Base-calling of the raw data was performed with GUPPY-FOR-GRIDION v3.0.6. The assembly and polishing were performed as described previously, using canu v.1.8 instead of v.1.6. The ONT data was assembled into 5 contigs, while the Illumina data were assembled into 87 scaffolds containing 310 contigs using NEWBLOK v2.8. After manual curation using CONSED, the complete genome of N. coensis DSM 45129, consisting of one circular chromosome of 9,073,954 bp (72.12% G + C) was obtained. Annotation was performed using PROKKA v1.11 resulting in the prediction of 8,398 coding sequences (CDS), 5 rRNA operons, 73 tRNAs, and 5 noncoding RNA elements. The annotated genome and ONT raw data were deposited at DDBJ/ENA/GenBank under the BioProject accession number PRJNA693185.

4.9. In Silico Analysis Tools and Approaches. Routine analysis of nucleotide and amino acid sequences was performed in GENEIOUS v4.8.5. Multiple sequence alignments, selection of the best models for the phylogenetic reconstruction and phylogenetic reconstruction itself were done with the MEGA X package. To reconstruct the multilocus phylogeny of Nonomuraea, orthologues of S. coelicolor house-keeping proteins (Table S3) were identified within the genomes of 34 Nonomuraea spp. (Table S2) using reciprocal best hit (RBH) BLAST. Sequences of these proteins from each Nonomuraea spp. were concatenated, and these concatenates were used for the upstream phylogenetic reconstruction.

ASSOCIATED CONTENT

† Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschemicalbiology.1c00170.

Table S1–S7 and Figures S1–S18 (PDF)

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Notes
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