Identification of the Actinomycin D Biosynthetic Pathway from Marine-Derived *Streptomyces costaricanus* SCSIO ZS0073

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**Abstract:** Bioactive secondary metabolites from *Streptomyces* are important sources of lead compounds in current drug development. *Streptomyces costaricanus* SCSIO ZS0073, a mangrove-derived actinomycete, produces actinomycin D, a clinically used therapeutic for Wilm’s tumor of the kidney, trophoblastic tumors and rhabdomyosarcoma. In this work, we identified the actinomycin biosynthetic gene cluster (BGC) acn by detailed analyses of the *S. costaricanus* SCSIO ZS0073 genome. This organism produces actinomycin D with a titer of ~69.8 µg mL$^{-1}$ along with traces of actinomycin X$_o$β. The acn cluster localized to a 39.8 kb length region consisting of 25 open reading frames (ORFs), including a set of four genes that drive the construction of the 4-methyl-3-hydroxy-anthranilic acid (4-MHA) precursor and three non-ribosomal peptide synthetases (NRPSs) that generate the 4-MHA pentapeptide semi-lactone, which, upon dimerization, affords final actinomycin D. Furthermore, the acn cluster contains four positive regulatory genes acnWU4RO, which were identified by in vivo gene inactivation studies. Our data provide insights into the genetic characteristics of this new mangrove-derived actinomycin D bioproducer, enabling future metabolic engineering campaigns to improve both titers and the structural diversities possible for actinomycin D and related analogues.

**Keywords:** actinomycin D; biosynthetic gene cluster; biosynthesis; bioproducer; *Streptomyces costaricanus*

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1. Introduction

Actinomycins are a group of chromopeptide lactone antibiotics. To date, 42 actinomycins have been isolated and identified from many species of *Streptomyces* (Supporting Information (SI), Table S1), including actinomycin D, N-demethylactinomycins, actinomycin C, actinomycin F, actinomycin Z, actinomycin G and actinomycin Y [1–3] (SI, Table S1). Some actinomycin analogues, such as methylated actinomycin D [4], an actinomycin Z analogue having an additional oxygen bridge between the chromophore and β-depsipentapeptide [5], actinomycins D1–D4 [6] and neo-actinomycins A and B [7], possess structurally modified cyclopeptide rings or chromophore (SI, Table S1). Initiatives to develop actinomycin analogues with superior bioactivities have been heavily rooted in precursor-directed biosynthesis [8]. Actinomycins, commonly employed clinically in anticancer therapeutic regimes, exhibit excellent antitumor activity. The core phenoxazinone chromophore intercalates between the
stacked nucleobases at guanine/cytosine sites of DNA whereas the pentapeptide elements bind to the minor groove; these binding interactions effectively inhibit duplication and transcription processes in tumor cells [9]. Exemplary in this fashion, actinomycin D is a highly effective chemotherapeutic used to treat Wilm’s kidney tumors, trophoblastic tumors and rhabdomyosarcoma [2]. Actinomycin D also specifically targets and down-regulates the expression of stem-cell transcription factor, Sox-2; this protein facilitates depletion of the stem-cell population resulting in the inability of breast cancer cells to initiate tumor progression [10]. Low doses of actinomycin D specifically activate p53-dependant transcription enhancing the activity of chemotherapeutic drug-induced killing of p53 positive human tumor cells [11]. Actinomycins are also able to inhibit some viruses such as the coxsackie virus B3 and HIV-1, the causative agent of AIDS [12,13]. The combination of these novel activities and potential in human health makes actinomycin D one of the most promising candidates for medicinal development campaigns. Moreover, the actinomycins often display strong antimicrobial activities. Potent antibacterial activities against human pathogens, human pathogenic fungi, as well as aquatic pathogenic bacteria are well known [14]. For instance, actinomycin X₂ kills both resting and budding spores of Bacillus megaterium and actinomycin X₃β has pronounced anti-tuberculosis activities [15]. Notably, despite these very favorable activities, clinical applications of actinomycin D are often limited by undesirable side-effects; liver and kidney damage rank high on this list of detrimental side-effects [16].

Recent progress in genome research has revealed the presence of the actinomycin D biosynthetic gene cluster (BGC) in S. chrysomallus. The gene cluster was found to encompass 50 kb of contiguous DNA on the chromosome of S. chrysomallus and to contain 28 biosynthetic genes bordered on both sides by IS (Insertion Sequence) elements [17]. Remarkably, 9 of the genes embedded within the actinomycin D BGC have two copies, all of which are in the same order but in the opposite orientation; such an arrangement of genes within a BGC is unprecedented. Sequencing of the actinomycin BGC in S. antibioticus IMRU 3720, which produces actinomycin D, also revealed 20 genes organized into a similar framework but without gene duplicates as in the actinomycin D biosynthetic gene cluster of S. chrysomallus [18]. In addition, the actinomycin G BGC in S. iakyrus was identified and reported in 2013 [19].

