Investigations of Pactamycin Biosynthesis

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Abstract

Pactamycin, a complex aminocyclitol compound produced by *Streptomyces pactum*, has significant antibiotic, antitumor, and antiplasmodial activity. However, this natural product has not been developed for clinical use due to its high cytotoxicity. Modulation of its biological properties by chemical synthesis has been difficult due to its complex chemical structure. Therefore, biosynthetic-based genetic engineering appears to be a feasible alternative approach to developing new analogs of pactamycin. While the pactamycin biosynthetic gene cluster is known, many parts of the pathway remain elusive. For example, the acyltransferase gene responsible for the transfer of 6-methylsalicylic acid to the aminocyclitol core has not yet been identified. As part of our effort to identify this gene, we used gene disruption to inactivate 1) a putative acyltransferase gene, located outside of the pactamycin gene cluster but near the pactamycin resistance gene, and 2) a putative ketoacyl-(ACP) synthase (KAS)-III gene (*ptmR*), located within the cluster. The results showed that PtmR is the enzyme responsible for the attachment of 6-methylsalicylic acid. We also hypothesized that pactamycin production could be increased through the modulation of global regulators. Inactivation of the global regulatory genes *afsA*, *arpA*, and *phoP*, which are known to affect secondary metabolism, revealed that ArpA is directly involved in the regulation of pactamycin biosynthesis. On the other hand, inactivation of *afsA* and *phoP* did not affect pactamycin production.
Introduction

Pactamycin, a member of the aminocyclitol family of antibiotics, is produced by the soil bacterium *Streptomyces pactum* (Figure 1 A). It contains a unique aminocyclopentitol unit and two aromatic rings: 3-aminoacetophenone and 6-methylsalicylic acid (6-MSA).\(^1\) Pactamycin has strong antibiotic activity against Gram-negative and Gram-positive bacteria, as well as antitumor activity. It inhibits the growth of bacteria by binding to the 16S rRNA of the 30S ribosomal subunit, thus inhibiting protein synthesis.\(^2\) Despite its superior biological activity, pactamycin has not been developed for clinical use. This is mainly due to its high toxicity and complex chemical structure, which has made modification by conventional chemistry techniques difficult. More recently, two research groups reported the total syntheses of pactamycin.\(^3,4\) However, the overall yields of the long and inflexible syntheses were low, limiting the usefulness of this approach to producing pactamycin and pactamycin analogs.

On the other hand, biosynthetic studies and genetic engineering have been successfully used to create analogs of pactamycin.\(^1\) Many of these analogs show increased antimalarial activity with reduced toxicity toward mammalian cells. For example, the genetically engineered pactamycin analogs 7-demethyl-7-deoxypactamycin (TM-026) and de-6MSA-7-demethyl-7-deoxypactamycin (TM-025) have shown increased selectivity against *Plasmodium falciparum* and decreased antibacterial activity and cytotoxicity (Figures 1 B and C).\(^5\) However, low production yields of these analogs in the mutant strains of *S. pactum* have hampered their further biological studies. Therefore, complete understanding of both the mode of formation of pactamycin in bacteria, and how it is regulated, is important to improve production yields.
Figure 1. Pactamycin and important pactamycin analogs. A) Pactamycin. B) 7-demethyl-7-deoxypactamycin (TM-026). C) de-6MSA-7-demethyl-7-deoxypactamycin (TM-025).[^5]

The biosynthetic gene cluster of pactamycin has been identified in *S. pactum* and, on the basis of the putative gene functions, the biosynthetic pathway to pactamycin has been proposed.[^1] However, some parts of the pathway are still elusive. For example, the gene encoding an acyltransferase enzyme that attaches 6-MSA to the aminocyclopentitol core has not yet been found in the gene cluster.[^1] Identification of this gene would lead to better understanding of how 6-MSA is attached to the core unit. This knowledge could be useful for generating new analogs of pactamycin chemoenzymatically by replacing 6-MSA with alternative side chains.

