Identification of Two Putative Acyltransferase Genes Potentially Implicated in Dithiolopyrrolone Biosyntheses in Saccharothrix algeriensis NRRL B-24137

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Abstract The dithiolopyrrolone class of antibiotics has been known to display bacteriostatic activity against both Gram-positive and Gram-negative bacteria and exert other biological activities. Acyltransferase activities are proposed to be responsible for the structural diversity of dithiolopyrrolones produced by *Saccharothrix algeriensis* NRRL B-24137. Moreover, two activities, pyrrothine *N*-acetyltransferase and pyrrothine *N*-benzoyltransferase, are reported to catalyze the formation, respectively, to thiolutin and benzoyl-pyrrothine (BEP) in this bacterium. In this study, two genes encoding two putative acyltransferases were identified in *S. algeriensis*. The first one, *actA*, was identified by bioinformatic analysis and by analogy to an acetyltransferase, *hlmA*, identified in holomycin biosynthetic gene cluster in *Streptomyces clavuligerus*. The second was identified by purification of both enzymes from the bacterial biomass which provided a semipurified extract. The microsequencing of tryptic peptides from the final protein preparation yielded sequences of eight different fragments, two of them encoded by one gene, *actB*, in *S. algeriensis* genome bank. The alignment of *actB* against the GenBank database revealed significant homology to acyltransferase family. Differential expression of these genes, *actA* and *actB*, was then investigated in three different media: (i) semisynthetic medium (SSM), which promotes the production of thiolutin; (ii) SSM supplemented by 1.25 mM benzoic acid (SSM + BA), which promotes the production of both thiolutin and BEP; and (iii) tryptic soy broth (TSB) in which no dithiolopyrrolone derivatives were detected.

Keywords *Saccharothrix algeriensis* · Dithiolopyrrolone · Acyltransferase · Protein purification · Gene expression

Introduction

Dithiolopyrrolones (DTPs) are members of the pyrrothine class of naturally occurring antibiotics. This class of compounds includes thiolutin (acetyl-pyrrothine), aureothricin (propionyl-

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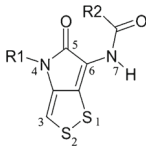
pyrrothine), isobuturyl-pyrrothine (2-methylpropanoyl-pyrrothine), butanoyl-pyrrothine (xenorhabdin VII), senecioyl-pyrrothine (3-methyl-2-butenoyl-pyrrothine), tigloyl-pyrrothine, propionyl-holothin, holomycin, xenorhabdins, xenorxides, and thiomarinols [1–6]. Dithiolopyrrolone antibiotics have strong inhibitory effects against a variety of Gram-positive and Gram-negative bacteria, yeasts, filamentous fungi, and amoeboid parasites [2, 7, 8]. Thiolutin was also reported to inhibit RNA polymerases [9–11] and the degradation of mRNA in yeast [12]. Recent studies showed that thiolutin also potently inhibits developmental angiogenesis in zebrafish and vascular outgrowth from tissue explants in 3D cultures [13]. Biological activity of dithiolopyrrolones is strongly influenced by the nature of its acyl groups [8, 14–16]. Dithiolopyrrolones have been initially isolated from the species of *Streptomyces* in the 1940s and from other microorganisms such as *Alteromonas rava*, *Xenorhabdus bovienii*, and *Xenorhabdus nematophilus* [1, 5, 17, 18]. *Saccharothrix algeriensis* NRRL B-24137 isolated from a south Algerian soil sample was found to naturally produce at least six pyrrothine derivatives characterized by their different *N*-acyl groups R_2: thiolutin (acetyl-pyrrothine), senecioyl-pyrrothine (SEP), tigloyl-pyrrothine (TIP), isobuturyl-pyrrothine (ISP), butanoyl-pyrrothine (BUP), and benzoyl-pyrrothin (BEP) [3, 8] (Fig. 1a). Precursors of pyrrothine and acyl-CoA have been successfully applied to induce the production of both dithiolopyrrolones and new dithiolopyrrolone derivatives. For example, cystine and benzoic acid increase the production of thiolutin and benzoyl-pyrrothin, respectively, while valeric acid and sorbic acid induce valeryl-pyrrothin and sorbyl-pyrrothin, respectively [19–24] (Fig. 1b).