A defining feature of the actinomycins is a central phenoxazinone chromophore which serves to bridge two pentapeptide lactones consisting of diverse amino acids. The pentapeptide precursor is biosynthesized by the non-ribosomal peptide synthetase (NRPS) assembly line with the 4-methyl-3-hydroxy-anthranilic acid (4-MHA) as the initiating unit [20,21]. The biosynthesis of 4-MHA differs in various actinomycin-producing strains. For example, in S. chrysomallus, Streptosporangium sibiricum and S. parvulus, tryptophan is implicated as a substrate for tryptophan-2,3-dioxygenase which primarily forms the important MHA precursor, N-formyl kynurenine (Figure 2). N-formyl kynurenine then serves as a substrate for kynurenine formamidase and kynurenine 3-monoxygenase; the actions of these two enzymes produce 3-hydroxykynurenine (3-HK) and this sequence of chemistries is apparent in all actinomycin producers. Methylation of 3-HK then affords 4-methyl-3-hydroxy-kynurenine (4-MHK). 4-MHK is then catalyzed by hydroxykynureninase to form 4-MHA. Some deviations in this chemistry are seen, however, based on the precise actinomycin producer in question. For instance, in S. antibioticus, tryptophan is converted to 3-hydroxyanthranilic acid (3-HA) which is subsequently processed by a methyltransferase to form 4-MHA [22].

Perhaps one of the more intriguing questions associated with actinomycin assembly has to do with the final step of actinomycin D biosynthesis and how two molecules of MHA-pentapeptide converge, via phenoxazinone assembly, to generate the intact actinomycin. Phenoxazinone synthase (PHS), a 650 aa, two copper-containing phenoloxidase, has long been theorized to mediate this final step [23]. Once cloned and expressed in vitro, PHS was characterized and anticipated to activate MHA-pentapeptide monomers for subsequent dimerization reactions [24,25]. However, in the actinomycin-producer S. antibioticus IMRU 3720, actinomycin D production persisted in an inactivated ΔphsA mutant [26], suggesting that the putative phenoxazinone synthase is not essential for actinomycin
assembly. Hence, the mechanism by which the two MHA-pentapeptide monomers come together to form intact actinomycins remains largely unclear.

Enabled by high quality genomic scanning and analyses, we report here the identification of a distinct 39.8 kb gene cluster from \textit{S. costaricanus} SCSIO ZS0073, a previously identified actinomycin D and actinomycin X\textsubscript{\text{O\beta}} producer [27]. We have defined the gene cluster boundaries and described the organization of the complete biosynthetic gene cluster as guided by the results of gene insertions and metabolic profile analyses. Interestingly, we have discovered that, within the upstream and downstream regions of the cluster, can be found regulatory genes \textit{acnW}, \textit{acnR}, \textit{acnU4} and \textit{acnO}. Our study expands insights into how actinomycin titers and structural diversities stand to be improved via the application of combinatorial biosynthetic approaches. Holistically speaking, actinomycin analogs with reduced toxicities and improved bioactivities represent important goals for the downstream application of new knowledge reported herein.

2. Results

2.1. Identification of the Actinomycin D Biosynthetic Gene Cluster

To identify the gene cluster, we sequenced the \textit{S. costaricanus} SCSIO ZS0073 genomic DNA using a combination of Hiseq 4000 and PacBio smart technologies [27]. \textit{S. costaricanus} SCSIO ZS0073 is a mangrove-derived \textit{actinomycete} which produces antibacterial secondary metabolites such as fungichromin, actinomycin D and actinomycin X\textsubscript{\text{O\beta}} [27,28]. We analyzed a 39.8 kb region containing 24 open reading frames (ORFs), suspected of coding for actinomycin D biosynthesis. We elucidated the gene organization as shown in Figure 1 and deduced its biosynthetic pathway (Figure 2) along with assigned gene product functions as noted below in Table 1. The gene cluster has been deposited in Genbank (with the accession number of MK234849). A cosmid library of \textit{S. costaricanus} SCSIO ZS0073 was constructed using the SuperCos 1 vector system and 2 positive clones (9C2, 9A7) covering the whole gene cluster were screened using targeted gene inactivations.