In addition to the pactamycin biosynthetic gene cluster, a 4.9 kb region of *S. pactum* chromosomal DNA located outside of the cluster has been previously reported to contain a pactamycin resistance gene.[^6] This is rather unusual, as most antibiotic resistance genes are located within or close to the biosynthetic gene clusters. The gene encodes a *S*-adenosylmethionine(SAM)-dependent methyltransferase, which transfers a methyl group to the 16S rRNA, resulting in inhibition of pactamycin binding.[^2,7] Located after the resistance gene is a putative acyltransferase gene. Therefore, it is tempting to speculate the potential involvement of this gene in 6MSA attachment.
We have identified the pactamycin biosynthetic gene *ptmR*, which encodes a putative ketoacyl-(ACP) synthase (KAS)-III, as another possible 6MSA transferase gene. KAS-III enzymes are known to catalyze a Claisen condensation between acetyl-CoA and malonyl-ACP in fatty acid biosynthesis. It has also been implicated in the transfer of certain activated aromatic carboxylic acids in natural products biosynthesis.[8,9] However, no direct evidence or biochemical data are available to support that notion. Therefore, in this study we set out to identify the gene that is responsible for 6-MSA transfer by disrupting the putative acyltransferase gene (*AT* gene), which is located next to the resistance gene, and the KAS-III gene, which is located within the pactamycin cluster.

It has been well documented that secondary metabolite production in microorganisms may be increased by modulating the transcription regulators that control the expression of biosynthetic genes.[10] In *Streptomyces* spp., disruption of global and/or pathway specific regulatory genes has enhanced production of certain natural products.[10] However, little is known about the global regulation of the pactamycin gene cluster. Therefore, we aim to identify possible global regulatory genes that may be involved in pactamycin biosynthesis. It is expected that by disrupting these regulatory genes, their impact on pactamycin biosynthesis can be observed through changes in production of pactamycin and its analogs. We identified three global regulatory genes from *Streptomyces* spp. were identified in the shotgun genome sequence of the *S. pactum* chromosome that could affect expression of the pactamycin biosynthetic gene cluster. The genes, *afsA*, *arpA*, and *phoP*, are known to affect secondary metabolism in other *Streptomyces* species.[11–13]
Two of the identified genes, *arpA* and *afsA*, encode proteins involved in the A-Factor Regulatory Cascade, a pathway that controls expression of genes involving morphological development and secondary metabolism.\[^{11,14}\] ArpA is a transcriptional repressor that only targets the *adpA* gene, a global transcriptional activator expressed by the pathway.\[^{15}\] AfsA is the unique enzyme involved in A-Factor synthesis. The A-Factor (a γ-butyrolactone), first found in *S. griseus*, binds to ArpA, which prevents transcriptional repression of *adpA*.\[^{11,12}\] In simple terms, AfsA and ArpA have opposing functions; AfsA is involved in activating *adpA* transcription and ArpA is involved in repressing *adpA* transcription. Therefore, through the disruption of the genes *afsA* or *adpA*, the entire A-Factor Regulatory Cascade can be turned on or off and the impact of the A-Factor Regulatory Cascade on pactamycin biosynthesis can be determined.

The third gene, *phoP*, encodes a protein that is an environmental phosphate response regulator.\[^{16}\] PhoP and PhoR form a two component regulatory system where PhoP is activated via phosphorylation mediated by the membrane bound PhoR.\[^{13}\] Phosphorylated PhoP forms dimers, which act as a transcriptional activator or repressor.\[^{17}\] Through transcriptional activity, PhoP links phosphate uptake and metabolism with other nutrients. PhoP has very broad effects in *Streptomyces* spp., and the disruption of *phoP* affects secondary metabolism differently in each species.\[^{17}\] The deletion of *phoP* in *S. coelicolor* and *S. lividans*, two closely related species, produced different results regarding the production of actinorhordin (ACT) and undecylprodigiosin (RED). *S. coelicolor* produces ACT and RED under most conditions, whereas *S. lividans* rarely produces ACT and RED. In strains lacking *phoP*, *S. coelicolor* production of ACT and RED was reduced, while *S. lividans* produced ACT and RED under conditions where the
Figure 2. DNA gel analysis of the acyltransferase gene. A) Separation of the pactamycin resistance gene from *S. pactum*. B) Identification of *E. coli* transformants with pTMA01. C) Confirmation of pTMA02 in *E. coli*. D) PCR analysis of the resistance gene after insertion into *S. lividans 1236*. 

