Published in final edited form as:
Chembiochem. 2009 January 26; 10(2): 366–376. doi:10.1002/cbic.200800646.

Investigations into Viomycin Biosynthesis Using Heterologous Production in *Streptomyces lividans*

John J. Barkei[a], **Brian M. Kevany**[a], **Elizabeth A. Felnagle**[a,b], and **Michael G. Thomas**[a,b,*]

[a] J. J. Barkei, Dr. B. M. Kevany, E. A. Felnagle, Prof. M. G. Thomas. Department of Bacteriology, University of Wisconsin-Madison, 1550 Linden Drive, Madison, WI 53706 USA

[b] E. A. Felnagle, Prof. M. G. Thomas. Microbiology Doctoral Training Program, University of Wisconsin-Madison, 1550 Linden Drive, Madison, WI 53706 USA

Abstract

Viomycin and capreomycin are members of the tuberactinomycin family of antituberculosis drugs. As with many antibacterial drugs, resistance to the tuberactinomycins is problematic in treating tuberculosis, making the development of new derivatives of these antibiotics to combat this resistance of utmost importance. To take steps towards developing new derivatives of this family of antibiotics, we have focused our efforts on understanding how these antibiotics are biosynthesized by the producing bacteria so that metabolic engineering of these pathways can be used to generate desired derivatives. Here we present the heterologous production of viomycin in *Streptomyces lividans* 1326 and the use of targeted-gene deletion as a mechanism for investigating viomycin biosynthesis as well as the generation of viomycin derivatives. Deletion of *vioQ* resulted in nonhydroxylated derivatives of viomycin, while strains lacking *vioP* failed to acylate the cyclic pentapeptide core of viomycin with β-lysine. Surprisingly, strains lacking *vioL* produced derivatives that had the carbamoyl group of viomycin replaced by an acetyl group. Additionally, the acetylated viomycin derivatives were produced at very low levels. These two observations suggested that the carbamoyl group of the cyclic pentapeptide core of viomycin was introduced at an earlier step in the biosynthetic pathway than previously proposed. We present biochemical evidence that the carbamoyl group is added to the β-amino group of L-2,3-diaminopropionate prior to this amino acid being incorporated by the nonribosomal peptide synthetases that form the cyclic pentapeptide cores of both viomycin and capreomycin.

Keywords

viomycin; antituberculosis; antibiotic; metabolic engineering; capreomycin

Introduction

It has been estimated that one-third of the world's population is infected with the bacterium *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB).[1] Of these infected people, approximately 10% will develop TB at some point in their life. In 2006, there were 14.4 million cases of TB, with 1.7 million deaths due to complications from TB.[2] TB is second only to HIV/AIDS in the number of deaths caused by an infectious agent.[3,4] One of the most significant challenges in treating patients suffering from TB is that *M. tuberculosis* strains are developing resistance to many of the currently used anti-TB drugs. Multidrug-

Corresponding author: Department of Bacteriology, University of Wisconsin-Madison, 6155 Microbial Sciences Building, 1550 Linden Drive, Madison, WI 53706 U.S.A. Phone: 608-263-9075; thomas@bact.wisc.edu.

resistant TB (MDR-TB), which is defined as an infection that proves resilient to isoniazid and rifampin treatment, has proven to be a significant challenge to eradicating TB. Another class of infections that causes more significant challenges to treatment is extensively drug-resistant TB (XDR-TB) that has been defined by the Center for Disease Control and Prevention as an MDR-TB infection that is also resistant to treatment with fluoroquiolones and at least one of the injectable drugs kanamycin, amikacin, or capreomycin.[4] To combat these resistant strains of *M. tuberculosis* it is important that we identify of new drugs or develop new derivatives of currently used drugs that are active in treating TB, MDR-TB, and XDR-TB.

The tuberactinomycins are a family of nonribosomal peptide antituberculosis drugs that target the ribosome of susceptible bacteria.[5,6] Viomycin (**1**) was the first member of this antibiotic family to be identified.[7] Viomycin was used to treat TB until it was replaced by the structurally related antibiotic capreomycin because this mixture of four metabolites (**2–5**) has reduced toxicity compared to **1** (Scheme 1).[8] Additional members of this antibiotic family include the tuberactinomycins (**6–9)** (Scheme 1),[9] of which tuberactinomycin N (**9**), also known as enviomycin, is used for the treatment of TB in Asia.[10] Capreomycin is the most commonly used antibiotic in this drug family for the treatment of TB (Scheme 1) and is on the World Health Organization's "List of Essential Medicines"[11] due to its activity against MDR-TB. Additionally, the recent analysis of second-line injectable drugs and the treatment outcomes of MDR-TB and XDR-TB determined that the development of capreomycin resistance results in a higher level of drug-treatment failure and subsequent death of the patient.[12] Based on these results, it is important that additional derivatives of members of the tuberactinomycin family of antibiotics be developed to ensure their continued use against drug-resistant *M. tuberculosis* infections.

We are interested in deciphering how members of the tuberactinomycin family of antibiotics are biosynthesized by the producing bacteria, with the long-term goal of using this information to generate new derivatives of these antibiotics through metabolic engineering. To this end, we have identified the biosynthetic pathways for **1** and **2–5**,[13,14] identified a new capreomycin resistance gene, and have investigated the biosynthesis of the unusual amino acid (2*S*,3*R*)-capreomycidine (L-Cam; residue five that is hydroxylated by VioQ in Scheme 2).[15] Zabriskie and colleagues have also investigated the biosynthesis of **1** and have sequenced some of the genes within the associated biosynthetic gene cluster.[16] They also investigated L-Cam biosynthesis, along with the incorporation and hydroxylation of this amino acid.[17–19] Hypotheses for how **1** and **2–5** are biosynthesized have been developed based on our results and those of Zabriskie and colleagues.

Each of these antibiotics consists of a central cyclic pentapeptide core that is likely to be assembled by a nonribosomal peptide synthetase (NRPS). For **1**, we proposed that the NRPS, consisting of the proteins VioA, VioF, VioI, and VioG, condenses and cyclizes two molecules of L-2,3-diaminopropionate (L-Dap), two molecules of L-serine (L-Ser), and one molecule of L-Cam. While the NRPS condenses and cyclizes these five residues, VioJ was proposed to catalyzes the α,β-desaturation of residue four.[13] To complete the synthesis of **1**, we proposed that three subsequent modifications occur (Scheme 2). One modification is the hydroxylation of the ring of residue five by VioQ, a proposal that was recently supported by experimental work by Zabriskie and colleagues.[19] A second modification is the N-acylation of residue one with β-Lysine (β-Lys) by the concerted actions of VioO and VioM, a monomodular NRPS. The third modification is the carbamoylation of residue four by the carbamoyltransferase homolog VioL. We have proposed a similar set of biosynthetic mechanisms for the assembly of **2–5**, with the subtle change of N-acylation of residue three with β-Lys by CmnO and CmnM, which are homologs of VioO and VioM, respectively. Also, the capreomycin biosynthetic pathway lacks a VioQ ortholog, which is consistent with the observation that **2–5** are not hydroxylated (Scheme 1).[14]

Here we present genetic and biochemical analyses of portions of the biosynthetic pathway for **1**. First, we show that *Streptomyces lividans* 1326 is a competent host for the heterologous production of **1**. This result provides additional evidence that we have identified all of the genes necessary for the biosynthesis of **1**. We subsequently used a series of gene deletions to investigate the three proposed modification steps in the biosynthesis of **1** and to generate alternative derivatives of **1**. These data confirmed the proposed functions of each of the encoded enzymes and support the hypothesis that metabolic engineering of the biosynthetic gene cluster for **1** can be used to generate alternative derivatives of **1**. Based on the results from the genetic experiments, the NRPSs involved in the formation of the cyclic pentapeptide core of **1** and **2–5** were re-evaluated, and we present new models for core assembly during the biosynthesis of these antibiotics.

RESULTS AND DISCUSSION

Heterologous production of viomycin in *S. lividans* 1326

We have previously shown that *S. lividans* 1326 is a competent host for the heterologous production of **2–5**.[14] Based on the structural similarity of **1** and **2–5** (Scheme 1) and the nearly identical organization of the associated biosynthetic gene clusters, [13,14] it was reasonable to hypothesize that *S. lividans* 1326 would also be competent for the heterologous production of **1**. As an alternative to reassembling the biosynthetic gene cluster for **1** from two overlapping cosmids that we previously isolated and characterized,[13] we focused on the identification of another cosmid that contained the entire biosynthetic gene cluster. Once identified, this cosmid could subsequently be introduced into *S. lividans* 1326 to test whether the resulting strain was able to heterologously produce **1**.

During our initial characterization of the biosynthetic gene cluster for **1**, we isolated a number of cosmids that contained the resistance gene *vph*, the biosynthetic gene *vioG*, or both genes. Upon screening by PCR amplification, one of the cosmids that contained *vph* and *vioG*, pVIO-P4C3RH, was also found to contain *vioA* and *vioQ*. These genes are at or near the 5′ and 3′ ends, respectively, of what we have proposed to be the complete biosynthetic gene cluster. [13] The presence of *vioA*, *vioG*, *vph*, and *vioQ* on pVIO-P4C3RH suggested all the genes necessary for the production of **1** were contained on this single cosmid. The 5′ and 3′ ends of the DNA inserted into the *Bam*HI site of the cosmid were sequenced to determine what region of the *Streptomyces* sp. strain ATCC11861 chromosome was carried by pVIO-P4C3RH. Analysis of this DNA sequence determined that the chromosomal DNA fragment contained on pVIO-P4C3RH consisted of four kb of DNA upstream of *vioA* through a portion of the *vioT* gene, which is three kb downstream of *vioQ*. Our initial hypothesis that *vioT* was involved in regulating the expression of the biosynthetic gene cluster for **1** was revised after we failed to find a *vioT* homolog in the biosynthetic gene cluster for the production of **2–5**.[14]

Our next goal was to investigate whether the introduction of this cosmid into *S. lividans* 1326 would enable this bacterium to heterologously produce **1**. The pVIO-P4C3RH cosmid was modified to enable conjugation between *E. coli* and *S. lividans* 1326 and stable integration into the *S. lividans* 1326 genome. The strain carrying the integrated cosmid was grown in viomycin-production medium for ten days, the culture supernatant was processed as previously described for **1** and **2–5** purification,[13,14] and the metabolites produced by this strain were compared by HPLC and MALDI-TOF MS to an authentic standard of **1** (Fig. 1, Table 2).

