Identification of an unusual type II thioesterase in the dithiolopyrrolone antibiotics biosynthetic pathway

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1. Introduction

Dithiolopyrrolone group antibiotics are a class of broad-spectrum antibacterial natural products characterized by an electronically unique dithiolopyrrolone heterocyclic core [1,2]. Since the first member of this class, aureothricin [3], was reported in the late 1940s, more than 30 naturally occurring dithiolopyrrolone compounds have been isolated from diverse soil- and marine-derived bacteria [2]. Various substituents at the two amide nitrogens lead to structural diversity of the compound class (Fig. 1). Either unsubstituted or N-methylated form appeared at the exo-cyclic pyrrolone nitrogen (R2), whereas varying length acyl chains of the exo-cyclic amide (R1) have been found, even with branching, unsaturation and benzylolation. In general, dithiolopyrrolones act as transcription inhibitors [4] to display relatively broad-spectrum antibiotic activity against a variety of Gram-negative and Gram-positive bacteria, fungi and even parasites [5–7]. In the last two decades, discovery of their antitumor properties by inhibition of endothelial cell adhesion, antiangiogenesis and antimitosis [8,9], has renewed interest in this structurally intriguing class of compounds.

Holomycin (desmethyl-acetyl-pyrrothine) [10], thiolutin (acetyl-pyrrothine) [11] and more recently identified marine antibiotic thiomarinol (desmethyl-pyrrothine linked with pseudomonic acid moiety by an amide bond) [12] are the best-known representatives of dithiolopyrrolones. The biosynthetic gene cluster for holomycin (hlm) was first identified through genome mining of the producing bacterium Streptomyces clavuligerus [13,14]. A minimum set of the cluster has been determined by heterologous expression and successful production of holomycin in different hosts, such as Streptomyces abulus [13], Streptomyces coelicolor [15] and Streptomyces avermitilis [16], in which 13 protein-coding genes hlmA-M are located. It contains an acyltransferase HlmA, a globin HlmG, four flavin-dependent oxidoreductases HlmB, D, F, I and four non-ribosomal peptide synthetase (NRPS)-related enzymes including a modular NRPS HlmE with three non-canononical domains (cyclization (Cy), adenylation (A) and thiolation (T)

Abstract

Dithiolopyrrolone group antibiotics characterized by an electronically unique dithiolopyrrolone heterocyclic core are known for their antibacterial, antifungal, insecticidal and antitumor activities. Recently the biosynthetic gene clusters for two dithiolopyrrolone compounds, holomycin and thiomarinol, have been identified respectively in different bacterial species. Here, we report a novel dithiolopyrrolone biosynthetic gene cluster (aut) isolated from Streptomyces thioluteus DSM 40027 which produces two pyrothine derivatives, aureothricin and thiolutin. By comparison with other characterized dithiolopyrrolone clusters, eight genes in the aut cluster were verified to be responsible for the assembly of dithiolopyrrolone core. The aut cluster was further confirmed by heterologous expression and in-frame gene deletion experiments. Intriguingly, we found that the heterogenetic thioesterase HlmK derived from the holomycin (hlm) gene cluster in Streptomyces clavuligerus significantly improved heterologous biosynthesis of dithiolopyrrolones in Streptomyces alb by coexpression with the aut cluster. In the previous studies, HlmK was considered invalid because it has a Ser to Gly point mutation within the canonical Ser-His-Asp catalytic triad of thioesterases. However, gene inactivation and complementation experiments in our study unequivocally demonstrated that HlmK is an active distinctive type II thioesterase that plays a beneficial role in dithiolopyrrolone biosynthesis.