Results and Discussion

Cloning and heterologous expression of the pactamycin resistance gene

The SAM-dependent methyltransferase resistance gene was previously identified in a 4.9 kb DNA fragment from the *S. pactum* chromosome. The SAM-dependent methyltransferase gene was amplified by polymerase chain reaction (PCR) (Figure 2 A). The resistance gene was inserted into the plasmid pRSET B, forming plasmid pTMA01 (Figure 2 B), and was sequenced. For heterologous expression in *S. lividans 1236*, the resistance gene was inserted into plasmid
pSET152, creating pTMA02 (Figure 2 C). The plasmid pTMA02 was transferred in *S. lividans* 1236, forming the mutant *S. lividans* +met. The gene inserted into *S. lividans* +met was the same as the gene in *S. pactum* (Figure 2 D).

Resistance to pactamycin was determined by Kirby-Bauer antibiotic testing. As shown in Figure 3, *S. lividans* +met was not inhibited by pactamycin or apramycin, in contrast to the wild type that did not grow near either. This suggests that, consistent with previous reports, the expression of the SAM-dependent methyltransferase gene by *S. lividans* conferred pactamycin resistance.[6]

![Figure 3](image)

**Figure 3.** Examination of pactamycin resistance in *S. lividans* +met and *S. lividans* 1236 WT. Disks arranged as follows: 1) 10 µL of 27 mM pactamycin; 2) 5 µL of 50 mg/mL apramycin; 3) 5 µL of 27 mM pactamycin. A) Bioassay of pactamycin in the *S. lividans* +met strain. B) Bioassay of *S. lividans* 1236 control.

**Characterizing the putative acyltransferase by in vivo inactivation of the acyltransferase gene**

The putative AT gene was identified as a 1.2 kb DNA sequence downstream of the SAM-methyltransferase gene. Part of the putative acyltransferase gene was amplified by PCR (Figure
The product was inserted into the plasmid pTMN002, to give pTMA03 (Figure 4 B). pTMA03 was transferred to S. pactum. Mutants with the acyltransferase gene disrupted were identified and designated S. pactum ΔAT (Figure 4 C).

Figure 4. DNA gel analysis of the acyltransferase gene. A) PCR products of acyltransferase gene disruption fragment. B) Identification of plasmid pTMA03. C) Identification of Streptomyces pactum ΔAT genome. Mixed primer pair: PCR product using one primer from aacIv and one primer from the acyltransferase gene fragment.

S. pactum ΔAT and S. pactum WT were cultured and metabolites were extracted. The extracts were concentrated and analyzed by mass spectrometry (MS). The results showed that the ΔAT mutant and the wild type strains produced pactamycin (m/z 559 ([M+H]+)) (Figure 5 A and B). The presence of pactamycin in the S. pactum ΔAT extract indicated that the AT gene is not involved in pactamycin biosynthesis. No other change in phenotype was detected by mass spectrometry. The putative AT gene is similar to many putative acyltransferases of unknown function.
function in biosynthetic gene clusters from *Streptomyces* spp. Disruption of this putative AT gene in *S. pactum* does not indicate a function for these acyltransferases.

It remains unclear as to why a pactamycin resistance gene is clustered with a putative acyltransferase gene outside of the pactamycin gene cluster. The 6.4 kb DNA fragment that contains the pactamycin resistance gene and the putative acyltransferase gene is most similar to a region from the lactonamycin gene cluster (78% identity) in *S. rishiriensis*. The pactamycin resistance gene is closely related to a putative lactonamycin resistance gene (75% identity), while the putative acyltransferase gene is most similar to a putative phosphatase/acyltransferase (71% identity) in the lactonamycin gene cluster. A lactonamycin-like gene cluster was also found in *S. sanglieri*, suggesting that other *Streptomyces* have