Two dominant metabolite peaks eluted at 5.1 and 12.1 min and had UV-visible adsorption spectra (λ_{max} = 268 nm) consistent with a derivative of **1**. The metabolite eluting at 5.1 min had an elution time equivalent to that of an authentic standard of **1** (Fig. 1, trace C versus trace A). Analysis of this metabolite by MALDI-TOF MS determined this sample was a mixture of two metabolites. One metabolite was **1**, while the second metabolite had a mass consistent with

dehydrodesoxyviomycin (**10**) (Scheme 3, Table 2). This derivative of **1** has been previously reported to be present in a sample of authentic **1**.[20] Additionally, we detected this derivative in **1** purchased from Research Diagnostics, Inc. (Table 2) and in **1** purified from *Streptomyces* sp. strain ATCC11861 (data not shown). Allmaier and colleagues proposed that this derivative is either an intermediate on the reaction pathway to **1** or is an artifact of the purification scheme.[20] Throughout our analysis of the production of **1** or derivatives of **1**, a mass for the dehydrodesoxy derivative was detected by MALDI-TOF MS unless the producing strain lacked *vioQ*. These data are consistent with the dehydrodesoxy derivatives being associated with the hydroxylation of the ring of residue five by VioQ.

A second metabolite eluting from the HPLC at 12.1 min had a mass consistent with desoxyviomycin, a derivative of **1** called tuberactinomycin O (**8**) that was previously isolated from the tuberactinomycin producer *Streptomyces griseoverticillatus* var. *tuberacticus*.[21] The production of **8** was likely due to a less efficient VioQ, an enzyme initially proposed to catalyze the hydroxylation of the ring of L-Cam.[13] Zabriskie and colleagues recently provided experimental evidence to support this proposal by showing that a strain of *Streptomyces* sp. strain ATCC11861 lacking *vioQ* produced **8** rather than **1**.[19] VioQ is a homolog of Rieske non-heme oxygenases that typically require a ferridoxin and a ferridoxin reductase to activate the oxygenase activity of the enzyme.[22] One hypothesis for why the hydroxylation of **8** is incomplete is that VioQ has evolved to interact most efficiently with the cellular ferridoxin and ferridoxin reductase from *Streptomyces* sp. strain ATCC11861, but works less efficiently with the comparable enzymes from *S. lividans* 1362. Thus, although **1** is produced by *S. lividans* 1326, VioQ functions less efficiently resulting in the accumulation of **8**.

The results from these data are important for two reasons. First, these data support the hypothesis that all of the genes required for the heterologous production of viomycin are present on pVIO-P4C3RH-436 and that *vio*T is not necessary for expression in *S. lividans* 1326. Second, since the genetic information is carried on a cosmid that can be conjugated from *E. coli* into *S. lividans* 1326, the extensive genetic tools developed for manipulating genes in *E. coli* can now be used to modify this biosynthetic gene cluster before production in *S. lividans* 1326. This provides an effective means of investigating and redirecting biosynthesis.

Deletion of *vioQ*, *vioP*, or *vioL*

Our proposed mechanism for the biosynthesis of **1** involves the synthesis of the cyclic pentapeptide core of the antibiotic followed by three modifications: 1) hydroxylation of residue five by VioQ; 2) β-Lys synthesis by VioP and subsequent attachment of β-Lys to the α-amino group of residue one by the concerted actions of VioO and VioM; and 3) carbamoylation of the β-amino group of residue four by VioL (Scheme 2). These hypotheses were tested by deleting the corresponding genes on p-VIO-P4C3RH-436 while present in *E. coli*, transferring the modified cosmid to *S. lividans* 1326, and monitoring heterologous antibiotic production by the resulting *S. lividans* 1326 strain.

Deletion of *vioQ*—We used the λred mutagenesis system [23,24] followed by restriction enzyme digestion and religation to generate an in-frame deletion of *vioQ* carried on pVIO-P4C3RH-436. Our prediction was that **8** would be produced when the resulting cosmid, pVIO-P4C3RH-436-Δ*vioQ*, was introduced into *S. lividans* 1326. Consistent with this hypothesis, **1** was not produced by the Δ*vioQ* strain, but a metabolite was observed to elute at 12.1 min (Fig. 1, trace D). The UV-vis spectrum of this metabolite was consistent with it being a derivative of **1**, and it was 16 mass units less than **1** as determined MALDI-TOF MS analysis (Table 2). This metabolite had the same retention time and mass as **8** produced by *S. lividans*

1326 when it contained the entire gene cluster (Fig. 1, compare trace C to D). Thus, the deletion of *vioQ* from the gene cluster results in the heterologous production of **8**.

To confirm that the deletion of *vioQ* was the only mutation causing the formation of **8** instead of **1**, we introduced pJJB501-*vioQ* into the Δ*vioQ* strain of *S. lividans* 1326. The presence of this plasmid restored the ability of the Δ*vioQ* strain to produce **1** (Fig. 1, trace E; Table 2), confirming that the accumulation of **8** is due to the loss of the function of VioQ. While the trace shown in Figure 3 suggests the presence of *vioQ* on a multicopy plasmid reduces amount of **8** that accumulates, this was true for only this particular strain. Additional isolates of the Δ*vioQ* strain of *S. lividans* 1326 carrying the pJJB501-*vioQ* plasmid typically generated **1** and **8** at levels comparable to those seen when the intact gene cluster was present in *S. lividans* 1326 (Fig. 1, trace C). The ability to restore the production of **1** to the Δ*vioQ* strain by adding pJJB501-vioQ supported the conclusion that the loss of VioQ abolishes the hydroxylation of residue five. These data showed that *S. lividans* 1326 with *vioQ* deleted from the integrated biosynthetic gene cluster behaves in a similar manner to *Streptomyces* sp. strain ATCC11861 that lacks a functional *vioQ*. [19]

Deletion of *vioP*—The addition of the β-Lys moiety to the α-amino group of residue one requires prior formation of this nonproteinogenic amino acid. The enzyme coded by *vioP* is a homolog of the well-studied lysine-2,3-aminomutases that convert L-Lys to β-Lys.[25] We hypothesized that by deleting *vioP* from the gene cluster, the resulting *S. lividans* 1326 strain would produce des-β-Lys-viomycin (tuberactinamine A, **14**) instead of **1**, along with the corresponding dehydrodesoxy derivative **11** since *vioQ* was present (Scheme 3). Additionally, since we observed the incomplete hydroxylation of the ring of residue five when entire cluster was present in *S. lividans* 1326, we anticipated that we would also observe desoxy-tuberactinamine A (tuberactinamine N, **15**).

As predicted, **1** was not produced by a strain lacking *vioP*. Instead, new metabolites with UV-visible spectra consistent with derivatives of **1** eluted at 3.8 and 8.2 min (Fig. 1, trace F). MALDI-TOF MS analysis of these metabolites determined that the metabolites eluting at 3.8 min were a mixture of **14** and **11**, while the mass of the metabolite eluting at 8.2 min was consistent with the formation of **15** (Table 2). The only other time **14** has been produced biosynthetically was through the addition of the lysine-2,3-aminomutase inhibitor (*S*)-2-aminoethyl-L-cysteine to the culture of a strain that produced **6–9**.[26] The construction of a Δ*vioP* strain provides a genetic means for biosynthetically producing this derivative of **1**.

The introduction of a plasmid expressing *vioP* into the *S. lividans* 1326 Δ*vioP* strain restored the ability of the strain to produce viomycin (Fig. 1, trace G). This complementation was incomplete based on the presence of detectable levels of **14** in the strain carrying *vioP* on a plasmid. This was true for multiple strains deleted for *vioP* that were carrying *vioP* on pJJB501. We have observed that in many cases, the genes expressed by the *ermEp** promoter on pJJB501 or its parent plasmid pSE34 do not fully complement the corresponding deletion mutant. Additionally we have observed variable levels of detectable protein produced from *ermEp** expression of the corresponding gene (data not shown). Regardless, the ability to restore the production of **1** in the Δ*vioP* strain by the addition of pJJB501-*vioP*, supported the conclusion that the loss of β-Lys attachment to residue one is due to the loss of VioP.

