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Short communication

Identification and heterologous expression of the biosynthetic gene cluster for holomycin produced by *Streptomyces clavuligerus*

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ABSTRACT

Holomycin is a dithiolopyrrolone antibiotic natural product produced by *Streptomyces clavuligerus*, ATCC 27064. This paper reports on the identification of a gene cluster from *S. clavuligerus* that directs holomycin biosynthesis. Heterologous expression of the cluster in *S. albus* then induced the production of holomycin. Bioinformatics analysis of the gene cluster revealed that holomycin was assembled by a single multidomain non-ribosomal peptide synthetase (NRPS) consisting of heterocyclization, adenylation, and thiolation domains (Cy-A-T), a free-standing condensation domain, two thioesterases, five tailoring enzymes involved in oxidative reactions and two regulatory and transcriptional genes. Knock out of the gene, HomI, completely abolished the production of holomycin, which is consistent with its role as a key biosynthetic gene and is likely involved in the formation of the disulfide bond generating the final precursor, holothin.

1. Introduction

Dithiolopyrroloness are a class of antibiotics that possess the unique pyrrolinonodithiole (4H-[1,2] dithiolo [4,3-b] pyrrol-5-one) skeleton linked to two variable groups, R1 and R2 (Fig. 1). The structural class includes the bioactive natural products, holomycin (**1**) [1], thiolutin (**2**) [2], and aureothricin (**3**) [3], which are products of various strains of *streptomyces*. The class also includes the more recently identified, thiomarinol (**4**) [4], which has been isolated from a marine bacterium. Generally, dithiolopyrrolone antibiotics have broad-spectrum antibacterial activity against various microorganisms, including Gram-positive and Gram-negative bacteria, and even parasites. It has been proposed that **1** is a prodrug, requiring intracellular conversion into an active species, which then inhibits RNA polymerase [5]. Moreover, some dithiolopyrrolone derivatives have demonstrated promising antitumor activities [6].

Despite the emerging importance of dithiolopyrrolones, the understanding of their biosynthesis is limited. **1** was isolated from various *streptomyces* strains, including an important industrial strain, *Streptomyces clavuligerus*, ATCC 27064. *S. clavuligerus* is renowned for its capacity to produce an array of natural products with a large degree of structural diversity, including three clinically important antibiotics, the β-lactam, cephamycin C (**5**), the β-lactamase inhibitor clavulanic acid (**6**), and **1**. Most recently, full genome sequence analysis of *S. clavuligerus* revealed novel features compared to other *streptomyces* strains [7]. First, the *S. clavuligerus* genome has a relatively small chromosome of 6.8 Mb in length; however, it possesses a megaplasmid, pSCL4, of 1.8 Mb in length, which is by far the largest linear megaplasmid sequenced. Second, 48 putative secondary metabolite gene clusters in *S. clavuligerus* have been identified by a homology comparison to known sequence data, which is an unprecedented number in any bacterium. Twenty-three of these gene clusters are on the chromosome, and 25 are on the megaplasmid. It has therefore been proposed that secondary metabolite production in *S. clavuligerus* is due to cross-regulation between the megaplasmid and the chromosome. Finally, only three gene clusters encoding known antibiotics were unambiguously identified within the genome assembly, including **5** and **6**. Approaches allowing the characterization of the rest of the gene clusters will undoubtedly hold potential for uncovering biosynthetic pathways for other known or unidentified metabolites from *S. clavuligerus*. Due to its potential use as a clinical antibiotic, and in

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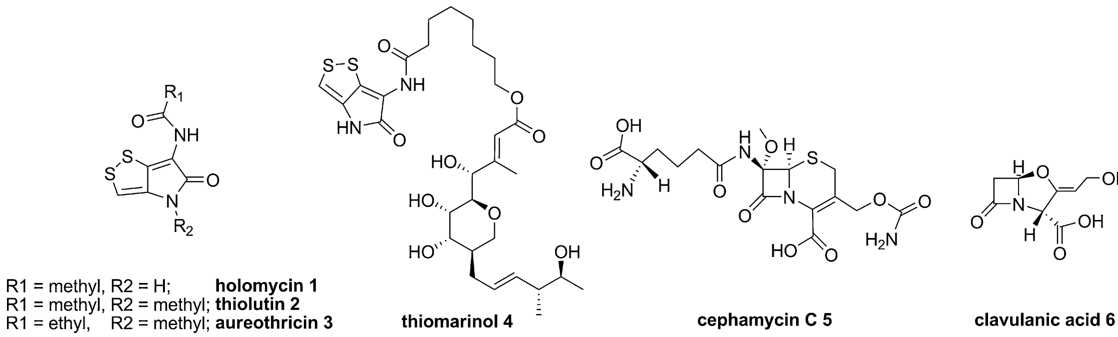

Fig. 1. Structures of dithiolopyrrolones and natural products isolated from *S. clavuligerus*.

light of a dearth of biosynthetic knowledge, we set out to identify the biosynthetic gene cluster for **1**. Herein, we report that heterologous expression of a previously uncharacterized non-ribosomal peptide synthetase (NRPS) gene cluster, SMC18, from *S. clavuligerus* led to good production of **1** in *S. albus*. Bioinformatic analysis of the gene cluster and gene disruption experiments suggested unique biosynthesis for the dithiopyrrolones.