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**Figure 5.** Identification of pactamycin using mass spectrometry. A) Pactamycin (m/z 559 ([M+H]+)) observed in the extract from *S. pactum ΔAT*. B) Pactamycin observed in the extract of *S. pactum WT*. 
similar gene clusters. *Streptomyces* spp. are known for producing many different secondary metabolites;\(^{[10]}\) *S. pactum* indeed produces other secondary metabolites: actinopyrones, NFAT-133, and piericidins.\(^{[19,20]}\) As *S. pactum* has many biosynthetic gene clusters, it is not unreasonable to suggest that *S. pactum* may have a lactonamycin-like gene cluster. If *S. pactum* does have a lactonamycin-like gene cluster, a homologous recombination event could occur between two similar SAM-dependent rRNA methyltransferase genes, removing the pactamycin resistance gene from the pactamycin gene cluster. Alternatively, there may have been two SAM-dependent rRNA methyltransferase genes with the same function in different biosynthetic gene clusters, and the pactamycin resistance gene was lost from the pactamycin biosynthetic gene cluster during the evolution of *S. pactum*. A lactonamycin-like gene cluster could also explain why no change in phenotype was observed in *S. pactum ΔAT*, as lactonamycin production is not consistent.\(^{[18]}\) Further sequencing of the *S. pactum* genome could identify this gene cluster.

**Investigating the catalytic function of PtM**

Since the putative AT gene was not involved in pactamycin biosynthesis, our next objective was to identify potential 6MSA transferases in the pactamycin gene cluster. A pactamycin biosynthetic gene called *ptmR* appeared to be a likely candidate. The *ptmR* gene encodes a putative Ketoacyl-(ACP) synthase (KAS)-III enzyme, which has been theorized to transfer activated aromatic carboxylic acids during biosynthesis of secondary metabolites.\(^{[8]}\) To test the role of *ptmR* as the 6MSA transferase gene, we disrupted *ptmR* in the *S. pactum* mutant that lacks the gene *ptmH*. *S. pactum ΔptmH* primarily produces the pactamycin analog TM-026, which has the 6MSA moiety attached, and the pactamycin analog TM-025, the direct
biosynthetic precursor to TM-026 that lacks 6MSA. Both TM-025 and TM-026 have reduced cytotoxicity and show increased antimalarial activity compared to pactamycin. TM-025 characterization has been hampered by low yields and a mutant lacking both the \textit{ptmH} and the 6MSA transferase gene could produce TM-025 as the major biosynthetic product. Therefore, if \textit{ptmR} was the 6MSA transferase we could obtain significant quantities of TM-025 to facilitate its characterization.

A \textit{S. pactum} double mutant lacking the genes \textit{ptmH} and \textit{ptmR} was constructed by a graduate student, Mostafa Abugreen. The \textit{S. pactum ΔptmRH} mutant was cultured and metabolites were extracted. MS analysis of the extracts revealed the presence of a pactamycin analog with an \textit{m/z} of 395 ([M+H]+), corresponding to the compound TM-025, a pactamycin analog lacking the 6MSA moiety (Figure 6). The compound (400 mg) was isolated by High Performance Liquid Chromatography (HPLC) and characterized by \textsuperscript{1}H nuclear magnetic resonance (NMR) (Figure 7 A). The \textsuperscript{1}H NMR spectra of the isolated compound matched that of TM-025 (Figure 7 B).\textsuperscript{[5]} The accumulation of TM-025 in the \textit{ΔptmRH} mutant suggests that PtmR is involved in either the synthesis of 6MSA or the attachment of 6MSA to the core pactamycin structure. However, our previous study demonstrated that an iterative type I polyketide synthase (PtmQ) is responsible for 6MSA synthesis.\textsuperscript{[1]} Therefore PtmR appears to be responsible for adding 6MSA to the pactamycin core.
Identification of TM-025 in the *S. pactum* ΔptmRH mutant is direct evidence that a ketoacyl-(ACP) synthase (KAS)-III is involved in the transfer of an aromatic carboxylic acid. PtmR shows similarity to other KAS-III acyltransferases, such as CerJ (35% identity) for cervimycin biosynthesis, ChlB6 (35% identity) for chlorothricin biosynthesis, and TiaF (20% identity) for tiacumicin B biosynthesis. CerJ, ChlB6, and TiaF were all proposed to transfer acyl groups to a sugar on a secondary metabolite.\(^8,9,21\) During pactamycin assembly, PtmR probably adds 6MSA after the sugar molecule is rearranged to form the aminocyclopentitol core, suggesting divergence of PtmR from other KAS-III acyltransferases. The rearrangement reaction is unlikely to occur with both 3-aminoacetophenone and 6MSA attached to the sugar. To further explore PtmR as a 6MSA transferase, future experiments will include a biochemical analysis of PtmR,
with TM-025 and 6MSA as substrates, which could contribute further evidence of 6MSA transferase activity by PtmR.