2.2. Determination of the Actinomycin D Biosynthetic Gene Cluster Boundaries

The upstream boundary of the actinomycin D gene cluster was preliminarily determined as being between \textit{orf}(−1) and \textit{acnW}; the downstream boundary was defined between \textit{acnT3} and \textit{orf}(+1). The \textit{orf}(−1) encodes Type VII secretion system protein EccC. Although \textit{orf}(−1) carries with it the function of enabling secretion, all the inactivation mutants of \textit{orf}(−1) failed to show any differences in actinomycin D production levels compared to wild-type (WT) (SI, Figure S7). The upstream boundary region was further delineated by a set of gene inactivations for \textit{orf}(−1), \textit{orf}(−2) and \textit{orf}(−3). None of the inactivation bearing mutants for these genes displayed discernible differences in actinomycin D biosynthesis rates or yields relative to WT producer (SI, Figure S7). Likewise, the downstream boundary was ascertained by bioinformatic analyses and the application of mutants bearing inactivated \textit{orf}(+1), \textit{orf}(+2), \textit{orf}(+3) genes. Although \textit{orf}(+1) gene encodes an N-acetyltransferase, it is shown by inactivation experiments, the mutant strains produce the same level of actinomycin D compared to wild type strains. Further evaluations proximal to the downstream boundary revealed that genes corresponding to \textit{orf}(+2) and \textit{orf}(+3) encode a hypothetical protein and a two-component hybrid sensor and regulator; comparative analyses of \textit{\Delta orf}(+2) and \textit{\Delta orf}(+3) inactivation mutants (vs. WT) revealed that neither of these genes and their respective products play a role in actinomycin D biosynthesis (SI, Figure S7).

2.3. NRPS Genes for Peptide Chain Assembly in Actinomycin D Biosynthesis

Following the biosynthesis of 4-MHA, this anthranilic acid derivative, along with other amino acids, by several NRPSs ultimately affording the 4-MHA-pentapeptide halves of actinomycin (Figure 2A). The pentapeptide is composed of five different amino acids: l-threonine, d-valine, l-proline, l-sarcosine and methyl-valine. The genes responsible for peptide chain assembly in \textit{S. costaricanus} SCSIO ZS0073 are \textit{acnD}, \textit{acnE}, \textit{acnN1}, \textit{acnN2}, \textit{acnN3}. AcnD was found to contain 66 amino acid residues arranged in a
manner that resembles the MbtH protein. MbtH-like proteins are found in many NRPS gene clusters [29] and researchers have demonstrated that MbtH-like proteins participate in some adenylation reactions by tightly binding to NRPS proteins containing adenylation domains [30, 31]. MbtH-like proteins, such as gene dptG in S. roseosporus participate in the biosynthesis of the cyclic lipotripeptide antibiotic daptomycin [32] and mbh in Mycobacterium spp. responsible for the siderophore biosynthesis [33]. The NRPS gene acnN1 encodes an adenylation domain protein and acnE encoding 4-MHA carrier protein. Two large multidomain NRPSs acnN1 and acnN3 are responsible for peptide chain assembly and release from the NRPS machinery. Gene deletions of acnD + acnE in combination, acnN1 and acnN3 were found to abolish actinomycin D biosynthesis (Figure 3) in the mutant producers bearing these gene inactivations.

![Figure 1. Gene organization of the actinomycin D cluster in S. costaricanus SCSIO ZS0073. The direction of transcription and the proposed functions of individual ORF are indicated.](image)