Pyrrothine *N*-acyltransferase in *S. algeriensis* was presumed to be responsible for the transfer of the acyl groups to the pyrrothin nucleus causing the diversity of dithiolopyrrolone derivatives [25]. The study of the enzymatic activity in the cell-free extract of *S. algeriensis* grown on semisynthetic medium supplemented or not supplemented by organic acid was already investigated by Chorin et al. [25]. The results obtained in this study suggested that two different enzymes could catalyze the acylation of pyrrothine core. This hypothesis was based on the fact that the ratio of both enzymatic specific activities was not constant throughout the culture. The difference between acetic acid and benzoic acid additions to the culture medium emphasized the hypothesis; 1.25 mM of benzoic acid increased benzoyltransferase activity by 2-fold and multiplied the benzoyl-pyrrothine production by 50-fold, with no effect on pyrrothine *N*-acetyltransferase activity. In contrast, 1.25 mM of acetic acid did not affect the



a

R1=CH3

R2

CH_3	Acetyl-pyrrothine (thiolutin)
C_6H_5	Benzoyl-pyrrothine
$CH(CH_3)$	Iso-buturyl-pyrrothine
CH_2CH_2-CH_3	Butanoyl-pyrrothine
$CH=C(CH_3)_2$	Senecioyl-pyrrothine
$C(CH_3)=CH(CH3)$	Tigloyl-pyrrothine

b

R1=CH3

R2

H	Formyl-pyrrothine
CH_2-CH_3	Propionyl-pyrrothine (aureothricin)
$CH=CH(CH_3)$	Crotonyl-pyrrothine
$CH_2CH_2CH_2CH_3$	Valeryl-pyrrothine
$CH(CH_3)(CH_2CH_3)$	Isovaleryl-pyrrothine
$CH=CHCH=CH(CH_3)$	Sorbyl-pyrrothine
$CH=CHCH_2CH_2(CH_3)$	2-hexonyl-pyrrothine
$CH=C(CH_3)(CH_2CH_3)$	2-methyl-3-pentenyl-pyrrothine

R1=H

R2=C_6H_5 Demethyl-benzoyl-pyrrothine (benzoyl-holothin)

Fig. 1 Dithiolopyrrolones produced by *S. algeriensis*: **a** natural dithiolopyrrolone products; **b** unnatural dithiolopyrrolone products using precursor-directed biosynthesis ("PDB method")

production of thiolutin nor *N*-acetyltransferase [25]. Recently, holomycin biosynthetic gene cluster was identified and characterized in *Streptomyces clavuligerus* [26, 27]. An acetyltransferase was proposed to be responsible for the transfer of the acyl group to the holothin core to produce holomycin [27]. Only one acetyltransferase was found in this cluster to catalyze the formation of holomycin, which is the sole dithiolopyrrolone derivative produced in vivo by *S. clavuligerus*. However, the purified form of this enzyme was able to utilize acetyl-CoA in vitro as well as the hexanoyl-, octanoyl-, and palmitoyl-CoA as substrates to form the corresponding product [27].

The role of acyltransferase in the diversification of dithiolopyrrolone in *S. algeriensis* led us to investigate the genes encoding these acyltransferases. In this study, we have identified two putative acyltransferase genes in *S. algeriensis*. The differential expression of these genes strongly suggests a role in dithiolopyrrolone biosynthesis. Based on the hypothesis of Chorin et al. [25], we also investigated the overexpression of acyltransferase genes in response to medium supplementation.

Materials and Methods

Strain and Culture Conditions

S. algeriensis NRRL B-24137 (=DSM 44581) was used for this study. *S. algeriensis* precultures were prepared in 250 mL Erlenmeyer flasks containing 50 mL culture medium and inoculated with 2.10^7 CFU mL^{-1}. Five milliliters of *S. algeriensis* precultured for 48 h was used to inoculate 100 mL of culture medium prepared in 500 mL Erlenmeyer flasks. The preculture and the culture were prepared in the same medium.

For enzyme purification, a semisynthetic medium (SSM) [20], supplemented by 1.25 mM of benzoic acid separately autoclaved and added before inoculation, was used. *S. algeriensis* was incubated at 30 °C at 240 rpm on a rotary shaker (New Brunswick Scientific Company, NJ, USA) for 48 h.