Deletion of *vioL*—We hypothesized that VioL catalyzed the carbamoylation of residue four of the cyclic pentapeptide core of **1** (Scheme 2).[13] This hypothesis was based on the homology of VioL with ornithine carbamoyltransferases involved in arginine biosynthesis. [27] Based on this homology, a strain deleted for *vioL* was predicted to produce descarbamoyl-viomycin. Introduction of the cosmid pVIO-P4C3RH-436-Δ*vioL* into *S. lividans* 1326 resulted in a strain that produced a low level of a metabolite that had a UV-visible spectrum similar to

1, but eluted at 9.5 min from the HPLC (Fig. 1, trace H). MALDI-TOF MS analysis of this metabolite determined it was 42 mass units larger than anticipated for descarbamoyl-viomycin (expected: $[M+H]^+ = 643.3$; observed: $[M+H]^+ = 685.3$). The observed mass was consistent with a derivative of **1** having an acetyl moiety replacing the carbamoyl moiety of residue four, or N-descarbamoyl-N-acetyl-viomycin (**16**) along with the corresponding dehydrodesoxy derivative (**12**) (Table 2). The proposal that the β-nitrogen of residue four has been acetylated is based on the failure to detect any acetylated version of **1** or derivatives of **1**, unless *vioL* was deleted. The acetylation of a nitrogen that becomes available after the inactivation of the gene coding for a modifying enzyme is not unprecedented. For example, Sherman, Liu, and colleagues noted that when the gene coding for an *N*-methyltransferase involved in methylating the amino group of the desosamine sugar in the methymycin/pikromycin biosynthetic pathway is inactivated, the amino group of the sugar is acetylated.[28]

These data provide important insights into the steps in the biosynthesis of **1**. First, the loss of the carbamoyl group from **1** in the Δ*vioL* strain confirmed our hypothesis that VioL introduces the carbamoyl group onto residue four. The introduction of a plasmid that expresses *vioL* restored production of **1** to the Δ*vioL* strain, confirming the conclusion that the observed phenotype was due to the loss of *vioL* (Fig. 1, trace I). Second, the low levels of **16** produced by the Δ*vioL* strain and the finding that only derivatives identified in strains lacking *vioL* (additional mutants are discussed below) have the carbamoyl group replaced by an acetyl group suggests that VioL functions at a step prior to the peptide release from the NRPS. We address this issue in more detail in a later section.

Combining Gene Deletions

The success of the single gene deletions in producing derivatives of **1** suggested that combining the individual mutations would lead to additional variations in the metabolites produced. The corresponding deletion cosmids were constructed in a sequential manner, resulting in four cosmid constructs with the following gene deletion combinations: 1) Δ*vioQ*Δ*vioP*; 2) Δ*vioQ*Δ*vioL*; 3) Δ*vioP*Δ*vioL*; and 4) Δ*vioQ*Δ*vioP*Δ*vioL*. These four constructs were individually conjugated into *S. lividans* 1326 and the resulting strains were analyzed for the production of the anticipated derivatives.

The deletion of *vioQ* and *vioP* was anticipated to result in a strain that the produced **15**, a derivative of **1** lacking both the hydroxylation of residue five and the β-Lys moiety attached to residue one. As predicted, the predominant metabolite produced by the Δ*vioQ*Δ*vioP* strain had an HPLC retention time of 8.2 min and had a mass consistent with **15** (Fig. 1 trace J, Table 2). The two remaining double mutant strains, Δ*vioQ*Δ*vioL* and Δ*vioP*Δ*vioL*, and the triple mutant,Δ*vioQ*Δ*vioP*Δ*vioL*, produced derivatives of **1** at low levels with each of these metabolites having a mass consistent with a descarbamoyl derivative that had been acetylated. The replacement of the carbamoyl group with an acetyl group was due to the loss of Δ*vioL* as previously discussed. The Δ*vioQ*Δ*vioL* strain produced a metabolite that eluted from the HPLC at 12.6 min that had a UV-visible spectrum consistent with a derivative of **1** (Fig. 2, trace B). MALDI-TOF MS analysis of this metabolite determined the mass was consistent with N-descarbamoyl-N-acetyl-tuberactinomycin O (**17**) (Table 2). The Δ*vioP*Δ*vioL* strain produced metabolites that eluted at 6.6 min that had masses consistent with 23-descarbamoyl-23-acetyl-tuberactinamine A (**18**) and the corresponding dehydrodesoxy derivative (**13**) (Fig. 2, trace C; Table 2). Finally, the triple mutant, Δ*vioQ*Δ*vioP*Δ*vioL*, produced a series of metabolites that eluted between 11.8 to 13.5 min from the HPLC one of which, eluting at 12.3 min, showed a UV visible spectrum similar to **1** (Fig. 2, trace D). Analysis of this metabolite by MALDI-TOF MS determined it had a mass consistent with N-descarbamoyl-N-acetyl-tuberactinamine N (**19**) (Table 2).

A number of conclusions can be made from the data discussed above. First, we have confirmed the proposed function of VioQ and VioL in the biosynthesis of **1** based on the accumulation of derivatives of **1** lacking the hydroxylation of residue five and the carbamoylation of residue four. Second, the data support the hypothesis that VioP catalyzes the formation of β-Lys since the absence of *vioP* results in the accumulation of des-β-Lys derivatives of **1**. Third, the use of gene deletions in the biosynthetic gene cluster of **1** provides an efficient means for generating structural derivatives. Of the nine derivatives of **1** produced by *S. lividans* 1326, four have not previously been observed in nature or synthesized chemically. One of the other derivatives, **15**, has only been generated semisynthetically.[29] Thus, metabolic engineering of the biosynthetic gene cluster provides a means for generating structural diversity in this important class of antibiotics. Finally, the low levels of antibiotic production in strains deleted for *vioL* and the detection of only acetylated versions of the metabolites purified from these strains suggested the proposed biosynthetic scheme for assembly of the cyclic pentapeptide core of **1** by the associated NRPS must be revisited.

Analysis of the amino acid specificity of VioF and CmnF

The genetic data analyzing the strains lacking *vioL* strongly suggested that the carbamoylation of residue four occurs prior to cyclic pentapeptide release from the NRPS involved in the biosynthesis of **1**. This suggestion was based on the observation that the removal of the carbamoyl group always resulted in this moiety being replaced by an acetyl group. One hypothesis was the acetyl group structurally mimicked the carbamoyl group, allowing the biosynthesis of the cyclic pentapeptide core to proceed. Without that modification, biosynthesis cannot proceed. Thus, when all the biosynthetic enzymes are present, carbamoylation must occur prior to cyclic pentapeptide release from the NRPS. There are two possible models for when this carbamoylation would occur. In one model, VioL would function in a concerted manner with the NRPS to carbamoylate residue four while the peptide is being synthesized. A second model is that the carbamoylation of L-Dap to generate β-ureidoalanine (β-Uda) occurs prior to the amino acid being recognized by the NRPS. In our initial proposal the NRPS involved in cyclic pentapeptide assembly would proceed in the order of VioF→VioA→VioI→VioG (Fig. 3A). This model was developed based on the amino acid specificity codes of both adenylation (A) domains of VioA suggesting they activate L-Ser, while the A domain specificity code of VioF suggested it recognized L-Gln, which is structurally similar to L-Dap.[13] VioF would aminoacylate its own PCP domain and that of VioI with L-Dap; therefore, VioF introduces the L-Dap components of residues one and four. VioA would incorporate the L-Ser at residues two and three, while VioG would incorporate L-Cam. In support of the proposed function of VioG, Zabriskie and colleagues have shown that this enzyme is specific for L-Cam.[19] At some point during the synthesis of the pentapeptide, VioL would catalyze the carbamoylation of residue four and VioJ would catalyze the α,β-desaturation of residue four. In this model, there is no clear mechanism for the incorporation of β-Uda.

If β-Uda is incorporated by the NRPS, an alternative model for how the NRPS functions would have to be developed. In this alternative model the NRPS components would function in the order of VioA→VioI→VioF→VioG to account for an A domain being required for the recognition of β-Uda. Thus, the first A domain of VioA would form a L-Dap-PCP intermediate on the first PCP domain of the enzyme, while the second A domain would load L-Ser onto the second PCP domain and also the PCP of VioI. VioF would then activate β-Uda, with VioG catalyzing the incorporation of L-Cam (Fig. 3). Again, VioJ would catalyze the α,β-desaturation of residue four in concert with the NRPS.

The two proposed models can be differentiated by analyzing the amino acid specificities of the A domains of VioA and VioF. Unfortunately, we have been unable obtain soluble and

functional full-length VioA when overproduced in *E. coli* or *S. lividans* 1326, and constructs containing the isolated A domains of VioA have proven inactive when overproduced in *E. coli* (data not shown). VioF, however, proved to be more amenable to *in vitro* characterization. VioF was overproduced in *E. coli* and purified using nickel-chelate chromatography (Fig. 4A). Standard (d)ATP/PPi exchange reactions were used to investigate whether the A domain of VioF was able to activate L-Ser, L-Dap, L-Ala, L-Cam, or β-Uda. We used ATP and dATP in parallel reactions to support the conclusion that the amino acid activation observed was due to the A domain of VioF. The A domains of NRPSs do not discriminate between ATP and dATP for amino acid activation, but other amino acyl-AMP-forming enzymes such as tRNA synthetases do.[30] Thus, if we observed amino acid activation in the presence of ATP and when ATP is preplaced by dATP we could be confident the activation was due to the catalytic activity of VioF. We determined that VioF specifically activated β-Uda but did not activate any of the other amino acids (Fig. 4B). These data support the second proposed model for cyclic pentapeptide assembly (Fig. 3B). To provide further support for this model, the homolog of VioF from the capreomycin biosynthetic pathway, CmnF, was overproduced in *E. coli*, purified by nickel-chelate chromatography (Fig. 4A), and analyzed for amino acid activation as done for VioF. We observed that CmnF also specifically activates β-Uda (Fig. 4B), similar to the results with VioF.