![Figure 2. Proposed model for actinomycin D assembly (A) and 4-MHA precursor production (B) in S. costaricanus SCSIO ZS0073. A: adenylation domain; C: condensation; PCP: peptidyl carrier protein; TE: thioesterase.](image)
Table 1. Deduced *orf* functions in the *acn* cluster.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Size</th>
<th>Protein Homolog and Origin</th>
<th>ID/SM (%)</th>
<th>Origin (Protein ID)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>orf</em> (−3)</td>
<td>492</td>
<td>type VII secretion protein EccB</td>
<td>96/97</td>
<td>SCF88120 Streptomyces sp. LamerLS-31b</td>
</tr>
<tr>
<td><em>orf</em> (−2)</td>
<td>464</td>
<td>type VII secretion integral membrane protein EccD</td>
<td>99/99</td>
<td>SCF8128 Streptomyces sp. LamerLS-31b</td>
</tr>
<tr>
<td><em>orf</em> (−1)</td>
<td>1289</td>
<td>Collagen triple helix repeat-containing protein</td>
<td>53/75</td>
<td>Clostridium uliginosum</td>
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<tr>
<td><em>acnW</em></td>
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<td>Hypothetical protein</td>
<td>99/100</td>
<td>AB905443.1 Streptomyces rochei 7434AN4</td>
</tr>
<tr>
<td><em>acnA</em></td>
<td>121</td>
<td>Protein of unknown function</td>
<td>93/96</td>
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<tr>
<td><em>acnB</em></td>
<td>415</td>
<td>Anti-anti-sigma regulatory factor</td>
<td>96/97</td>
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</tr>
<tr>
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<td>121</td>
<td>hypothetical protein</td>
<td>99/100</td>
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<tr>
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<td><em>acnC</em></td>
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<td>acyl-CoA dehydrogenase</td>
<td>91/95</td>
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<tr>
<td><em>acnU4</em></td>
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<tr>
<td><em>acnD</em></td>
<td>66</td>
<td>protein mbtH</td>
<td>91/95</td>
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<td><em>acnE</em></td>
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<td>4-MHA carrier protein</td>
<td>73/85</td>
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<td><em>acnN1</em></td>
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<td>Adenylation domain protein</td>
<td>51/65</td>
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<td><em>acnN2</em></td>
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<td>non-ribosomal peptide synthase</td>
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<td>Streptomyces anulatus WP_064726364</td>
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<td><em>acnN3</em></td>
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<td>non-ribosomal peptide synthetase</td>
<td>78/85</td>
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<td>hypothetical protein</td>
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<td><em>acnG</em></td>
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<td>aryIformamidase</td>
<td>79/85</td>
<td>Streptomyces sp. Cmuel-A718b OOQ48467</td>
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<td><em>acnH</em></td>
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<td>tryptophan 2, 3-dioxygenase</td>
<td>84/88</td>
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<td><em>acnL</em></td>
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<td>kynureninase</td>
<td>86/90</td>
<td>Streptomyces antibioticus WP_030594248</td>
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<tr>
<td><em>acnM</em></td>
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<td>methyltransferase</td>
<td>88/91</td>
<td>Streptomyces antibioticus WP_030594247</td>
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<tr>
<td><em>acnP</em></td>
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<td>cytochrome P450</td>
<td>86/91</td>
<td>Streptomyces antibioticus ADG27350</td>
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<td><em>acnO</em></td>
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<td>LmbU-like protein</td>
<td>73/82</td>
<td>Streptomyces antibioticus OOQ48074</td>
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<tr>
<td><em>acnR</em></td>
<td>282</td>
<td>TetR family transcriptional regulator</td>
<td>78/86</td>
<td>Streptomyces antibioticus WP_030594239</td>
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<td><em>acnQ</em></td>
<td>294</td>
<td>siderophore-interacting protein</td>
<td>78/87</td>
<td>Streptomyces antibioticus OOQ48072</td>
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<tr>
<td><em>acnT1</em></td>
<td>346</td>
<td>ABC transporter</td>
<td>87/92</td>
<td>Streptomyces antibioticus OOQ48071</td>
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<tr>
<td><em>acnT2</em></td>
<td>255</td>
<td>multidrug ABC transporter permease</td>
<td>92/95</td>
<td>Streptomyces antibioticus AMV28079</td>
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<tr>
<td><em>acnT3</em></td>
<td>753</td>
<td>UvrABC system protein A</td>
<td>60/77</td>
<td>Gemmatia sp. SH-PL17 UN35851</td>
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<tr>
<td><em>orf</em> (+1)</td>
<td>191</td>
<td>GCN5-related N-acetyltransferase</td>
<td>81/87</td>
<td>Streptomyces venezuelae WP_052876109</td>
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<tr>
<td><em>orf</em> (+2)</td>
<td>109</td>
<td>hypothetical protein</td>
<td>74/83</td>
<td>Streptomyces sp. NRRL F-4335 SBO98167</td>
</tr>
<tr>
<td><em>orf</em> (+3)</td>
<td>1130</td>
<td>Two-component hybrid sensor and regulator</td>
<td>55/68</td>
<td>Nonomuraea sp. ATCC 39727</td>
</tr>
</tbody>
</table>

*a* Size in units of amino acids (aa); ID/SM: identity/similarity; *acn*: the BGC of actinomycin from *S. costaricanus SCSIO Z50073*. 
which shows high similarity to TetR family members and is involved in tirandamycin production.

The inactivation of essential role as a and important positive regulator of actinomycin D biosynthesis (Figure 3, trace of transporters, which help bacteria acclimate to their environment [37,38]. In some cases, members motif and a C-terminal ligand regulatory domain. Many of these regulators control the expression system and generally possess a two-domain structure composed of an N-terminal HTH DNA-binding and metabolite regulation [35,36]. TetR family transcriptional regulators belong to a one-component transcriptional repressor or activator in many biological processes such as cell-cell communication and siderophore-interacting systems often constitute transport systems by which microbes control intracellular concentrations of specific second ary metabolites. As with the other genes noted construction appear nonessential. Inactivations for all these genes have no impact on actinomycin D synthesis compared to the WT producer (Figure 3). Consequently, we concluded that these three transporter genes are not necessary in actinomycin D production compared to the WT producer (Figure 3).