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domains), a freestanding condensation (C) domain HlmL and two standalone thioesterase (TE) domains, HlmC and HlmK. Thereinto, hlmK was believed to be an invalid gene based on a key amino acid residue mutation at the conserved catalytic active sites of thioesterases [1,13]. It has been proposed that holomycin is a prodrug which requires enzymatic or chemical reduction into its active dithiol form to produce biological effects in the cell [7]. The thio-redoxin reductase-like enzyme HlmI was discovered to play an important role in self-protection of the producing strains by oxidizing the reduced dithiol form of holomycin to the disulfide form [17,18]. Whereafter, two other holomycin biosynthetic gene clusters have also been found in the genome sequences of fish pathogen Yersinia ruckeri [19] and marine bacterium Photobacterium halotolerans [20]. The analogs of HlmI, L, K, however, are missing in these gene clusters, suggesting that they are not essential for holomycin biosynthesis or alternative mechanisms exist. For instance, instead of HlmI, a RNA methyltransferase Hom12 is recruited to methylate RNA for preventing inhibition by holomycin in Y. ruckeri [19]. So far the detailed biosynthetic pathway of the dithiolopyrrolone heterobicyclic core is yet to be deciphered experimentally, and moreover, there are a lot of unsolved problems on the complex regulation network and multifarious self-resistance modes of this family of natural products left.

In this paper, we report: (i) cloning and sequence analyses of a new dithiolopyrrolone biosynthetic gene cluster (aut) from Streptomyces thioluteus DSM 40027 [21] which produces two pyrrothine derivatives, aureothricin (4) and thiolutin (3); (ii) identification of the aut cluster by heterologous expression and in-frame gene deletion experiments; (iii) significant improvement of heterologous biosynthesis of dithiolopyrrolones through coexpression of the aut cluster and the heterogenetic thioesterase HlmK derived from the hlm cluster; (iv) identification of HlmK as an active type II thioesterase of the unexpected.

2. Materials and methods

See Supplementary data for details.

3. Results

3.1. Cloning and sequence analyses of the aureothricin biosynthetic gene cluster

Since genomes of Streptomyces strains usually carry a lot of NRPS and polyketide synthase (PKS) gene clusters for secondary metabolism, we used two different pairs of primers to probe the putative aureothricin biosynthetic gene cluster from S. thioluteus DSM 40027. Besides of a pair of NRPS-specific primers, a pair of degenerated primers was designed based on universally conserved thioredoxin reductase motifs. A single PCR product of ca. 300 bp was amplified from the genomic DNA of S. thioluteus and proved to be a homolog of hlmI by subcloning and sequence analysis. Subsequently, a pair of specific primers targeting the identified thio-redoxin reductase gene region was designed for PCR-based screening of the S. thioluteus genomic library, and then 7 positive cosmids were selected out. By PCR with the NRPS-specific primers, 5 of them were demonstrated to possess a modular NRPS gene as well as the thioredoxin reductase gene, which indicated that these five cosmids may harbor the putative aureothricin (aut) biosynthetic gene cluster. On the basis of cosmid mapping and sequencing results, the cosmid 18D3 which contains the biggest DNA insert region was subjected to both directed and shotgun subcloning, sequencing and assembling. This allowed us to define a 22 kb of DNA region consisting of 18 open reading frames (ORFs) whose deduced products are likely involved in dithiolopyrrolone biosynthesis. The gene organization of the aut cluster (GenBank accession number KU753007) is graphically presented in Fig. 2, and sequence similarity and deduced function of each gene are

![Dithiolopyrrolone compounds isolated from different bacteria.](image)
summarized in Table S2.

Bioinformatic analyses revealed that eight ORFs of the aut cluster show high sequence homology to those in the hlm cluster from *S. clavuligerus*, including four flavin-dependent oxidoreductases AutB, D, F, I, one acetyltransferase AutA, one globin AutG, one thioesterase AutC and one multidomain NRPS AutE. Their homologs were identified in other dithiolopyrrolone biosynthetic pathways as well (Fig. 2), which indicated that they are responsible for the assembly of dithiolopyrrolone core.

In the upstream region of aut, four putative regulatory genes, *orf1, 3, 4, 5*, were readily discerned. Orf1 shows high homology to the PCS-14 regulatory protein in the planosporicin lantibiotic biosynthetic gene cluster of *Planomonospora alba* [22]. It has a cyclic nucleotide-binding domain, characteristic of the Crp/Fnr family transcriptional regulators. The best studied member of this family is the prokaryotic catabolite gene activator Crp, also named as cAMP receptor protein. Orf3 presumably encodes a RNA polymerase sigma factor because the deduced 300 aa gene product contains the highly conserved sigma-70 region 2 and 4. The protein Orf4 belongs to NmrA family transcriptional regulators, which contains a NAD(P)H-binding signature and has significant sequence homology to the FlsQ1 regulatory protein in the fluostatin gene cluster from the marine-derived *Micromonospora rosaria* SCSIO N160 [23]. Orf5 contains an N-terminal XRE-family helix-turn-helix (HTH) DNA binding motif and a C-terminal cupin superfamily domain, which was annotated as an analog of the DNA-binding transcriptional repressor PuuR. All of these four ORFs seem likely to be involved in transcriptional regulation, but their exact roles in aureothricin biosynthetic pathway remain obscure.