For gene expression, three different media, SSM, SSM supplemented by 1.25 mM benzoic acid (SSM + BA), and tryptic soy broth (TSB, BD Bacto™), were used. Incubation time was 96 h.

For the effect of PDB on benzoyltransferase activity, benzoic acid, shikimic acid, cinnamic acid, L-phenylalanine, and benzoyl-CoA were separately prepared. The pH was adjusted to 7 using NaOH, and then the acids were sterilized using PVDF syringe filter (0.22 μm, 10 mm, Millipore™ Millex™) and added to the SSM medium at a final concentration of 5 mM. Benzoyl-CoA was added at a final concentration of 0.05 mM.

Cell-Free Extract Preparation (Crude Extract)

S. algeriensis was grown in SSM + BA for 48 h. The biomass was then pelleted by centrifugation at 5,000×*g* for 15 min at 4 °C (4 K15, Sigma, Osterode am Harz, Germany). The biomass was washed twice with saline solution (0.9 % NaCl) and once with buffer (Tris-HCl 50 mM, pH 8). Wet cells were finally recovered by filtration on 0.2 μm membrane filters (Advantec, Dublin, Ireland), crushed in liquid nitrogen, and stored at −80 °C. The final 15 g of frozen biomass was disrupted by a mechanical grinder for 1 min. The subsequent powder was suspended in Tris-HCl buffer at a rate of 1 mL for 0.6 g biomass. The suspension was centrifuged for 30 min at 5,000×*g* at 4 °C. The supernatant, which constitutes soluble cell-free extract of *S. algeriensis*, was used immediately for the assay of pyrrothine *N*-

acyltransferase activities. The remaining supernatant was frozen at −80 °C for further protein assays. The proteins were determined in the crude extract by the method of Bradford [28].

Dry Cell Weight, Dithiolopyrrolone, and Enzymatic Activity Determination

The dry cell weights (DCW) were determined as described by Bouras et al. [19, 20] and expressed in grams per liter. The dithiolopyrrolone and enzymatic activities were quantified as described by Chorin et al. [25]. Enzymatic activity was identified as either acetyltransferase or benzoyltransferase activity according to the acyl group donor used during the assay, i.e., acetyl-CoA and benzoyl-CoA, respectively. An enzyme unit is defined as the enzyme activity producing 1 μmol of thiolutin or BEP per minute. Specific enzymatic activity was expressed in microunits per milligram of protein.

Purification Protocol

The crude extract was successively saturated with ammonium sulfate, and five protein fractions were obtained: 0–20, 20–40, 40–60, and 60–80 % and the supernatant. The precipitates were recovered in pellet form by centrifugation for 20 min at 5,000×*g* at 4 °C. The protein pellet (one volume) was then dissolved in 5.5 mL of Tris-HCl buffer 50 mM, pH 8. Five milliliters of precipitated $(NH_4)_2SO_4$ fraction was loaded onto a hydrophobic interaction chromatography column (HiTrap Phenyl Fast Flow, 25 by 7 mm). The mobile phase consisted of two buffers: a high ionic strength buffer A of sodium phosphate 50 mM + ammonium sulfate 0.7 mM and a low ionic strength buffer B of sodium phosphate 50 mM. The pH is adjusted to 7. The proteins were eluted with 100 % of buffer A for 40 min, reaching 50 % in 20 min, then from 50 to 100 % of buffer B in 36 min, using a flow rate of 1 mL min^{-1}. The optical density (OD) at 280 nm was followed and fractions of 3 mL were collected. For the ion exchange chromatography, 3.6 mL of the desired hydrophobic interaction chromatography (HIC) fraction (named pool1) was loaded onto a column (UNOsphere Q column Bio-Rad, 40 mm by 12.6 mm). The mobile phase consisted of buffer A Tris-HCl 50 mM pH 8 and buffer B Tris-HCl 50 mM pH 8 added with NaCl 1 M. The proteins were eluted with 100 % of buffer A for 20 min, reaching 50 % in 34 min, and then from 50 to 100 % buffer B in 25 min, using a flow rate of 1.5 mL min^{-1}. The OD at 280 nm was followed and fractions of 3 mL were collected.