These results are surprising based on their implications not only on the biosynthesis of **1**, but also on the production of the cyclic pentapeptide core of **2–5**. We have previously shown that the biosynthetic gene cluster for **2–5** is nearly identical to that for **1**.[14] The data presented here strongly suggest that the functional order of the NRPS components for **2–5** production is CmnA→CmnI→CmnF→CmnG (Fig. 3C). This NRPS subunit order requires that the second A domain of CmnA does not aminoacylate the PCP domain of CmnI as seen for the function of VioA and VioI. Instead the first A domain of CmnA must aminoacylate the PCP domain of CmnI. Thus, although the NRPS components of the two systems are strikingly similar, different A domains aminoacylate the PCP domain involved in the incorporation of the amino acid at residue three. Furthermore, the second A domain of VioA must be specific for L-Ser while the second A domain of CmnA must activate either L-Ser or L-Ala to generate the structural diversity seen in **2–5** (Fig. 3C). These results suggest a more thorough analysis of the NRPSs involved in **1** and **2–5** biosynthesis is needed to fully define the function of these enzymes in cyclic pentapeptide assembly.

Conclusions

We have shown that *S. lividans* 1326 is a competent host for the heterologous production of **1**. These data support our previous hypothesis that all of the genes essential for production of **1** are contained on the region of the *Streptomyces* sp. strain ATCC11861 chromosome cloned into pVIO-P4C3RH. We have shown that the genetic manipulation of the pathway in *E. coli* followed by heterologous production in *S. lividans* 1326 is an efficient means for investigating the biosynthesis of **1**. In particular, we showed that VioQ, VioP, and VioL are involved in introducing the hydroxylation, β-Lys addition, and carbamoylation of the cyclic pentapeptide core of **1**, respectively. In the process, we have shown that the power of *E. coli*-based genetic manipulation of the biosynthetic gene cluster of **1** is an efficient means of generating derivatives of **1**. Based on these genetic results, the mechanism for cyclic pentapeptide assembly for **1** and **2–5** biosynthesis by their respective NRPSs was re-evalulated. This revision results in the unusual observation that two structurally similar nonribosomal peptides are assembled using different *in trans* aminoacylations that result in structural differences in the peptide sequence generated.

Experimental Section

Bacterial strains and media

Bacterial strains used in this study are listed in Table 1. *E. coli* strains were propagated in LB medium or on LB agar plates with the appropriate antibiotic selection. *S. lividans* 1326 strains were propagated on ISP2 plates with the appropriate antibiotic selection when needed. For *S. lividans* 1326 derivatives, alternative media used for conjugations and antibiotic production are discussed below.

Identification of pVIO-P4C3RH

The cosmid pVIO-P4C3RH was isolated using *vph* and *vioG* primers as previously described.[31] Primers specific for an internal fragment of *vioA*[31] and the complete ORF of *vioQ* (vioQNdeI: 5′ AAGGGCCCCCATATGCCGACGTGTCGAGTC 3′; vioQHindIII: 5′ GGTGTGTGAAGCTTATGTCGCGACTGCCCA 3′) were used in standard PCR amplification reactions to determine whether pVIO-P4C3RH contained these two portions of the biosynthetic gene cluster of **1**. The pVIO-P4C3RH cosmid was positive for both *vioA* and *vioQ* based on this analysis. The ends of the DNA insert were sequenced to determine the region of the *Streptomyces* sp. strain ATCC11861 genome carried by this cosmid.

Construction of pVIO-R4C3RH-436

The pVIO-P4C3RH cosmid was digested with *Nsi*I and the *Nsi*I ends were blunt-ended using Klenow Fragment following the manufacturer's instruction (New England Biolabs, Inc.). The 6.7 kb *Dra*I fragment of pOJ436[32] containing the *oriT*, *aac(3)IV* apramycin resistance gene, φC31*attP*, and φC31 integrase was cloned into the blunt-ended *Nsi*I site of pVIO-P4C3RH to generate pVIO-P4C3RH-436.

Construction of Δ*vioQ*, Δ*vioP*, and Δ*vioL* derivatives of pVIO-R4C3RH-436

Standard protocols for λred-mediated mutagenesis of SuperCos1-based cosmids in BW25113/pIJ790 (REDIRECT©, John Innes Centre)[24] were used to generate in-frame deletions of *vioQ*, *vioP*, and *vioL* in pVIO-R4C3RH-436. Briefly, pIJ778 was used as the DNA template for PCR amplification of the spectinomycin resistance gene (*aadA*), and the resulting PCR amplicon was electroporated into BW25113/pIJ790 strains carrying pVIO-R4C3RH-436 derivatives. The desired electroporants were selected on LB containing spectinomycin (50 μg/ml) and apramycin (100 μg/ml). An alternative to the REDIRECT© protocol for removing the *aadA* gene was utilized to create each deletion. For each primer set used, the 5′ primer contained an *Nhe*I restriction site immediately upstream of the 20 nucleotide priming sequence, while the 3′ primer contained an *Xba*I restriction site immediately upstream of the 19 nucleotide priming sequence. These restriction sites were added to allow the resulting *aadA*-containing cosmids (pVIO-P4C3RH-436-*vioQ::aadA*, pVIO-P4C3RH-436-*vioP::aadA*, and pVIO-P4C3RH-436-*vioL::aadA*) to be digested with *Nhe*I and *Xba*I and religated to remove *aadA*, leaving a six bp "scar" sequence that could not be digested with *Nhe*I or *Xba*I. The primers used were as follows: for Δ*vioQ*, (vioQ F: 5′ ACGTGTCGAGTCATCAGAAACTGGGGTGTGACGATGGATGCTAGCATTCCGGGG ATCCGT CGACC 3′ and vioQ R: 5′ GCTTTCCTTGAAATTCATCAGCAGTTCCCCGTACACCTTTCTAGATGTAGGCTGG AGCTGCTTC 3′); for Δ*vioP*, (vioP F: 5′ AGGCCGACCGCCCCTCCCCCGCCCGAGCCGGCATCCTGCGCTAGCATTCCGGGG ATCCG TCGACC 3′ and vioP R: 5′ GCATGCCTGGCCCTCACGCGCCGCGACCTGCCGTTCCCATCTAGATGTAGGCTG GAGCTGCTTC 3′); for Δ*vioL* (vioL F: 5′ GAACCCCGCGGCCTGTACTCCTTGGCCGACCTGGACGACGCTAGCATTCCGGGG

ATCCG TCGACC 3′ and vioL R: 5′ CGTGACCACCCATTCCAGGACGGCCATCGCGCCGGCGGCTCTAGATGTAGGCTG GAGCTGCTTC 3′). Each primer set was designed to leave the first and last 13 codons of the gene separated by the six bp scar, which proved not to have polar effects on transcription of downstream genes. To confirm each deletion, the DNA flanking the putative site of the deletion was PCR amplified and the PCR product was sequenced. The primers used were as follows: for Δ*vio*Q confirmation (vio-29436: 5′ ATCACCACATGGTGGAACTGCC 3′ and vio-31220: 5′ GACCGAAAGGAGTTGGTTCACC 3′); for Δ*vio*P confirmation: (vio-27134: 5′ ATCTGACGTTTCAGGACGTGTT 3′ and vio-29079: 5′ GTACCGAACTGTTCCCGCTGAT 3′); for Δ*vioL* confirmation: (vio-22558: 5′ CCACGTATCCCTGGACGATCTG 3′ and vio-24255: 5′ AGATGTGGTGCAGTGAGCAGAC 3′). The resulting cosmids were named: pVIO-P4C3RH-436-Δ*vioQ*, pVIO-P4C3RH-436-Δ*vioP*, and pVIO-P4C3RH-436-Δ*vioL* to denote the gene deleted from the cosmid.

Construction of Δ*vioQ*Δ*vioP*, Δ*vioQ*Δ*vioL*, Δ*vioP*Δ*vioL*, and Δ*vioQ*Δ*vioP*Δ*vioL* derivatives of pVIO-R4C3RH-436

The λred-mediated mutagenesis approach was used to generate double and triple mutants. To make the first of the multiple deletions, the cosmid containing *aadA* inserted into *vioQ* was used as template DNA for PCR amplification with the same primer pairs listed above used for confirmation of the *vioQ* deletion. These primers provided an increased amount of DNA flanking *vioQ* that was available for homologous recombination mediated by the λred system. This increase in flanking DNA minimized undesired recombination with the region of the pVIO-P4C3RH-436 cosmid that was homologous to a portion of the *aadA* disruption cassette. The PCR amplicon from the *vioQ::aadA*-containing cosmid was used in the λred-mediated disruption of *vioQ* in the pVIO-P4C3RH-436-Δ*vioP* and pVIO-P4C3RH-436-Δ*vioL* cosmids. The analogous approach was used to introduce the *vioL::aadA* insertion into pVIO-P4C3RH-436-Δ*vioP*. Each construct containing the *aadA* insertion was digested with *Nhe*I and *Xba*I, religated, and screened for sensitivity to spectinomycin (50 μg/ml). The resulting cosmids were: pVIO-P4C3RH-436-Δ*vioQ*Δ*vioP*, pVIO-P4C3RH-436-Δ*vioQ*Δ*vioL*, and pVIO-P4C3RH-436-Δ*vioP*Δ*vioL*. The cosmid lacking both *vioP* and *vioQ* was modified by λred-mediated mutagenesis using the *vioL::aadA* cassette, followed by restriction digestion to remove *aadA*, resulting in the formation of the triple mutant cosmid pVIO-P4C3RH-436-Δ*vioQ*Δ*vioP*Δ*vioL*. The deletions were confirmed by PCR amplification and sequencing.