In the downstream region of aut, there are three putative NAD- or NADP-dependent oxidoreductase genes. Orf8 encodes an alcohol dehydrogenase or a quinone reductase, which usually catalyzes the interconversion of alcohols and aldehydes, or ketones, using NAD(P) as a cofactor. Both gene products of orf9 and orf10 belong to the classical short-chain dehydrogenases/oxidases (SDR) family of oxidoreductases. Members of this family mainly catalyze a wide range of activities including the metabolism of steroids, cofactors, carbohydrates, lipids, aromatic compounds and amino acids, and act in redox sensing. Three oxidoreductase genes possibly transcribed together were identified only in the aut cluster, thus whether they are involved in aureothricin biosynthesis needs further investigation.

### 3.2. Heterologous expression of the aut cluster

Because all attempts to introduce foreign DNA into the aureothricin producing strain *S. thiolutes* DSM 40027 failed, the cosmid 18D3 which carries the aut cluster was introduced into *Streptomyces albus* by intergeneric conjugation for heterologous expression, and the empty cosmid vector pTU2554 (as negative control) and the cosmid 10A3 which harbors the entire holomycin biosynthetic gene cluster from *S. clavuligerus* (as positive control) likewise. The resulting recombinant strains were cultivated for 3 days and then subjected to secondary metabolites detection and analysis. HPLC and LC-MS results revealed that characteristic signals of dithiolopyrrolone compounds appeared in the extract of *S. albus*::18D3, but the low production rate were very depressing (Fig. 3a). Conversely, the positive control strain *S. albus*::10A3 produced holomycin at a titer comparable with the wild type while no dithiolopyrrolone was detected in the negative control strain *S. albus*::pTU2554, which reconfirmed that 13 genes of hlmA-M are sufficient for high yield production of dithiolopyrrolone core [16]. Comparison with the hlm cluster, we found the absence of the hlmL and hlmK homologs in the aut cluster (Fig. 2). Prior to detection of transcription difference between two gene clusters, hlmL and hlmK were respectively cloned into the *Streptomyces* self-replicative vector pWHM4s under the control of a constitutive promoter PermB and introduced into the heterologous expression strain *S. albus*::18D3 for coexpression. Surprisingly, HlmK, rather than HlmL, greatly enhanced the production rate of dithiolopyrrolone compounds in *S. albus*::18D3 (Fig. 3a, c) so that two products were readily isolated and identified as holomycin (1) (Fig. S11) and N-propionylholothin (2) (Fig. S12), which are the desmethyl derivatives of two dithiolopyrrolone products (aureothricin (4) and thiolutin (3)) isolated from the wild-type strain, respectively.
3.3. Identification of the aut cluster by in-frame gene deletion

In order to further verify the involvement of the aut cluster in aureothricin biosynthesis, the key catalytic enzyme gene autE (multidomain NRPS) was in-frame deleted from the cosmid 18D3 via λ-RED mediated recombination technology [24]. The resulting cosmid 18D3ΔautE was introduced into S. albus/pWHM4s-hlmK for coexpression with hlmK. The heterologous expression mutant strain S. albus::18D3ΔautE+hlmK was unable to produce dithiolopyrrolones (Fig. S9). In-frame deletions of the thioredoxin reductase gene autI and the phosphopantothenoylcysteine decarboxylase gene autF, likewise, abolished the production of dithiolopyrrolone compounds. In contrast, the mutant S. albus::18D3Δorf10+hlmK displayed the same production profile as the high yield strain S. albus::18D3+hlmK, excluding the essential role of orf10 in dithiolopyrrolone core biosynthesis. A thiol reductase thioredoxin coding gene orf6 located between autI and autG was also excluded by using the same strategy (Fig. S9). Considering that no other similar gene cluster was found in the genome sequence of S. thioluteus, the identified aut cluster has been proved to be the only candidate responsible for aureothricin biosynthesis.