Native polyacrylamide gel electrophoresis (PAGE) was performed following the protocol of Ornstein and Davis [29, 30] in a Tetracell 4 (Bio-Rad). The separation gel was performed with 10 % acrylamide and a gel concentration of 3 %. The migration was carried out at 200 V for 45 min at 4 °C. Detection of proteins on the gel was achieved with Coomassie blue R-250 (detection limit ranging from 36 to 47 ng protein per band).

The enzymatic activity was measured in the native PAGE as follows: the gel was cut into six parts according to the retardation factor (Rf). Then, each gel fraction was, instead of crude extract in the enzymatic reaction, carried out in 200 μL (20 μL pyrrothine, 20 μL acyl-CoA solution with the same concentration indicated above, and 160 μL Tris-HCl buffer pH 8). The reaction mixture was incubated 15 h at 30 °C, and the dithiolopyrrolones were quantified by HPLC.

Automatic Amino Acid Sequence Determination and Localization

The protein sample (F2 obtained from native PAGE) was digested by proteolytic enzyme [31]. These peptides were analyzed by NanoLC-MS/MS using an Ultimate 3000 system (Dionex,

Amsterdam, Netherlands) coupled with an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) [32]. Amino acid sequences were compared against the *S. algeriensis* databank contigs (i.e., tblastn) and the GenBank database.

Nucleic Acid Extraction and Complementary DNA Synthesis

Total RNA was extracted from *S. algeriensis* using the RNeasy Mini Kit (Qiagen, France) with little modification. Seven hundred microliters of *S. algeriensis* homogenate cultures were mixed with the same volume of solution I without washing or centrifugation. The quality and quantity of the RNA were checked by the A_{260}/A_{280} ratio via a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and 1 % agarose gels were run to visualize the integrity of the RNA according to standard protocols [33]. Complementary DNAs (cDNAs) were obtained through reverse transcription of 2 μg of DNAse I-treated total RNA using Advantage RT-for-PCR Kit (BD Biosciences) and random hexamer primers according to the manufacturer's instructions. The cDNA products were diluted 1:10 and stored at −80 °C.

Primer Design and RT-qPCR Conditions

Primers were designed using Clone Manager 9 software (Sci-Ed Software, USA) to produce amplicons between 100 and 250 bp. Primer synthesis was performed by GeneCust (Luxembourg) (Table 1). The PCR was performed with the *Taq* DNA polymerase kit (Qiagen, France) according to the user manual provided. PCR conditions were as follows: 96 °C for 10 min (96 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s)×30 cycles followed by incubation at 72 °C for 10 min. The amplified products were examined by agarose gel electrophoresis.

The 7500 Real-Time PCR System (Applied Biosystems, USA) was used for RT-qPCR amplification and detection. Samples for RT-qPCR were prepared in 25 μL reaction mixture in MicroAmp optical 96-well reaction plates and sealed with optical adhesive covers (Applied Biosystems, Foster City, CA, USA). Each reaction contained 2 μL of template cDNA, 12.5 μL of 2× Quantitect SYBRR® Green I Mix (Qiagen, France), 1.25 μL each of the forward and reverse primers (10 μM), and 0.05 μL of ROX solution 5 μM (fermentase 50 μM). Reactions were run using the manufacturer's recommended cycling parameters of 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Negative controls (no template controls) were included in all RT-qPCR assays, and each experiment was performed in triplicate. Dissociation curves for each amplicon were then analyzed to verify the specificity of each amplification reaction; the dissociation curve was

Table 1 Oligonucleotides used for PCR and RT-PCR

Primer name	Sequence (5′–3′)	Gene	PCR product size (base pair)
actA-F	GAACACCTCGGCGGCGATCC	*actA*	94
actA-R	TCACCGGCTACGGCGTGCTG		
actB-F	CAGCACGCCGTCCCTGGTCATC	*actB*	90
actB-R	GTGCACCGGGTGGAGTGGTTGG		
FtsZ-F	CGCCGTGAACCGGATGATCG	*FtsZ*-like	94
FtsZ-R	GTCGGCGTCGGACATCAGCAG		

obtained by heating the amplicon from 60 to 95 °C. SYBR fluorescence was analyzed by StepOne software version 2.0 (Applied Biosystems). The quantification cycle (C_q, previously known as the threshold cycle C_t) was automatically determined for each reaction by the Applied Biosystems 7500 Real-Time PCR System set with default parameters, and then, the C_q value was calculated and reported using the $2^{-\Delta\Delta Cq}$ method [34]. A *FtsZ*-like gene (FtsZ is homologous to tubulin, the building block of the microtubule cytoskeleton in eukaryotes [35]) was used as the reference gene to quantify the relative expression of target genes, as it is expressed constantly throughout cellular growth.