2.5. Nonessential Genes of Unknown within the acn Cluster

We identified six genes, designated as acnG, acnH, acnL and acnM, that show high functional similarities to enzymes involved in the biosynthesis of 4-MHA, the critical building block of the actinomycin chromophore. These genes are homologous to a set of four counterparts in actinomycin-producing strains S. antibioticus IMRUS720 and S. chrysomallus [17,18]. These genes encode for arylformamidase (acnG), tryptophan-2, 3-dioxygenase (acnH), kynureninase (acnL) and methyltransferase (acnM). The gene acnM shows 88% identities with the 3-hydroxy kynurenine methyltransferase acmL, which is responsible for the methylation of 3-HK [34]. However, the vital step entailing kynurenine conversion to 3-hydroxykynurenine appears to be catalyzed by a kynurenine 3-monoxygenase, the gene for which is not found within the acn gene cluster. Inactivation of acnGHLM in combination, followed by metabolite analysis of the mutant strain revealed that these genes play essential roles in actinomycin D biosynthesis (Figure 3).

2.6. Regulator and Self-Resistance Genes

We identified six genes acnW, acnU4, acnR, acnT1, acnT2 and acnT3 with apparent regulatory and/or protective functions. AcnR encodes a TetR family transcriptional regulator and usually acts as a transcriptional repressor or activator in many biological processes such as cell-cell communication and metabolite regulation [35,36]. TetR family transcriptional regulators belong to a one-component system and generally possess a two-domain structure composed of an N-terminal HTH DNA-binding motif and a C-terminal ligand regulatory domain. Many of these regulators control the expression of transporters, which help bacteria acclimate to their environment [37,38]. In some cases, members of the TetR family suppress the biosynthesis of antibiotics. For instance, disruption of calR3, a TetR family member in the calcimycin BGC, was found to improve calcimycin titers [39]. Moreover, TrdK, which shows high similarity to TetR family members and is involved in tirandamycin production from marine-derived Streptomyces sp. SCSIO 1666 was found by gene inactivations, to suppress tirandamycin biosynthesis; trdK inactivation led to dramatically improved tirandamycin titers [40]. The inactivation of acnR completely abolished actinomycin D production, consistent with this gene’s essential role as a and important positive regulator of actinomycin D biosynthesis (Figure 3, trace

Figure 3. HPLC analysis of S. costaricanus strains fermentation extracts. (i) WT producer; (ii) ΔacnDE mutant; (iii) ΔacnN1 mutant; (iv) ΔacnN3 mutant; (v) ΔacnGHLM mutant; (vi) ΔacnW mutant; (vii) ΔacnU4 mutant; (viii) ΔacnR mutant; (ix) ΔacnO mutant; (x) ΔacnT1 mutant; (xi) ΔacnT2 mutant; (xii) ΔacnT3 mutant. 1, actinomycin D; 2, actinomycin Xoβ; 3, actinomycin X2.
Gene acnW is located downstream of the boundary gene orf(−1) and appears to encode for a hypothetical protein; acnW disruption impaired, but did not abolish, production of actinomycin D. The gene acnU4 also encodes a hypothetical protein and, as with acnW, inactivation of acnU4 correlates to diminished actinomycin D production (Figure 3). On the basis of these findings, AcnW and AcnU4 appear to function as positive regulators of actinomycin D production although the structural families to which they belong are not yet apparent. AcnO encodes a LmbU-like protein. LmbU is a regulatory gene involved in licomycin biosynthesis in S. lincolnensis 78-11. It always contains a TTA codon close to the N-terminal end of its ORF. The codon is often found in genes involved in the regulation of differentiation or secondary metabolism [41], in neither case is a specific role in actinomycin D assembly known, S. costaricanus SCSIO ZS0073 inactivation mutant strain of ΔacnO was not able to produce even trace amounts of actinomycin D (Figure 3, traces ix). Thus, acnO might serve as a positive regulator in actinomycin D biosynthetic pathway.

Beyond these gene–function correlations, we also identified three putative transporter genes acnT1, acnT2 and acnT3. The acnT1 gene is predicted to encode an ATP-binding cassette (ABC) transporter which uses the energy of ATP to transport molecules through the membrane. Bioinformatics analyses suggest that acnT2 encodes a multidrug ABC transporter permease whereas acnT3 encodes a UvrA-like protein, which is a DNA binding protein involved in the excision repair of DNA. Somewhat surprisingly, inactivations of acnT1 acnT2 and acnT3 all produced mutant strains able to generate actinomycin D with efficiencies rivaling those seen with the WT producer (Figure 3). Consequently, we concluded that these three transporter genes are not necessary in actinomycin D biosynthesis.