Heterologous production of 1 and derivatives of 1

Each pVIO-P4C3RH-436 derivative was conjugated into *S. lividans* 1326 following established protocols.[33] Conjugation was either from *E. coli* S17-1λ*pir* directly or from *E. coli* DH10B in a triparental mating reaction using the helper plasmid pRK2013 in *E. coli* HB101. Apramycin (100 μg/mL) was used to select for *S. lividans* 1326 carrying the integrated pVIO-P4C3RH-436 derivative while nalidixic acid (50 μg/mL) was used to counter select against the *E. coli*. Exconjugants of each strain were streaked for isolation on ISP2 medium containing apramycin (100 μg/mL). To obtain a starter culture for antibiotic production, the strains were grown in tryptic soy broth containing apramycin (100μg/mL). A minimum of two exconjugants per strain were grown in viomycin production medium for 7 to 10 days and **1** and derivatives of **1** were purified as previously described.[13,14]

For complementation studies, the vector pSE34 was modified by insertion of the origin of transfer, *oriT*, from pIJ778 into the *Eco*RI site of pSE34. This was accomplished by PCR-based cloning of the *oriT* with primers containing *Mfe*I sites that, when digested, were compatible with the *Eco*RI digested plasmid. The resulting plasmid was named pJJB501. While this plasmid was able to be conjugated into *S. lividans* 1326 derivatives, in this study pJJB501

derivatives were introduced into *S. lividans* 1326 derivatives using standard PEG-assisted transformation in addition to conjugation using established protocols.[33] Successful transformants were selected for by the addition of thiostrepton at 50 μg/mL.

Construction of *vioQ*, *vioP*, or *vioL* complementing clones

Each gene was PCR amplified with a primer set consisting of a 5′ primer with an *Nde*I site and a 3′ primer containing a *Hind*III site and cloned into the corresponding restriction sites of pET-37b. The cloned gene was then excised with X*ba*I and *Hind*III and subcloned into the corresponding sites of pJJB501. The primers used were: for *vioQ* (vioQNdeI: 5′ AAGGGCCCCCATATGCCGACGTGTCGAGTC 3′ and vioQHindIII: 5′ GGTGTGTGAAGCTTATGTCGCGACTGCCCA 3′); *vio*P (vioPNdeI: 5′ GAACATATGGAACCTGACTGGCGCAGACTGCCC 3′ and vioPHindIII: 5′ GATCAAGCTTTCAGGCGCATGCCTGGCCCT 3′); for *vioL* (vioLNdeI: 5′ AGACATATGACCGAACCCCGCG 3′ and vioLHindIII: 5′ AGTCAAGCTTTCACGTCGTGACCACCCATT 3′). Each of the complementing clones was conjugated into the appropriate strain an analyzed for viomycin production.

HPLC and MALDI-TOF MS analysis of 1 and derivatives of 1

The purified metabolites from the culture supernatants of the *S. lividans* 1326 strains were analyzed by reverse phase high performance liquid chromatography (HPLC). A 250 mM small pore C18 column (Vydac) was used on a Beckman-Coulter machine with a 126 solvent module and 168 detector to separate derivatives of **1**. UV-visible spectra were monitored to detect derivatives of **1** that showed the characteristic λ_{max} at 268 nm. The separation method was 5 min of H_2O + 0.1 %TFA followed by a linear gradient over 5 min to 20% ACN + 0.1 % TFA and a 10 min reequilibration in H_2O + 0.1 %TFA. MALDI-TOF data on a Voyager-DE Pro Workstation (PerSeptive Biosystems) were obtained at the University of Wisconsin-Madison Biophysics Instrumentation Facility, which was established with support from the University of Wisconsin-Madison and grants BIR-9512577 (NSF) and S10 RR13790 (NIH). The MALDI-TOF MS data were calibrated with peptides of known mass during each analysis.

Overproduction and purification of VioF and CmnF

The genes *vioF* and *cmnF* were cloned into the *E. coli* expression vector pET28b (Novagen) using PCR-based cloning. The primers used to amplify *vioF* from the pVIO-P4C3RH were: VioFNdeI 5′ AAGCGACATATGACGGATCTGGACTTCACC 3′ and VioFHindIII 5′ GGGCGGAAGCTTCTCCGTTGCGGCCAGGCG 3′. The corresponding PCR amplicon was cloned into the *Nde*I/*Hin*dIII sites of pET28b. The primers used to amplify *cmnF* were: cmnFNdeIwas cloned into pET28b using PCR-based cloning. The primers used to amplify *cmnF* from pCMN-P4C8RF-436[14] were: cmnFNdeI 5′ GGAGAGCATATGACCCAGGTCGACTTCACCCGG 3′ and cmnFHindIII 5′ CGCCCACCAAGCTTAGCCAGTGCGGGTCCGGCG 3′.

The *vioF*-overexpression clone pET28b-VioF was transformed into BL21(DE3) selecting for kanamycin (50 μg/mL) resistance. The *cmnF*-overexpression clone pET28b-cmnF was transformed into BL21(DE3)/pRARE (the Rosetta strain, Novagen) selecting for chloramphenicol (15 μg/mL) and kanamycin (50 μg/mL) resistance. To overproduce VioF, 6 × 1L cultures of the overexpression strain were grown at 25°C until an OD_{600} of 0.5 was reached. The cells were shifted to 15°C, and after 2 hours at this temperature IPTG (100 μg/mL) was added and cells were grown 14 hours at 15°C. To overproduce CmnF, 3 × 1 L cultures of the overexpression strain were grown at 25°C until an OD_{600} of 0.5 was reached. The cells were shifted to 15°C, after 1 hr at this temperature IPTG (100 μg/mL) was added, and the cells were grown an additional 14 hr at 15°C. The purification of VioF and CmnF from these cells followed the same protocol as previously described for VioC and VioD purification.[15]

(d)ATP/PPi exchange assays

(d)ATP/PP_i-exchange assays were performed as previously described.[31] Briefly, 3μg of purified VioF and CmnF were assayed in Tris-HCl (pH 7.5; 75 mM), $MgCl_2$ (10 mM), DTT (5 mM), dATP (or ATP) (3.5 mM), [^{32}P]-PP_i (1 mM, 1.8 Ci/mol) along with 500 μM of the indicated amino acid. Reactions were incubated at 23°C for 1.5 h before being quenched with stop solution containing activated charcoal. Charcoal was washed twice and counted in a scintillation counter. The β-Uda was purchased from Acros, the L-Cam was enzymatically synthesized as previously described.[15] All other components were purchased from Sigma-Aldrich or Fisher Scientific.

Acknowledgments

The authors thank M. R. Rondon for cloning of *vioQ*, S. Pellett for guidance with λred mutagenesis, and members of the Thomas laboratory for helpful discussions concerning this work. EAF was an NIH-sponsored Biotechnology Training Program predoctoral fellow. This work was funded, in part, by the NIH (RO1 AI065850) and support from an Alfred Toepfer Faculty Fellow to MGT.

References

1. Dye C, Scheele S, Dolin P, Pathania V, Raviglione MC. J Am Med Assoc 1999;282:677.
2. World Health Organization. 2008. available at: http://www.who.int/tb/publications/global_report/2008/download_centre/en/index.html
3. Joint United Nations Programme on HIV/AIDS (UNAIDS). Vol. 2008. World Health Organization; 2007. available at: http://www.unaids.org/en/KnowledgeCentre/HIVData/EpiUpdate/EpiUpdArchive/2007/default.asp
4. Fauci AS. J Infect Dis 2008;197:1493. [PubMed: 18426366]
5. Yamada T, Mizugichi Y, Nierhaus KH, Wittmann HG. Nature 1978;275:460. [PubMed: 211438]
6. Johansen SK, Maus CE, Plikaytis BB, Douthwaite S. Mol Cell 2006;23:173. [PubMed: 16857584]
7. Bartz QR, Ehrlich J, Mold JD, Penner MA, Smith RM. Am Rev Tuberc 1951;63:4. [PubMed: 14799784]
8. Herr EBJ. Antimicrob Agents Chemother 1962:201.
9. Nagata A, Ando T, Izumi R, Sakakibara H, Take T. J Antibiot (Tokyo) 1968;21:681. [PubMed: 5752384]
10. Tsukamura M, Ichiyama S, Miyachi T. Chest 1989;95:1056. [PubMed: 2539957]
11. World Health Organization. Vol. 15. Vol. 2008. World Health Organization; 2007.
12. Migliori GB, Lange C, Centis R, Sotgiu G, Mutterlein R, Hoffmann H, Kliiman K, De Iaco G, Lauria FN, Richardson MD, Spanevello A, Cirillo DM, Group TS. Eur Respir J 2008;31:1155. [PubMed: 18515555]
13. Thomas MG, Chan YA, Ozanick SG. Antimicrob Agents Chemother 2003;47:2823. [PubMed: 12936980]
14. Felnagle EA, Rondon MR, Berti AD, Crosby HA, Thomas MG. Appl Environ Microbiol 2007;73:4162. [PubMed: 17496129]
15. Ju J, Ozanick SG, Shen B, Thomas MG. ChemBioChem 2004;5:1281. [PubMed: 15368582]
16. Yin X, O'Hare T, Gould SJ, Zabriskie TM. Gene 2003;312:215. [PubMed: 12909358]
17. Yin X, Zabriskie TM. ChemBioChem 2004;5:1274. [PubMed: 15368580]
18. Yin X, McPhail KL, Kim K-j, Zabriskie TM. ChemBioChem 2004;5:1278. [PubMed: 15368581]
19. Fei X, Yin X, Zhang L, Zabriskie TM. J Nat Prod 2007;70:618. [PubMed: 17302456]
20. Pittenauer E, Zehl M, Belgacem O, Raptakis E, Mistrik R, Allmaier G. J Mass Spectrom 2006;41:421. [PubMed: 16604520]
21. Yoshioka H, Aoki T, Goko H, Nakatsu K, Noda T, Sakakibara H, Take T, Nagata A, Abe J, Wakamiya T, Shiba T, Kaneko T. Tetrahedron 1971;23:2043.