![Figure 7. ¹H NMR analysis of TM-025. Crossed out peaks are impurities, solvent, or water. A) Spectrum for TM-025 isolated from the *S. pactum ΔptmRH*. B) Reference spectrum for TM-025.][5]

**Inactivation of the regulatory genes afsA, arpA, and phoP**

Gene fragments for each regulatory gene were amplified by PCR (Figure 8). Each gene fragment was separately inserted into plasmid pTMN002, forming plasmid PTMA04 (containing the *afsA* gene fragment) and pTMA05 (containing the *arpA* gene fragment), and pTMA06 (containing the *phoP* gene fragment) (Figure 9). Each plasmid was transferred to *S. pactum ΔptmH* via *E. coli* mediated conjugation. Disruption of each gene was confirmed (Figure 10).
Figure 8. Gel analysis of gene disruption fragments for the regulatory genes afsA, arpA, and phoP. PCR products of: A) afsA gene fragment. B) arpA gene fragment. C) phoP gene fragment.

Figure 9. Identification of pTMN002-derived plasmids for regulatory gene disruption. A) PCR of pTMA04. B) Restriction digest of cloned pTMA05. C) Restriction digest of pTMA06.
Evaluating TM-026 production in global regulatory gene mutants

Each mutant was cultured in production media and metabolites were extracted using ethyl acetate; MS analysis of the extract revealed the presence of TM-026. Relative quantification of TM-026 production was performed by creating a standard curve using HPLC from a known pure TM-026 sample. Due to small sample sizes, no statistical analysis was performed. The ΔarpA mutant was calculated to produce TM-026 at a slightly higher rate than that of the control ΔptmH (Figure 11 A). This trend suggests that genetic disruption of the transcriptional repressor ArpA increases production of pactamycin compounds, presumably through increased expression of the protein AdpA. However, there is evidence to suggest that ArpA is not the only transcriptional regulator of adpA. First, AdpA is also known as an autorepressor. Second,
other γ-butyrolactone receptors, such as the ScbR (same protein family as ArpA) may repress adpA transcription.\[10\] And finally, pleiotropic regulators like the gene bldD from S. coelicolor may also repress transcription of adpA.\[23\] Other modes of adpA repression likely contribute to decreased pactamycin production. Overexpression of AdpA in S. pactum may increase pactamycin production if adpA is transcribed by a non-AdpA-repressible promoter.

The ΔafsA mutant did not produce TM-026 at a consistent rate, and no conclusions can be made (Figure 11 B). Since the disruption of the arpA gene appeared to increase TM-026 production, the ΔafsA mutant was expected to produce less TM-026 than the control; however, this trend was not observed. A fragment of the afsA gene was found in the S. pactum genome and only 230 base pairs of the afsA gene were used for gene disruption. It is possible that this gene is an afsA-like gene and not the afsA gene itself, affecting pactamycin biosynthesis differently. It is also possible that a functional AfsA was still transcribed.

The ΔphoP mutant did not produce more TM-026 than the (Figure 11 C). The ΔphoP mutant was expected to affect pactamycin production, as the amount of phosphate in the culture media affects pactamycin production (unpublished data). The impact of PhoP on secondary metabolite production is unclear,\[10\] and this result does not settle the debate. Future plans involve further confirmation of the ΔphoP mutant, growing the mutant with varying levels of phosphate in the media, and measuring pactamycin production
Conclusions

Through gene inactivation and heterologous expression, we confirmed that a SAM-dependent methyltransferase gene, which is located outside of the pactamycin biosynthetic gene cluster, is responsible for pactamycin resistance and the neighboring acyltransferase gene is not involved in pactamycin biosynthesis. The region of the *S. pactum* chromosome that contains the pactamycin resistance gene and the putative acyltransferase gene is very similar to a region in the lactonamycin biosynthetic gene cluster. However, it is not clear whether this similarity is significant.