2.6. Nonessential Genes of Unknown within the acn Cluster

The BGC for actinomycin D in S. costaricanus SCSIO ZS0073 houses a number of genes not associated with actinomycin D assembly. For instance, close to the upstream boundary reside genes acnA and acnB; all of these encode for hypothetical proteins. Additionally, acnU1, acnU2, acnU3 also encode for proteins whose functions have not been deciphered and whose roles in actinomycin D construction appear nonessential. Inactivations for all these genes have no impact on actinomycin D biosynthesis relative to WT production efficiencies (Figure 4). Bioinformatics have revealed that acnC resides between acnU2 and acnU3 and codes for an acyl-CoA dehydrogenase. This gene’s inactivation also appears to have no impact upon actinomycin D synthesis compared to the WT strain. This too is the case for acnQ. Located downstream of the acnR, acnQ was proposed, based on bioinformatics, to code for a siderophore-interacting protein. Such small molecule siderophore-interacting systems often constitute transport systems by which microbes control intracellular concentrations of specific secondary metabolites. As with the other genes noted however, inactivation of acnQ was found to have no bearing whatsoever on actinomycin D production compared to the WT producer (Figure 4).
2.7. Cytochrome P450 Gene acnP Is Responsible for the Hydroxylation of Proline in Actinomycin X_β

X-type actinomycins contain 4-hydroxyproline (actinomycin X_β) or 4-oxoproline (actinomycin X_β) in their β-pentapeptide lactone rings whereas their α-ring contains proline. Previous studies demonstrated the importance of a 4-oxoproline synthase within the actinomycin BGC of *S. antibioticus*. This enzyme catalyzes proline oxidation in each of the actinomycin halves (prior to dimerization) to form 4-hydroxyproline or 4-oxoproline; condensation of each of the hydroxylated actinomycin halves affords actinomycin X_β or actinomycin X_β [42]. Within the actinomycin BGC in *S. costaricanus* SCSIO ZS0073, we identified a cytochrome P450 gene, acnP, that encodes a 436 aa protein. We hypothesized that AcnP is responsible for proline hydroxylation *en route* to actinomycin X_β. This speculation was validated by targeted inactivation of acnP; fermentations and metabolite analyses of the ΔacnP mutant revealed the complete abrogation of actinomycin X_β production and concomitant production of actinomycin D with titers rivaling those seen with the WT producer (Figure 5). These data make clear that AcnP is responsible for the 4-oxoproline found in actinomycin X_β:

![Figure 4](https://example.com/figure4.png)

**Figure 4.** HPLC analyses of *S. costaricanus* strains fermentation extracts. (i) WT producer; (ii) ΔacnA mutant; (iii) ΔacnB mutant; (iv) ΔacnC mutant; (v) ΔacnU1 mutant; (vi) ΔacnU2 mutant; (vii) ΔacnU3 mutant; (viii) Δphs mutant; (ix) ΔacnP mutant; (x) ΔacnF mutant. 1, actinomycin D; 2, actinomycin X_β.

![Figure 5](https://example.com/figure5.png)

**Figure 5.** HPLC analysis of *S. costaricanus* strains fermentation extracts. (i) WT producer; (ii) ΔacnP mutant. 1, actinomycin D; 2, actinomycin X_β.

The PHS gene outside the *acn* cluster is not necessary for actinomycin D biosynthesis. PHS has been presumed to catalyze the oxidative condensation of two 4-MHA-pentapeptide lactone “monomers”
in the last step of actinomycin D biosynthesis. Previous studies had, in fact, demonstrated that PHS
is involved in a variety of enzymatic condensations of ortho-aminophenols to form phenoxazinones.
Within the genome of *S. costaricanus* SCSIO ZS0073, we identified a *phs* orf encoding a 627 aa, two
copper-containing phenoloxidase separated from the actinomycin biosynthetic cluster by 5.77 Mbp.
This *phs* gene shows sequence similarity (84% identity) to *phsA* in *S. antibioticus* IMRU3720. To validate
this gene’s involvement in actinomycin D assembly, we generated a Δ*phs* mutant strain and assessed
its biosynthetic capacity relative to the WT producer. Surprisingly, *phs* inactivation in the *S. costaricanus*
SCSIO ZS0073 strain had no detectable impact upon actinomycin D production relative to the WT
producer (Figure 4). This result is consistent with the previous gene inactivation result for *phsA* in *S.
antibioticus* IMRU3720 [26] and indicated also, that for *S. costaricanus* SCSIO ZS0073 actinomycin D
assembly does not appear to require a PHS.