22. Ferraro DJ, Gakhar L, Ramaswamy S. Biochem Biophys Res Commun 2005;338:175. [PubMed: 16168954]
23. Datsenko KA, Wanner BL. Proc Natl Acad Sci USA 2000;97:6640. [PubMed: 10829079]
24. Gust B, Challis GL, Fowler K, Kieser T, Chater KF. Proc Natl Acad Sci USA 2003;100:1541. [PubMed: 12563033]
25. Frey PA, Ballinger MD, Reed GH. Biochem Soc Trans 1998;26:304. [PubMed: 9765869]
26. Morse BK, Brown MS, Gagne JW, McArthur HA, McCormick EL, Murphy TK, Narrol MH, Perry DA, Smogowicz AA, Wax RG, Wong JW. J Antibiot 1997;50:698. [PubMed: 9315086]
27. Xu Y, Labedan B, Glansdorff N. Microbiol Mol Biol Rev 2007;71:36. [PubMed: 17347518]
28. Zhao L, Ahlert J, Xue Y, Thorson JS, Sherman DH, Liu H-w. J Am Chem Soc 1999;121:9881.
29. Wakamiya T, Shiba T. J Antibiot (Tokyo) 1975;28:292. [PubMed: 808523]
30. Pavela-Vrancic M, Van Liempt H, Pfeifer E, Freist W, Von Dohren H. Eur J Biochem 1994;220:535. [PubMed: 8125112]
31. Thomas MG, Burkart MD, Walsh CT. Chem Biol 2002;9:171. [PubMed: 11880032]
32. Bierman M, Logan R, O'Brien K, Seno ET, Rao RN, Schoner BE. Gene 1992;116:43. [PubMed: 1628843]
33. Kieser, T.; Bibb, MJ.; Buttner, MJ.; Chater, KF.; Hopwood, DA. Practical Streptomyces genetics. The John Innes Foundation; Norwich, UK: 2000. p. 229
34. Grant SG, Jessee J, Bloom FR, Hanahan D. Proc Natl Acad Sci USA 1990;87:4645. [PubMed: 2162051]
35. Simon R, Priefer U, Puhler A. Bio/Technology 1983;1:784.
36. Boyer HW, Roulland-Dussoix D. J Mol Biol 1969;41:459. [PubMed: 4896022]
37. Figurski DH, Helinski DR. Proc Natl Acad Sci USA 1979;76:1648. [PubMed: 377280]
38. Smirnova N, Reynolds KA. J Bacteriol 2001;183:2335. [PubMed: 11244075]

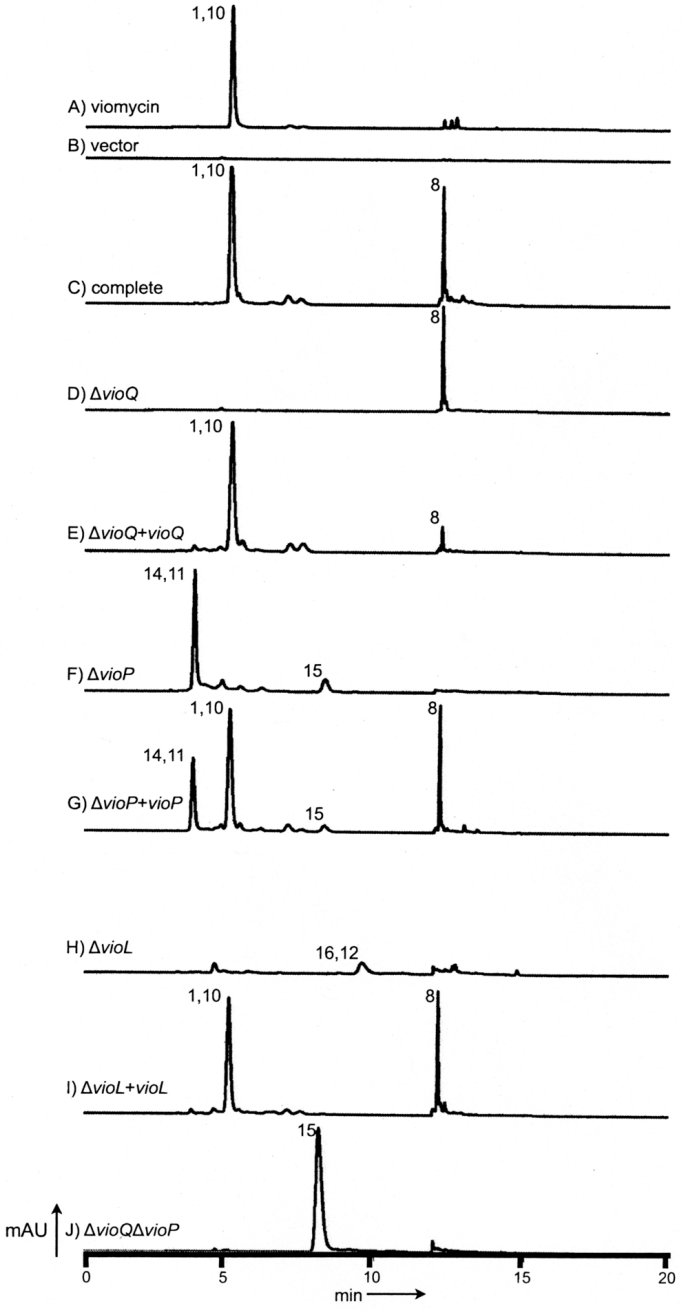


Figure 1.

Representative HPLC traces showing the separation of **1** and derivatives of **1** that were produced by the engineered strains of *S. lividans* 1326. The traces show metabolite elutions monitored at 268 nm (y-axis) versus time (x-axis). The number(s) to the top, left of the relevant metabolite elution peaks identifies the derivative of **1** that is associated with that peak as depicted in Figs. 1 and 4. In trace C, "complete" identifies the *S. lividans* 1326 strain carrying the intact biosynthetic gene cluster of **1**.

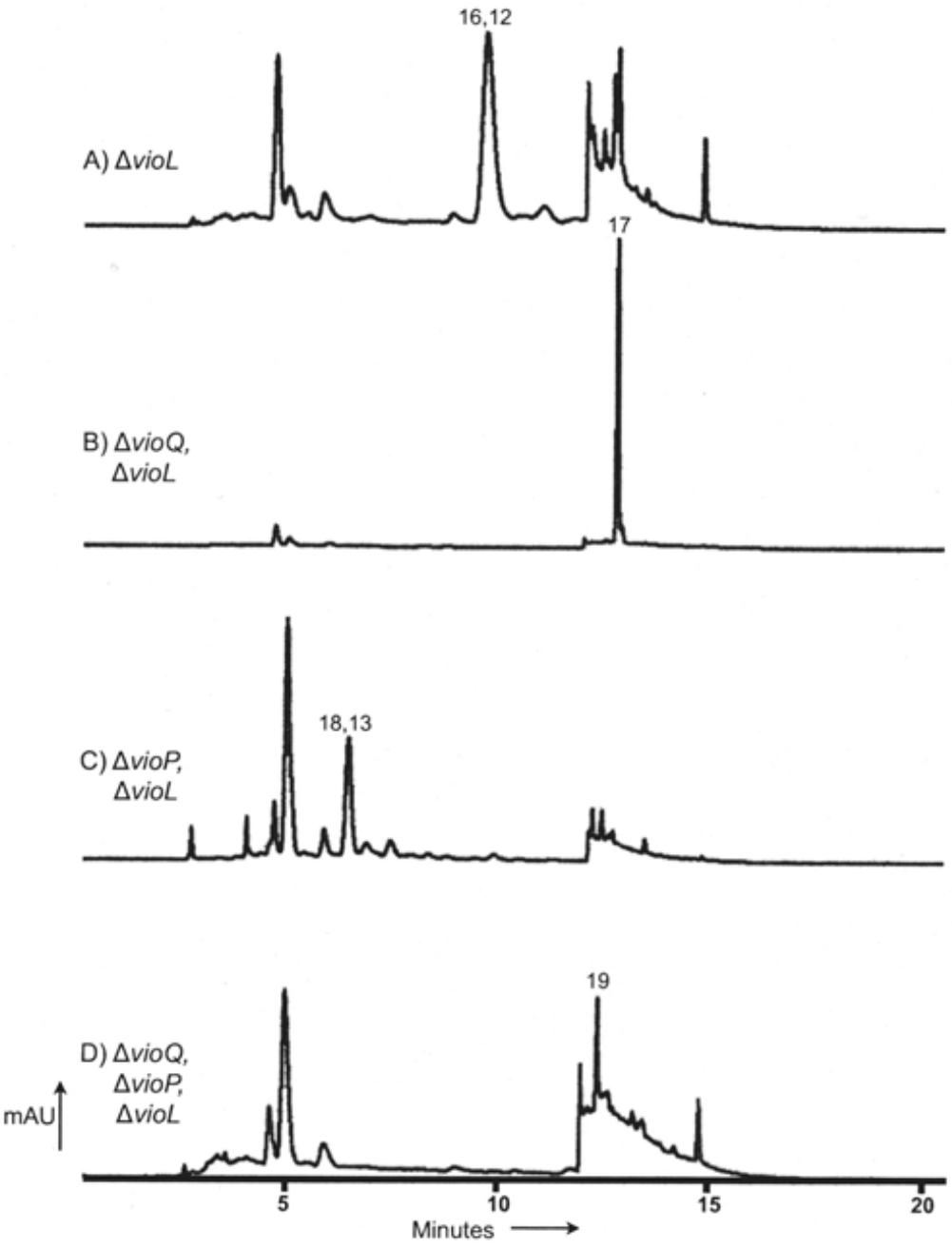


Figure 2.