Figure 11. Comparison of TM-026 production in the regulatory gene mutants. A) ΔafsA mutant produced slightly more TM-026 than the control, but not consistently. B) ΔarpA mutant produced more TM-026 than the control. C) ΔphoP mutant did not produce significantly more TM-026 than the control.
On the other hand, we discovered that the putative Ketoacyl-(ACP) synthases (KAS)-III PtmR is responsible for the attachment of 6MSA to the pactamycin core structure. (KAS)-III enzymes have been involved in fatty acid biosynthesis, but their involvement in natural product biosynthesis is not clear. It has been speculated that (KAS)-III enzymes may be responsible for transferring aromatic carboxylic acids during the biosynthesis of natural products.\[8,9,21\] However, no experimental evidence has demonstrated this function. Our result presents direct evidence that a (KAS)-III enzyme can facilitate the transfer of aromatic carboxylic acids during biosynthesis of secondary metabolites.

Finally, through inactivation of the global regulatory genes afsA, arpA, and phoP, and a semiquantitative analysis of pactamycin production, we discovered that the arpA gene is directly involved in regulation of pactamycin production. This is significant, as we can inactivate the arpA gene in other mutants to increased pactamycin production. Interestingly, inactivation of afsA and phoP did not affect pactamycin production.

**Experimental Section**

**Bacterial strains and culture conditions**

Plasmids and strains used are shown in Table 1. All *S. pactum* derivatives were maintained on BTT medium (10 g/L Glucose, 1 g/L Yeast Extract, 1 g/L Beef Extract, 2 g/L Casein Hydrolysate) with the appropriate antibiotic at 28°C for 5 days. Single colonies were used to inoculate BTT media. A modified BTT medium was used as a pactamycin production medium (10 g/L Glucose, 1 g/L Yeast Extract, 1 g/L Beef Extract, 2 g/L Soytone, 1% v/v metal ion solution). Production cultures were inoculated with 10% V/V culture from BTT medium. *E. coli* strains were cultured
on LB media with the appropriate antibiotic. *E. coli* mediated conjugation was facilitated on MS agar (20 g/L Soy flour, 2.03 g/L MgCl2, 20 g/L Mannitol, 20 g/L Agar). *S. lividans* strains were cultured on YMG media (10 g Malt Extract, 4 g Yeast Extract, 4 g Yeast).

**Table 1. Bacterial strains and plasmids used.**

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<th>Strains</th>
<th>Genotype/Comments</th>
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<tr>
<td><em>E. coli</em> DH10B</td>
<td>General cloning strain</td>
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<tr>
<td><em>E. coli</em> ET12567 (pUZ8002)</td>
<td>dam dcm hsdS, pUZ8002; Conjugative strain</td>
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<td><em>Streptomyces pactum</em> ATCC 27456</td>
<td>Pactamycin-producing strain</td>
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<td><em>S. pactum</em> ΔptmH</td>
<td><em>ptmH</em> disruption mutant</td>
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<tr>
<td><em>S. pactum</em> ΔptmRH</td>
<td><em>ptmH</em> and <em>ptmR</em> disruption mutant</td>
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<td><em>S. pactum</em> ΔAT</td>
<td>Putative acyltransferase disruption mutant</td>
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<td><em>S. pactum</em> ΔptmHΔafsA</td>
<td>Strain with disrupted <em>ptmH</em> and <em>afsA</em> genes</td>
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<tr>
<td><em>S. pactum</em> ΔptmHΔarpA</td>
<td>Strain with disrupted <em>ptmH</em> and <em>arpA</em> genes</td>
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<tr>
<td><em>S. pactum</em> ΔptmHΔphoP</td>
<td>Strain with disrupted <em>ptmH</em> and <em>phoP</em> genes</td>
</tr>
<tr>
<td><em>Streptomyces lividans</em> 1236</td>
<td>Wild-type strain</td>
</tr>
<tr>
<td><em>S. lividans</em> +met</td>
<td>Strain expressing the pactamycin resistance gene</td>
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</tbody>
</table>

**Plasmids**

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
</tr>
</thead>
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<tr>
<td>pRSET B</td>
<td>High copy number cloning/expression vector</td>
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<tr>
<td>pSET152</td>
<td><em>Streptomyces</em> expression vector</td>
</tr>
<tr>
<td>pTMN002</td>
<td>pJTU1278 derivative containing the apramycin resistance gene</td>
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<td></td>
<td>aac(3)IV</td>
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<td>pRSET B containing the complete pactamycin resistance gene</td>
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<tr>
<td>pTMA02</td>
<td>pSET 152 containing the complete pactamycin resistance gene</td>
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<td>pTMA03</td>
<td>pTMN002 containing gene disruption fragment for the putative acyltransferase gene</td>
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<td>pTMN002 containing gene disruption fragment of the <em>afsA</em> gene</td>
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<td>pTMA05</td>
<td>pTMN002 containing gene disruption fragment of the <em>arpA</em> gene</td>
</tr>
<tr>
<td>pTMA06</td>
<td>pTMN002 containing gene disruption fragment of the <em>phoP</em> gene</td>
</tr>
</tbody>
</table>