*acnF* is essential to actinomycin D biosynthesis. Within the actinomycin cluster, *acnF* encodes for
a hypothetical protein with 212 aa. Gene inactivation for *acnF*, revealed that *acnF* is indispensable
for actinomycin D construction (Figure 4). *S. costaricanus* SCSIO ZS0073 inactivation mutant strain
of Δ*acnF* was not able to produce even trace amounts of actinomycin D (Figure 4, traces x), thus
showcasing the importance of its gene products to actinomycin D biosynthesis. We proposed *acnF*
might be involved in the dimerization of the 4-MHA-pentapeptide monomer *en route* to actinomycin
(Figure 2). Efforts to identify the exact roles played by *acnF* products are currently ongoing and will be
reported in due course.

3. Materials and Methods

3.1. General Experimental Procedures

Reagents for polymerase chain reactions (PCR) were purchased from Takara Co. (Dalian, China)
and Trans Gene Co. (Beijing, China). The plasmid kit and gel extraction kit were from Promega. Unless
otherwise indicated, other biochemicals and chemicals were purchased from standard commercial
sources and used without further purification. All DNA manipulations were conducted according to
the standard procedures or manufactures’ instruction. DNA and aa sequence analyses were performed
with the seqmen and editsequence in the Lasergene software package (DNASTAR, Madison, WI, USA).
All primers and reagents used in this work were purchased from Sangong Bio-Pharm Technology Co.,
Ltd., Shanghai, China.

3.2. Bacterial Strains, Plasmids and Culture Conditions

*S. costaricanus* SCSIO ZS0073, a marine actinomycete isolated from the red sand park of Guangxi
(China), was used to identify the actinomycin D gene cluster [27]. The strain and mutants were grown
at 30 °C on ISP-2 medium (with 3% sea salt added) for cultivation. *E. coli* was used for DNA cloning
and sequencing. *E. coli* ET12567/pUZ8002, which is methylation deficient, was employed as the donor
cell for conjugal transfer of DNA into *S. costaricanus* SCSIO ZS0073. All *E. coli* strains were grown
in liquid lysogeny broth (LB) at 37 °C or 30 °C and 200 rpm. When used, antibiotics were added
at the following concentrations: chloramphenicol (Chl, 25 µg mL⁻¹), apramycin (Apr, 50 µg mL⁻¹),
kanamycin (Kan, 50 µg mL⁻¹), ampicillin (Amp, 100 µg mL⁻¹). The plasmids SuperCos 1 and pIJ773
were used for *S. costaricanus* SCSIO ZS0073 genomic library construction and the *aac(3)IV-oriT*
resistance gene amplifying, respectively.

3.3. Whole Genome Scanning and Sequence Analysis

*S. costaricanus* SCSIO ZS0073 genomic DNA was isolated according to the protocol with slight
modification [43]. Whole-genome scanning was achieved using both 454 and Solexa technology at
Macrogen (Seoul, Korea). Assembly, annotation and bioinformatics analyses allowed us to define
the correct contiguous fragments corresponding to the acn cluster. Assignments of ORFs and their
functional predictions were accomplished using FramePlot 4.0 (http://nocardia.bih.go.jp/fp4) and Blast (http://blast.ncbi.nlm.nih.gov/) software packages.

3.4. Genomic Library Construction and Screening

Genomic DNA was partially digested with Sau3AI and 30–40 kb fragments were ligated into XbaI/BamHI digested and dephosphorylated SuperCos 1. The resulting ligation mixture was packaged with Gigapack III gold and transduced into E. coli LE392 to generate the genomic library, according to the manufacturer instructions.