2. Materials and methods

2.1. Genomic library construction and screening

A genomic library of *S. clavuligerus*, ATCC 27064, was constructed in pJTU2554 according to the previous protocol [8]. *E. coli* LE392 and the Packagene Lambda DNA Packaging System (Promega Corporation, Madison, WI USA) were used for library construction following the manufacturer's instructions. The genomic library (1×10^4) was screened by PCR using primers homI-int-F (5′-CGG GAA TTC GGC GGA GTT CAT ACG GGG TGA GGT CG-3′, EcoRI site underlined) and homI-int-R (5′-CGT AAG CTT CGA CCA GTT GGT GGT TGA GCG CAA ACG C-3′, HindIII site underlined) for the identification of SCLAV_5275.

2.2. Sequence analysis

The open reading frames (ORFs) were deduced from the sequence by performing FramePlot 4.0 beta program (http://nocardia.nih.go.jp/fp4/). The corresponding deduced proteins were compared with other known proteins in the databases using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/). Amino acid sequence alignments were performed with the CLUSTALW algorithm from BIOLOGYWORKBENCH 3.2 software (http://workbench.sdsc.edu). The prediction of the amino acid specificities of the domain and the analysis of the conserved motifs in the C domain and TE were performed by using the BLAST server provided at http://www.nii.res.in/searchall.html.

2.3. HomI inactivation in holomycin high-yield strain HZ01

To inactivate *homI*, a 680 bp internal fragment of *homI* was amplified from pMSB101 using the primers homI-int-F and homI-int-R (see above). The PCR product was digested with HindIII and EcoRI, then cloned into the same sites on pKC1139, yielding the gene disruption plasmid, pMSB106. We use conjugation transfer to introduce pMSB106 into HZ01. The colonies that were apramycin-resistant at 37 °C were identified as the recombinant strain, HZ02, whose genotype was confirmed by Southern hybridization (data not shown).

2.4. Fermentation of S. clavuligerus and S. albus

Wild type and mutant *S. clavuligerus* were grown for sporulation on ISP medium number 3 (Difco, Detroit, MI), while *S. albus* and recombinant strains were propagated on SFM medium (mannitol 2%, soybean meal 2%, agar 2%, pH 7.2).

To produce **1**, 100 μL of *S. clavuligerus* or mutant strains spores was inoculated into 50 mL of seed medium (Trypticasein soy broth, TSB), and was incubated at 28 °C, shaking at 220 rpm for 48 h. Five milliliters of seed culture was then transferred into 100 mL of SA medium [9] and was grown at 28 °C, shaking at 220 rpm for 4 days. The fermentation conditions for *S. albus* and recombinant stains were the same as *S. clavuligerus*, except that the second phase fermentation medium is semi-synthetic [10].

2.5. Identification of holomycin

To isolate **1**, the fermentation broth was centrifuged (6000 rpm, 10 min), and the supernatant was extracted twice with ethyl acetate. The organic phase was collected and dried with anhydrous sodium sulfate. The extracted product was desiccated under vacuum, and dissolved in methanol for further analysis.

HPLC analysis of the extract samples was performed on a Diamonsil C18 column (4.6 mm × 250 mm, Dikma Technologies, Beijing, China), which was equilibrated with 90% solvent A (H_2O) and 10% B (CH_3CN) and was developed with the following program: 5 min, linear gradient from 90% A/10% B to 70% A/30% B; 20 min, linear gradient from 70% A/30% B to 100% B; and 3 min, linear gradient from 100% B to 90% A/10% B, using a 0.8 mL min^{-1} flow rate and a 390 nm UV detection wavelength.

The identities of dithiopyrrolones were confirmed by HPLC–ESIMS analysis, using Waters 1525 (Waters Corporation) for HPLC and Thermo Fisher LTQ Fleet (Thermo Fisher Scientific Inc.) for ESI-MS, performed under the same conditions.

2.6. High resolution LC–ESIMS analysis

High-resolution mass spectral data were obtained from a Thermo Instruments MS system (LTQ XL/LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler and Accela Pump). The following conditions were used: 45 V capillary voltage, 260 °C capillary temperature, auxiliary gas flow rate set at 10–20 arbitrary units, sheath gas flow rate set at 40–50 arbitrary units, 4.5 kV spray voltage, and 100–2000 amu mass range (maximum resolution 30,000). HPLC separations were carried out using a Phenomenex reversed-phase column (C18, 250 mm × 10 mm, L × i.d.) connected to an Agilent 1100 series binary pump and monitored using an Agilent photodiode array detector.

3. Results and discussion

3.1. Construction of S. clavuligerus HZ01 for the high-production of holomycin

For isolation of **1,** an *S. clavuligerus* mutant, HZ01, was constructed, using a previously described method [11]. Construction involved in-frame deletion of the *orf15* gene associated with the biosynthetic pathway of **6** on the chromosome (see Supplemental data). Disruption successfully abolished the production of **5** and **6** and resulted in good production of **1**. Unambiguous identification of **1** produced by the mutant strain HZ01 was verified by LC–HR-ESIMS (Fig. 2A and E).

3.2. Identification of the gene cluster responsible for holomycin production

It is rare for molecular scaffolds of bacterial natural products to contain disulfide bonds, and the mechanism of disulfide bond formation in these products is poorly understood. A recent study indicated that DepH, a FAD-dependent oxidoreductase, is involved in disulfide bond formation in FK228, a disulfide-containing natural product produced by the soil bacterium, *Chromobacterium violaceum*, number 968 [12]. DepH represents a new subclass of the thioredoxin protein superfamily and contains a signature redox motif, CPYC, a conserved FAD-binding domain, and an $NADP^+$/NADPH-binding domain.

Interestingly, a BLAST search of the *S. clavuligerus* genome database indicated that one gene, SCLAV_5275 (designated as *homI* later), which was annotated as a putative thioredoxin reductase, possesses 36% identity and 55% similarity to DepH. This gene is