3.4. Heterologous expression of two methyltransferase genes in the holomycin producing strain S. clavuligerus

On the basis of substituent species at the endo-cyclic pyrrolone nitrogen (R2), all dithiolopyrrolone compounds could be classified into two types: holomycin-type and thiolutin-type. N$_4$-methylation was supposed to be the last step in the biosynthetic process of thiolutin-type dithiolopyrrolones [25]. Although two SAM-dependent methyltransferase coding genes, orf2 and orf7, were found in the aut cluster, only unexpected desmethyl form of dithiolopyrrolones (holomycin-type) were detected in heterologous expression of the cluster. In order to investigate their biological functions, orf2 and orf7 were respectively cloned into the integrative vector pWS052 and located downstream of the promoter P$_{ermE}$. The constructed plasmids were transferred into the holomycin producing strain S. clavuligerus to test if they could catalyze the N$_4$-methylation conversion of holomycin into thiolutin. Product analysis showed that neither heterogenous methyltransferases could induce the production of methylated dithiolopyrrolone compounds (Fig. S10), suggesting that they are not involved in N$_4$-methylation of thiolutin-type dithiolopyrrolones.

3.5. Identification of HlmK as an active type II thioesterase

There are two thioesterase genes, hlmC and hlmK, identified in the hlm cluster, but only HlmC was proposed to be involved in holomycin biosynthesis. The hlmK gene was generally believed to encode for an inactive enzyme with an aberrant catalytic triad [2,13]. To our surprise, expression of the heterogenous hlmK gene in the heterologous expression strain of the aut cluster significantly stimulated high-yield production of dithiolopyrrolones. It suggested that HlmK is actually active and plays an important role in the biosynthesis of two products. Therefore, the re-sequencing work of hlmK was conducted. The entire encoding region of the gene was amplified by PCR respectively from the cosmid 10A3 and the genomic DNA of the wild type producing strain S. clavuligerus, and then submitted for sequencing. The recaptured nucleotide sequence is identical to that shown in the NCBI database, which demonstrated that a point mutation at the most crucial active site...
of thioesterases indeed occurred. Whereafter, *hlmK* was in-frame eliminated from the cosmid 10A3 and the recombinant cosmid 10A3*delhlmK* was introduced into *S. albus* to observe its function in holomycin biosynthesis. We found that the mutant strain *S. albus::10A3delhlmK* still produced holomycin but the yield significantly decreased 97% compared with that of the entire *hlm* cluster expression strain *S. albus::10A3* (Fig. 3b). When a copy of *hlmK* was complemented into the deletion mutant, production of holomycin in the complementation strain *S. albus::10A3delhlmK+delhlmK* was restored in part. In contrast, inactivation of the other thioesterase gene *hlmC* completely abolished dithiolopyrrolones production, as did *autC*, the analogous gene of *hlmC* in the *aut* cluster (Fig. 3a). Obviously, *HlmC* as well as *AutC* is the essential offloading enzyme in dithiolopyrrolone biosynthesis while *HlmK* serves as an active type II thioesterase that improves heterologous biosynthesis of dithiolopyrrolones in *S. albus*.

4. Discussion

Since the first cluster was reported in 2010, more than four dithiolopyrrolone biosynthetic clusters have already been discovered by mining of sequenced genomes [26,27]. It is a coincidence...
that all of them are responsible for the biosynthesis of holomycin-type compounds. Since thiolutin-type dithiolopyrrolones display better biological activities, we cloned the biosynthetic gene cluster of aureothricin, which is the first identified thiolutin-type compound, from \textit{S. thioluteus} DSM 40027. While the work was progressing, Yu et al. has reported a putative biosynthetic cluster (\textit{dtp}) for thiolutin from \textit{Saccharothrix algeriensis} NRRL B-24137 in 2015 [25]. Heterologous expression of the \textit{dtp} cluster in \textit{S. albus} only led to production of holomycin. Similarly, two holomycin-type products, rather than thiolutin-type compounds, were found in heterologous expression of the \textit{aut} cluster. Although there are methyltrasferase genes located in both clusters, their expression experiments demonstrated that they could not induce conversion of holomycin-type dithiolopyrrolone into its thiolutin-type form, which implied that the genes required for N\_methylthiolopyrrolones might be located outside of these two clusters.