Representative HPLC traces showing the separations of derivatives of **1** that are produced when the strain lacks *vioL*. The traces show metabolite elutions monitored at 268 nm. The number (s) above peaks identifies the derivative of **1** that is associated with the peak. The only metabolites showing UV-visible spectra consistent with a derivative of **1** are numbered. Trace A is the same as shown in Figure 3, trace H, but the Y-axis as been scaled for clarity. The same scale was used for traces B-D due to the low abundance of the metabolites.

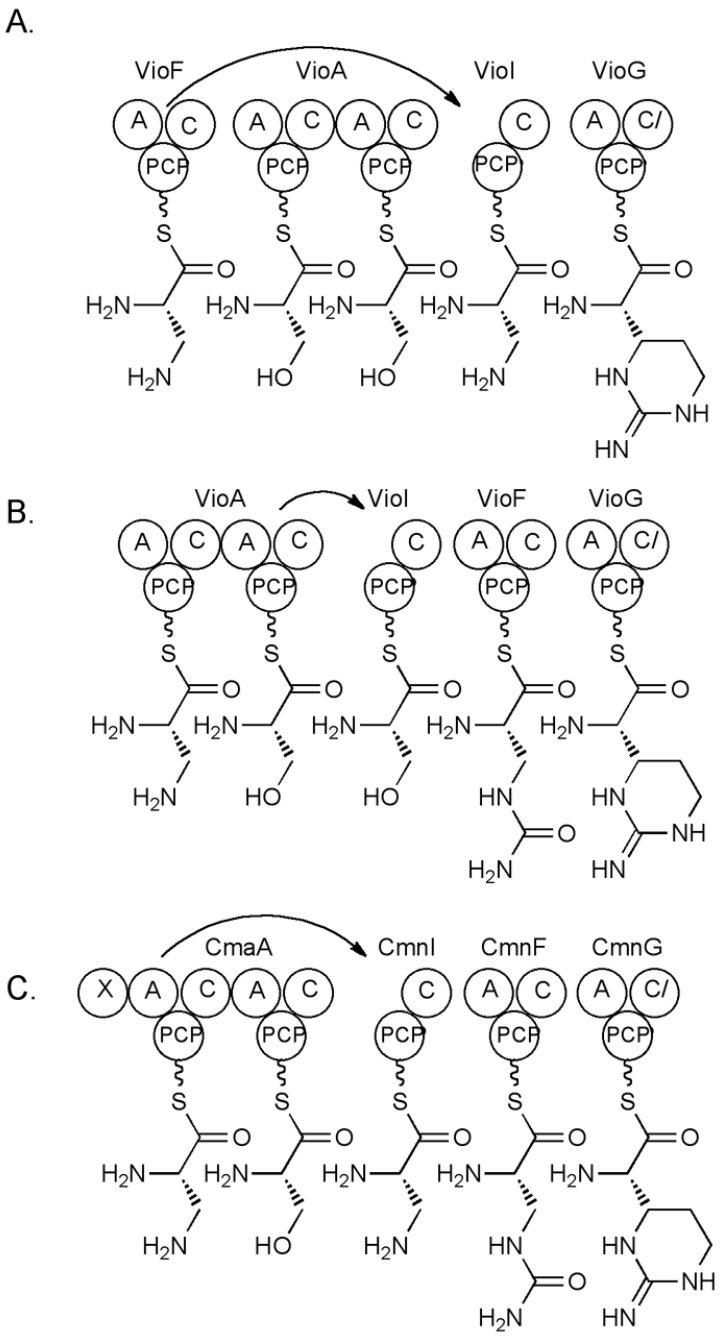


Figure 3.

Models for the order the NRPS components function for the production of **1** and **2–5**. A) Initially proposed model for the order in which the NRPS subunits function for the production of **1**. B) Alternative model for the order in which the NRPS subunits function for the production of **1**. C) Alternative model for the order in which the NRPS subunits function for the production of **2–5**. Each circle represents a catalytic domain. Abbreviations: A, adenylation; PCP, peptidyl carrier protein; C, condensation; C/, truncated condensation domain; X, domain of unknown function. The arrow in each model indicates the A domain that aminoacylates the PCP domain of VioI or CmnI.

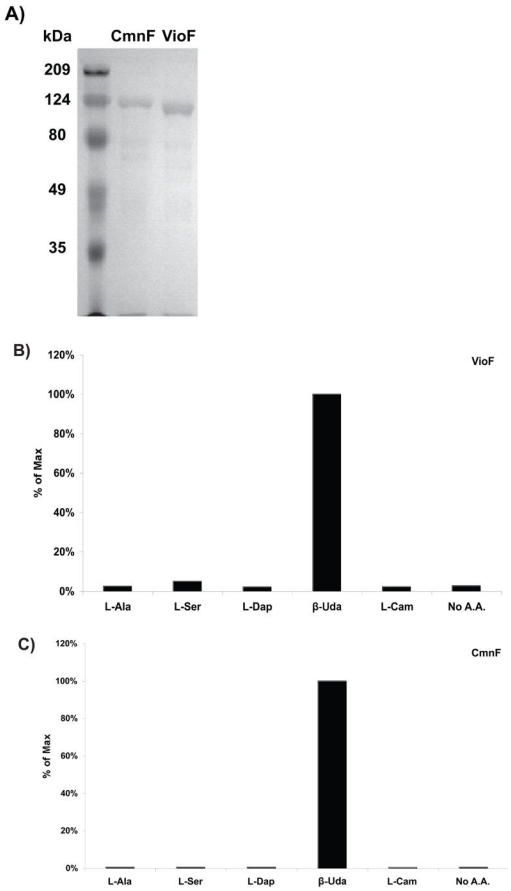
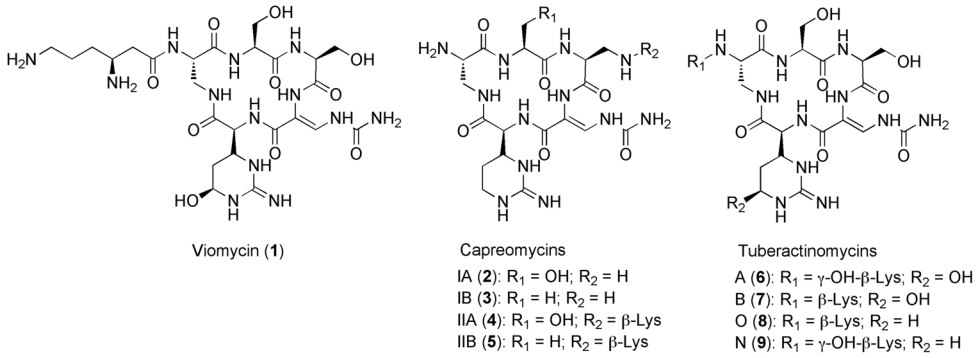


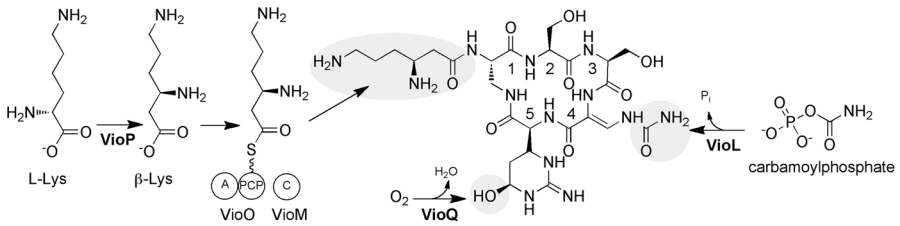
Figure 4.

In vitro analysis of VioF and CmnF. A) 10% SDS-PAGE gel of recombinant his-tagged CmnF and VioF stained with Coomassie blue. 3 μg of each protein was loaded in each lane. B) Amino acid activation of recombinant VioF by dATP/PP_i exchange assays. C) Amino acid activation of recombinant VioF by dATP/PP_i exchange assays. All assays were performed in triplicate and data are presented as percent of the maximally activated amino acid. Assays performed with ATP instead of dATP also showed specificity for β-Uda (data not shown).



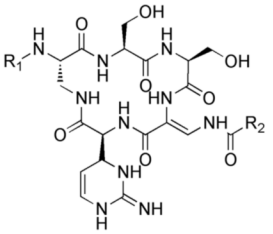
Scheme 1.

Chemical structures of the members of the tuberactinomycin family of anti-TB drugs. We note that *Streptomyces griseoverticillatus* var. *tuberacticus* produces viomycin (**1**) as part of the mixture of tuberactinomycins (**6–9**); however, it has been called tuberactinomycin B (**7**).

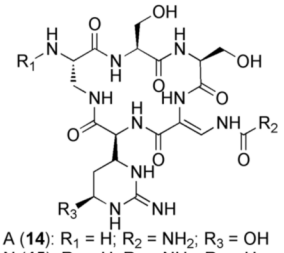


Scheme 2.