3.5. Inactivation of S. costaricanus SCSIO ZS0073 by λ-RED-Mediated PCR-Targeting Mutagenesis

Targeted genes in the S. costaricanus SCSIO ZS0073 biosynthetic gene cluster were inactivated using λ-mediated PCR-targeting methodology [44]. Two cosmids were used to disrupt target genes. An apramycin resistance cassette aac(3)IV-oriT fragment obtained by PCR (digested pIJ773 was used as template), with primer pairs containing 39-nucleotide extensions derived from the 5′- and 3′-ends of the targeted genes, was used to replace an internal region of each of the targeted genes. Each of the mutated genes was verified by PCR with primers designed to be 10–300 bp outside of the disruption region, with verification by restriction enzyme digestion. The constructed mutated cosmids were introduced into non-methylating E. coli ET12567/pUZ8002 for conjugal transfer. For conjugation, harvested S. costaricanus SCSIO ZS0073 spores were suspended in TSB medium and incubated for 3–5 h at 28 °C and 200 rpm after heating for 10 min at 50 °C. The culture was then centrifuged to harvest the germinated spores as the conjugation recipients. At the same time, LB supplied with Kan (50 µg mL⁻¹), Chl (25 µg mL⁻¹) and Apr (100 µg mL⁻¹) was inoculated with E. coli ET12567/pUZ8002 containing each mutated cosmid. After the culture OD₆₀₀ increased to 0.6–0.8, the cells were harvested, washed three times with LB, resuspended in 500 µL LB medium and mixed with the previously germinated spores. The mixture was plated on modified ISP-4 medium containing MgCl₂ (60 mM). The plates were incubated at 28 °C for an additional 6–7 days until exconjugants appeared. Double-crossover mutants were primarily selected on the basis of Kan<sup>S</sup>Apr<sup>R</sup> phenotypes and the desired double crossover mutants were further verified by PCR with primers listed in Table S3 (SI).

3.6. Fermentation and Analysis of S. costaricanus SCSIO ZS0073 WT and Mutant Strains

S. costaricanus SCSIO ZS0073 WT and mutant strains were inoculated into 250 mL flasks with 50 mL liquid ISP-2 medium and incubated on a rotary shaker at 28 °C, 200 rpm. After 7 days fermentation, each of the 50 mL cultures was added to 100 mL butanone and then vigorously mixed for 30 min. The butanone phase was separated and then evaporated to dryness to yield a residue. The residue was dissolved in 2 mL methanol and centrifuged and the resulting supernatant then subjected to HPLC analysis. Analytical HPLC was performed on an Agilent 1260 HPLC system (Agilent Technologies Inc., USA) equipped with a binary pump and a diode array detector using a Phenomenex Prodigy ODS column (150 × 4.60 mm, 5 µm) with UV detection at 254 nm. The mobile phase was comprised of solvents A and B. Solvent A consisted of 15% CH₃CN in water supplemented with 0.1% TFA whereas solvent B consisted of 85% CH₃CN in water supplemented with 0.1% TFA. Samples were eluted with a linear gradient from 5% to 90% solvent B in 20 min, followed by 9–100% solvent B for 5 min, then eluted with 100% solvent B for 3 min, at a flow rate of 1 mL/min and UV detection at 254 nm. LC-ESI-MS data were obtained using an amaZon SL ion trap mass spectrometer (Bruker, Billerica, MA, USA). The mobile phase comprises solvents A and B. Solvent A consists of 100% ddH₂O supplemented with 0.1% methanoic acid whereas solvent B consists of 100% CH₃CN supplemented with 0.1% methanoic acid. Samples were eluted with a linear gradient from 5 % to 90% solvent B in 20 min, followed by 9–100% solvent B for 5 min, then eluted with 100% solvent B for 3 min, at a flow rate of 1 mL/min and UV detection at 254 nm.
4. Conclusions

Actinomycin D is a vital antibiotic in the treatment of Wilms’ tumor, trophoblastic tumors and rhabdomyosarcoma. Here, we have identified and characterized a new actinomycin D BGC (acn) in marine derived producer S. costaricanus SCSIO ZS0073 by carrying out whole genome sequencing and systematic gene disruptions. Relative to the gene cluster for actinomycin of S. chrysomallus and S. antibioticus IMRU3720, the size of the acn cluster in S. costaricanus SCSIO ZS0073 is smaller. In silico analysis, gene inactivation and metabolomics data enabled us to deduce the biosynthetic pathway leading to actinomycin D in S. costaricanus SCSIO ZS0073. We furthermore determined the acn cluster boundaries and elucidated the functions of positive regulatory genes acnWU4RO along with the cytochrome P450 gene acnP which is responsible for installing the 4-oxoproline seen in actinomycin Xβ. The discovery of this new actinomycin BGC advances initiatives to engineer new actinomycin D analogues for clinical use and to explore the still elusive mechanism of actinomycin 4-MHA-pentapeptide monomer dimerization en route to intact actinomycins.

Supplementary Materials: The following are available online at http://www.mdpi.com/1660-3397/17/4/240/s1, Figures S1–S28. Disruption genes in actinomycin biosynthetic gene cluster in WT S. costaricanus SCSIO ZS0073 via PCR-targeting. Table S1, Structure of actinomycins; Table S2, Strains and plasmids used in this study.

Author Contributions: J.J. designed experiments. M.L. and Y.J. performed the experiments. Y.X., Z.C., J.M., C.S. and J.J. analyzed data. M.L. and J.J. wrote the manuscript. All authors read and approved the final manuscript.

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References