The heterologous expression strain \textit{S. albus}::18D3+HlmK produced not only holomycin but also \textit{N-propionylholothin}, corresponding with fermentation result of two dithiolopyrrolones production in the wild type strain. It suggested that the substrate specificity of the acyltransferase AutA differs from that of HlmA [14] and its analogs in other holomycin gene clusters. The optimum substrate for AutA is propionyl-CoA, instead of acetyl-CoA, due to much more aureothricin than thiolutin produced by the wild type strain \textit{S. thioluteus}. The experimental evidences will be supplied by genetic and biochemical studies in our future plan.

Thioesterases are frequently involved in the biosynthesis of fatty acids, polyketides and nonribosomal peptides. A common feature in biosynthetic processes of these three families of compounds is the covalent attachment of the growing compound to a carrier protein through a thioester bond. Cleavage of completed acyl or peptide chains from the enzymatic templates is catalyzed by the well-studied type I thioesterases (TEIs), which are typically found at the C-terminus of modular PKSs and NRPSs as essential biosynthetic enzymes [28]. On the contrary, the external stand-alone type II thioesterases (TEIIs) have been proved to be nonessential, although they are important for efficient biosynthesis of the final products [29]. We found that AutC and HlmC had the typical conserved Ser-His-Asp catalytic triad in the alignment with sequences of other thioesterases. Genetic studies revealed the essential roles of HlmC and AutC in dithiolopyrrolone biosynthesis, so we proposed that they act as TEIs to release synthesized products from the NRPSs by hydrolytic reaction. Interestingly, phylogenetic analysis revealed that HlmC and its analogs (blue area in Fig. 4) cluster away from canonical type I (green area) and type II (yellow area) thioesterases. As the freestanding essential offloading enzymes, HlmC and AutC are genetically distinct from other typical integrated TEIs. They seem more closely related to TEIIs, but their biosynthetic roles and hydrolase signature sequence (GHSFG) are different from those of characterized TEIIs (GHSMG) [30], suggesting that they might represent a new type of thioesterases.

TEIs play a corrective (editing) role by removal of misloaded substrates and aberrant intermediates from carrier protein and render the assembly lines active again. It has been reported that disruption of the corresponding TEI genes in the producer strains led to 80–90% reduction of products [29]. Coexpression of native or heterologous TEIs increased the production level of final compounds. For example, Li et al. recently reported that the cognate TEII improved heterologous biosynthesis of the nonribosomal peptide antibiotic valinomycin in \textit{Escherichia coli} through coexpression with valinomycin synthetase [31]. Now similar result is presented in our study as well. The heterologous biosynthesis of dithiolopyrrolones was significantly enhanced by coexpression of the \textit{aut} cluster and the heterologous TEII HlmK. HlmK used to be ignored by scientists because it has a Ser to Gly point mutation within the canonical Ser-His-Asp triad. To our knowledge, the Ser is the actual catalytic site for substrate loading and release mechanisms of thioesterases, while the His\textendash Asp dyad activates the Ser site by deprotonation to increase its nucleophilicity. So far, few thioesterases have been found to deviate from the conserved Ser-His-Asp triad [28]. Thioesterases containing a Cys in place of the Ser were isolated from the pyochelin biosynthetic pathway in \textit{Pseudomonas aeruginosa} [32] and \textit{Streptomyces venezuelae} [33]. Mutation of the native Ser site to Cys in the lipopeptide antibiotic surfactin TEII also produced a catalytically active thioesterase with marginally reduced activity [34]. Here, our study demonstrated another distinctive TEII HlmK in which the catalytic site Ser is replaced by a Gyl. Six similar examples were identified in the database of GenBank (red area in Fig. 4), such as a TEII from the hybrid nonribosomal peptide-polyketide antitumor antibiotic leu- namycin gene cluster [35]. However, there is no experimental evidence to support their activities as of yet. Biochemical and structural biology studies of HlmK will not only elucidate its enzymatic characteristics but also help us for intensive understanding of detailed catalytic mechanisms of TEIIs with different active sites.

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Appendix A. Supplementary data

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Transparency document

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