**DNA manipulation of *S. pactum* and *S. lividans***

DNA isolation and manipulations were performed using standard methods for *E. coli* and *Streptomyces*.\(^{[24]}\) PCR was performed for 35 cycles using a Mastercycler Gradient Thermocycler (Eppendorf, Germany). PCR primers were purchased from Sigma-Aldrich, USA. PCR products
and plasmids were purified using ethanol precipitation or Omega Biotek E.Z.N.A Plasmid/Gel Extraction DNA kits. PCR products and plasmids were digested with the appropriate restriction enzymes at 37°C for 1 hour. Digested products were ligated and transferred to *E. coli* DH10B via electroporation for cloning. Cloned products were transferred to *E. coli* ET12567 (pUZ8002) for conjugation with *S. pactum* or *S. lividans*. ORFs were assigned functions according to Frameplot 4.0 and Blastn.[24,25] DNA sequencing was performed at the Center for Genome Research and Biocomputing (CGRB) Core Laboratories, Oregon State University.

**Confirmation of pactamycin resistance in *S. lividans* +met**

A Kirby-Bauer Antibiotic Test was performed to confirm pactamycin resistance of the *S. lividans* +met strain. Two antibiotic disks of pactamycin (5 µL and 10 µL of 27 mM pactamycin) and one disk of apramycin (5 µL of 50 mg/mL apramycin) were place on YMG agar media after inoculation with *S. lividans* +met or the *S. lividans* WT. The strains were allowed incubated at 28°C for 5 days. The absence or presence of growth around the antibiotic disks indicated sensitivity or resistance to pactamycin and apramycin.

**Isolation and identification of pactamycin compounds**

*S. pactum* culture was grown in pactamycin production medium (described above) for 6 days. The culture was adjusted to pH 7.1. Mycelia were centrifuged and the culture supernatant was extracted with one volume of ethyl acetate twice and one volume of butanol. The combined organic layers were concentrated and the resulting organic extracts dissolved in methanol. MS of *S. pactum* ΔAT metabolites was performed on a 3200 Qtrap (AB Sciex, USA) using ESI+. MS of *S. pactum* ΔptmRH metabolites was performed on a ThermoFinnigan LCQ Advantage (Thermo Fisher Scientific, USA) ESI+. Reverse-phase HPLC was used to isolate TM-02S from the butanol
extract on a C-18 semipreparative column (YMC-pack ODS-A, 250 x 10 mm), purchased from YMC America, Inc. The solvent system used water (0.1% Trifluoroacetic acid, solvent A) and acetonitrile (0.1% Trifluoroacetic acid, solvent B). For elution of TM-025, a flow rate of 3.33 mL/min was used with the following gradient: 5-100% B (40 min). TM-025 was monitored using UV detection at 254 nm. $^1$H NMR of TM-025 was performed on an Avance III 700 MHz NMR (Bruker, USA). The $^1$H NMR spectrum of the isolated TM-025 matched the published spectrum for TM-025 (Figure 7).$^5$

Reversed-phase HPLC was used to quantify production of TM-026 in the ethyl acetate extracts of the regulatory gene mutants on an analytical C-18 column (YMC-pack ODS-A, 250 x 4.6 mm) purchased from YMC American, Inc. The solvent system was the same as for TM-025. The gradient used was: 25% (5 min), 25-95% B (30 min) and the flow rate was 0.7 mL/min. TM-026 was also monitored with UV detection at 254 nm. A standard curve was created using a pure sample of TM-026 and was based on the area under the TM-026 peak. Three cultures for each regulatory gene mutant and control were made in BTT medium. Controls and mutants were inoculated into the production media with the same cell mass. After extraction, crude extracts were dissolved in 0.5 mL methanol and TM-026 production was quantified according to the standard curve. TM-026 production was then converted to mg/L produced and compared to the controls.
References