Schematic of the three enzymatic steps in the biosynthesis of **1** addressed in this study. The reactions catalyzed by enzymes that are the focus of this study are highlighted. The moieties of **1** that these enzymes are involved in are shaded. The numbers within the core of **1** identify the amino acid residue number discussed in the text.



dehydrodesoxyviomycin (**10**): R_1 = β-Lys; R_2 = NH_2
dehydrodesoxytuberactinamine A (**11**): R_1 = H; R_2 = NH_2
dehydrodesoxy-N-descarbamoyl-
N-acetyl-viomycin (**12**): R_1 = β-Lys; R_2 = CH_3
dehydrodesoxy-N-descarbamoyl-
N-acetyl-tuberactinamine A (**13**): R_1 = H; R_2 = CH_3



tuberactinamine A (**14**): R_1 = H; R_2 = NH_2; R_3 = OH
tuberactinamine N (**15**): R_1 = H; R_2 = NH_2; R_3 = H
N-descarbamoyl-
N-acetyl-viomycin (**16**): R_1 = β-Lys; R_2 = CH_3; R_3 = OH
N-descarbamoyl-
N-acetyl-tuberactinomycin O (**17**):R_1 = β-Lysine; R_2 = CH_3; R_3 = H
N-descarbamoyl-
N-acetyl-tuberactinamine A (**18**): R_1 = H; R_2 = CH_3; R_3 = OH
N-descarbamoyl-
N-acetyl-tuberactinamine N (**19**): R_1 = H; R_2 = CH_3; R_3 = H

Scheme 3.
Chemical structures of derivatives of **1** produced by engineering *S. lividans* 1326 strains.

Table 1

Bacterial strains and plasmids used in this study

Strain or Plasmid	Relevant characteristic(s)[a]	Source or reference
Escherchia coli		
DH5α	F-/φ80Δ*lacZ*M15(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_{k-},m_{k+}) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	[34]
XL1-Blue MR	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac.*	Stratagene
BW25113/pIJ790	(Δ(*araD-araB*)*567*, Δ*lacZ4787*(::*rrnB-4*), *lacIP-4000*, λ^-, *rpoS369*(Am), *rph-1*, Δ(*rhaD-rhaBI*)568, *hsdR514*/pIJ790 *oriR101*, *repA101*(ts), *araBp-gam-be-exo*	John Innes Centre
S17-1λ*pir*	*recA*, *thi*, *pro*, *hsd*R^-M^+, RP4:2-Tc:Mu:Km Tn7, λ*pir*, Tc^R, Sm^R	[35]
DH10B	F^- *endA1*, *recA1*, *galE15*, *galK16*, *nupG*, *rpsL*, Δ*lacX74*, Φ80*lacZ*Δ*M15*, *araD139*, Δ(*ara*,*leu*)7697, *mcrA*, Δ(*mrr-hsdR*MS- *mcrBC*), λ^-	[34]
HB101	F^- D(*gpt-proA*)*62*, *leuB6*, *glnV44*, *ara-14*, *galK2*, *lacY1*, D(*mcrC-mrr*), *rpsL20*(Sm^R), *xyl-5*, *mtl-1*, *recA13*	[36]
BL21(DE3)	F^-, *ompT*, *gal*, *dcm*, *lon*, *hsd*SB(rB^- mB^-), λ(DE3 [*lacI*, *lacUV5-T7 gene 1*, *ind1*, *sam7*, *nin5*])	Novagen
BL21(DE3)/pRARE	BL21(DE3) containing a plasmid expressing rare tRNAs.	Novagen
Streptomyces lividans 1326	Wild type	A. Gehring
EAF1003	*S. lividans* with SuperCOS1-436-2 integrated into φC31 *attB* site	[14]
JJB1001	*S. lividans* with pVIO-P4C3RH-436 integrated into φC31 *attB* site	This study
JJB1002	*S. lividans* with pVIO-P4C3RH-436- Δ*vioQ* integrated into φC31 *attB* site	This study
JJB1003	*S. lividans* with pVIO-P4C3RH-436- Δ*vioP* integrated into φC31 *attB* site	This study
JJB1004	*S. lividans* with pVIO-P4C3RH-436-Δ*vioL* integrated into φC31 *attB* site	This study
JJB1005	*S. lividans* with pVIO-P4C3RH-436- Δ*vioQ*Δ*vioP* integrated into φC31 *attB* site	This study
JJB1006	*S. lividans* with pVIO-P4C3RH-436- Δ*vioQ*Δ*vioL* integrated into φC31 *attB* site	This study
JJB1007	*S. lividans* with pVIO-P4C3RH-436- Δ*vioP*Δ*vioL* integrated into φC31 *attB* site	This study
JJB1008	*S. lividans* with pVIO-P4C3RH-436- Δ*vioQ*Δ*vioP*Δ*vioL* integrated into φC31 *attB* site	This study
Plasmids		
pRK2013	Conjugation helper plasmid	[37]
SuperCos1	Kn^R Ap^R cloning cosmid	Stratagene
SuperCos1-436.2	SuperCos1 with *aac(3)IV*, *oriT*, φC31 *int*, φC31 *attP* or pOJ436 cloned into blunt- ended *Nsi*I site	This study
pOJ436	*aac(3)IV* (Ar^R), *oriT*, φC31 *int*, φC31 *attP* cosmid vector	[32]
pVIO-P4C3RH	*Streptomyces* sp. strain ATCC11861 genomic DNA cloned into SuperCos1; contains viomycin gene cluster	This study
pVIO-P4C3RH-436	pVIO-P4C3RH with Ar^R, *oriT*, φC31 *int*, φC31 *attP* *Dra*I fragment of pOJ436 inserted into blunt-ended *Nsi*I site	This study
pVIO-P4C3RH-436- *vioQ::aadA*	pVIO-P4C3RH-436 with *aadA* inserted into *vioQ*	This study
pVIO-P4C3RH-436-Δ*vioQ*	pVIO-P4C3RH-436 with *vioQ* deleted	This study
pVIO-P4C3RH-436- *vioP::aadA*	pVIO-P4C3RH-436 with *aadA* inserted into *vioP*	This study
pVIO-P4C3RH-436-Δ*vioP*	pVIO-P4C3RH-436 with *vioP* deleted	This study
pVIO-P4C3RH-436- *vioL::aadA*	pVIO-P4C3RH-436 with *aadA* inserted into *vioL*	This study
pVIO-P4C3RH-436-Δ*vioL*	pVIO-P4C3RH-436 with *vioL* deleted	This study
pVIO-P4C3RH-436- Δ*vioPvioQ::aadA*	pVIO-P4C3RH-436-Δ*vioP* with *aadA* inserted into *vioQ*	This study
pVIO-P4C3RH-436- Δ*vioQ*Δ*vioP*	pVIO-P4C3RH-436-Δ*vioP* with *vioQ* deleted	This study
pVIO-P4C3RH-436- Δ*vioLvioQ::aadA*	pVIO-P4C3RH-436-Δ*vioL* with *aadA* inserted into *vioQ*	This study
pVIO-P4C3RH-436- Δ*vioQ*Δ*vioL*	pVIO-P4C3RH-436-Δ*vioL* with *vioQ* deleted	This study
pVIO-P4C3RH-436- Δ*vioPvioL::aadA*	pVIO-P4C3RH-436-Δ*vioP* with *aadA* inserted into *vioL*	This study
pVIO-P4C3RH-436- Δ*vioP*Δ*vioL*	pVIO-P4C3RH-436-Δ*vioP* with *vioL* deleted	This study
pVIO-P4C3RH-436- Δ*vioP*Δ*vioLvioQ::aadA*	pVIO-P4C3RH-436-Δ*vioL*Δ*vioP* with *aadA* inserted into *vioQ*	This study
pVIO-P4C3RH-436- Δ*vioQ*Δ*vioP*Δ*vioL*	pVIO-P4C3RH-436-Δ*vioL*Δ*vioP* with *vioQ* deleted	This study
pET28b	T7 overexpression vector	Novagen
pET28b-vioF	pET28b expressing *vioF*	This study
pET28b-cmnF	pET28b expression *cmnF*	This study
pSE34	*Streptomyces* sp. expression vector	[38]
pJJB501	pSE34 containing an *oriT*	This study
pJJB501-vioQ	pJJB501 expressing *vioQ*	This study
pJJB501-vioP	pJJB501 expressing *vioP*	This study
pJJB501-vioL	pJJB501 expressing *vioL*	This study

A Tc^R, tetracycline resistant; Sm^R, streptomycin resistant; Ar^R, apramycin resistant; Kn^R, kanamycin resistant; Ap^R, ampicillin resistant.

Table 2

MALDI-TOF MS Analysis of Viomycin Derivatives

Sample	HPLC Elution Time (min)	Viomycin Derivative(s)	Theoretical Mass $[M+H]^+$	Observed Mass $[M+H]^+$
Viomycin	5.1	**1, 10**	686.3, 668.3	686.3, 668.3
S. lividans + vio gene cluster	5.1	**1, 10**	686.3, 668.3	686.3, 668.3
	12.1	**8**	670.3	670.4
S. lividans + Δ*vioQ*	12.1	**8**	670.3	670.5
S. lividans + Δ*vioQ* + *vioQ*	5.1	**1,10**	686.3, 668.3	686.4, 668.4
	12.1	**8**	670.3	670.4
S. lividans + Δ*vioP*	3.8	**14, 11**	558.2, 540.2	558.2, 540.3
	8.2	**15**	542.2	542.2
S. lividans + Δ*vioP* + *vioP*	3.8	**14, 11**	558.2, 540.2	558.4, 540.3
	5.1	**1, 10**	686.3, 668.3	686.4, 668.4
	12.1	**8**	670.3	670.4
S. lividans + Δ*vioL*	9.5	**16, 12**	685.3, 667.3	685.4, 667.4
S. lividans + Δ*vioL* + *vioL*	5.1	**1, 10**	686.3, 668.3	686.3, 668.3
	12.1	**8**	670.3	670.3
S. lividans + Δ*vioQ*Δ*vioP*	8.2	**15**	542.2	542.3
S. lividans + Δ*vioP*Δ*vioL*	6.5	**18, 13**	557.2, 539.2	557.3, 539.5
S. lividans + Δ*vioQ*Δ*vioL*	12.6	**17**	669.3	669.4
S. lividans + Δ*vioQ*Δ*vioP*Δ*vioL*	12.3	**19**	541.2	541.3