Data Analysis and Sequence Accession Number

The protein-protein Blast (Blastp) searches were conducted at the GenBank database (http://www.NCBI.nlm.nih.gov/) using the parameters proposed by default. The multi-alignments were conducted using the site http://multalin.toulouse.inra.fr/multalin/multalin.html. For local Blast (tblastn and tblastx), BioEdit Sequence Alignment Editor Version 7.1.3.0 was used. GeneMark™ Free gene prediction software (Version 2.8) was used for contigs-gene prediction. For the functional annotation of proteins, the Conserved Domain Database (NCBI) was used. For statistical analysis, the SPSS 16.0.2 program was used. The sequence of ActA and ActB was deposited in GenBank under the accession numbers AHB86989.1 and AHA46881.1, respectively.

Results

Identification of a Putative Acyltransferase ActA

A local Blast search through BioEdit application was performed against *S. algeriensis* genome (our data) by using protein (tblastn of HlmA) or gene sequence (tblastx of *hlmA*) extracted from *S. clavuligerus* genome (NCBI). We identified an amino acid sequence sharing 51 % identity and 60 % similarity with HlmA and located in one contig whose size is 7.4 Kb. Using GeneMark™ software and *Streptomyces coelicolor* as reference, nine open reading frames (ORFs) were found in these contigs, and further, the previous amino acid sequence was found to be encoded by one ORF which was designated as *actA*. For ActA putative function, a protein-protein Blast search revealed that ActA has an acetyltransferase conserved domain with best specific hit of pfam13508, and this domain belongs to the *N*-acyltransferase superfamily. Furthermore, ActA showed low identity and similarity (about 28/30 %) to Hom3 and HolE, which code for an acetyltransferase and have been already identified in biosynthetic gene clusters of holomycin in *Yersinia ruckeri* and of pyrrothin derivatives in *Pseudoalteromonas* sp., respectively (Fig. 2).

Enzyme Purification

Ammonium sulfate precipitation of the crude extract of *S. algeriensis* yielded five fractions (Fig. 3). The higher quantity of protein was obtained in the fraction 40–60 % (31.2 mg). This fraction also presented the highest specific benzoyltransferase activity, while specific acetyltransferase activity was maximal in the fraction 60–80 %. The first purification step of benzoyltransferase activity had a maximum yield and purification factor of 69.9 % and 2.02, respectively (Table 2). The ammonium sulfate precipitation yield of acetyltransferase activity was 11.7 % with a purification factor of less than 1 (data not shown).

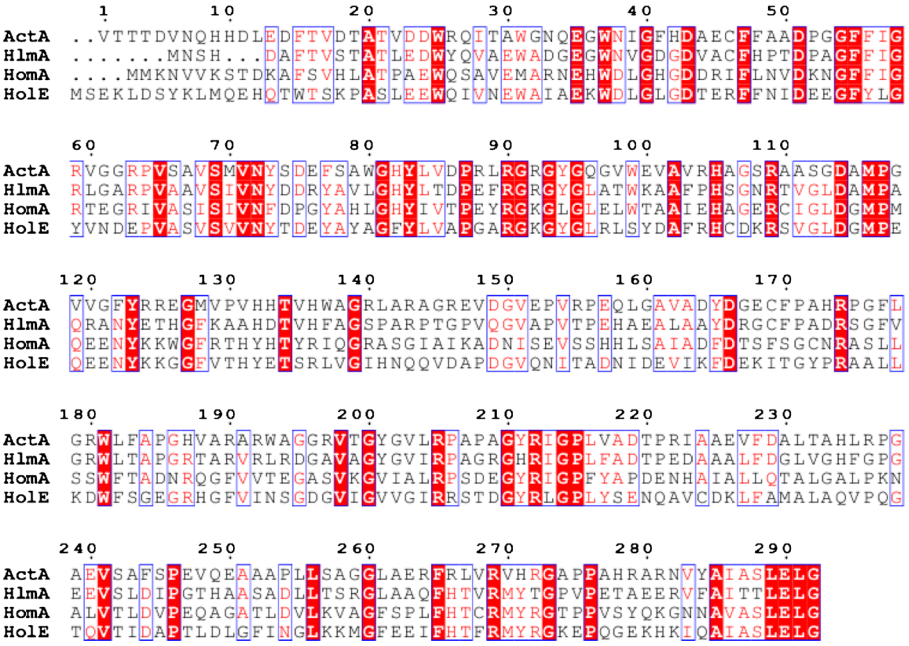


Fig. 2 Alignment of *S. algeriensis* ActA (accession no. AHB86989.1) with HlmA in *S. clavuligerus* (WP_003955866.1), HlmA in *S. clavuligerus*, Hom3 in *Y. ruckeri* (WP_004721324.1), and HolE in *Pseudoalteromonas* sp. (YP_004661191.1). Similarity significance value of 60 % is *colored in red* and *framed in blue*

The fraction 40–60 % was further purified by HIC. The two fractions that presented maximal benzoyltransferase activity were grouped as pool1. Acetyltransferase activity was no longer detected in this step. Anion exchange chromatography (AEC) was performed on pool1. Benzoyltransferase activity was detected only in four fractions (F18 to F21). This activity was maximal in fractions 19 and 20, and it was recovered with a yield of 0.4 and 0.6 % and purified to 8.1 and 12.2, respectively (Table 2).

Native PAGE shows that, even after AEC chromatography, a large number of contaminating proteins were still present in the extract, and the activity is far to be purified to

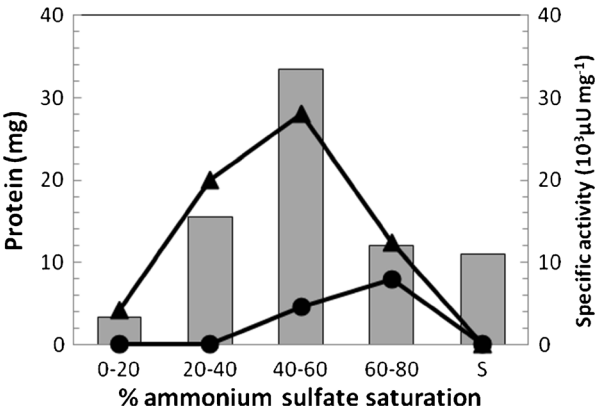


Fig. 3 Protein quantity and acyltransferase specific activity in the fractions according to the saturation with ammonium sulfate. Total protein (*gray bar*) and specific activity for acetyltransferase (*black circle*) and benzoyltransferase (*black triangle*) versus the percentage saturation of ammonium sulfate

Table 2 Purification of benzoyltransferase activity

Purification step	Protein (mg)	Activity (mU)	Activity specific (μU/mg)	Yield (%)	Purification (fold)
Crude extract	90.2	1,150	12,749	100	1
40–60 $(NH_4)_2SO_4$ fraction	31.2	804	25,769	69.9	2.02
HIC	1.18	105	88,983	9.1	6.79
AEC	0.05	7.8	156,000	0.6	12.23

homogeneity (Fig. 4). The presence of benzoyltransferase activity was tested by following the enzymatic activity in the native PAGE which had been previously cut in six fractions. The enzyme of interest was localized in fractions F2 and F3 with Rf of 0.16 to 0.32, and its molecular weight could be estimated about 66.2 kDa.

Peptide Microsequencing

The F2 fraction was excised from a Coomassie-stained gel and digested with trypsin, and the resulting peptides were analyzed yielding the sequences of eight different fragments. The alignment of these peptides against the databank of *S. algeriensis* contigs (tblastn) showed that two of them YTGLADAVTDLASSR and RLGMTRDGVL were located in the same contig whose size is 4.3 Kb. The GeneMark™ software for gene prediction using *Streptomyces coelicolor* as a reference allowed the prediction of four ORFs in this contig. The two previous fragments were also found to be encoded by the same ORF, which was designated as *actB*. Amino acid sequence comparison of ActA and ActB revealed that they are completely different.

The alignment of ActB against the GenBank database indicated that it shares a high homology to other acyltransferases in several bacteria, which are already known as

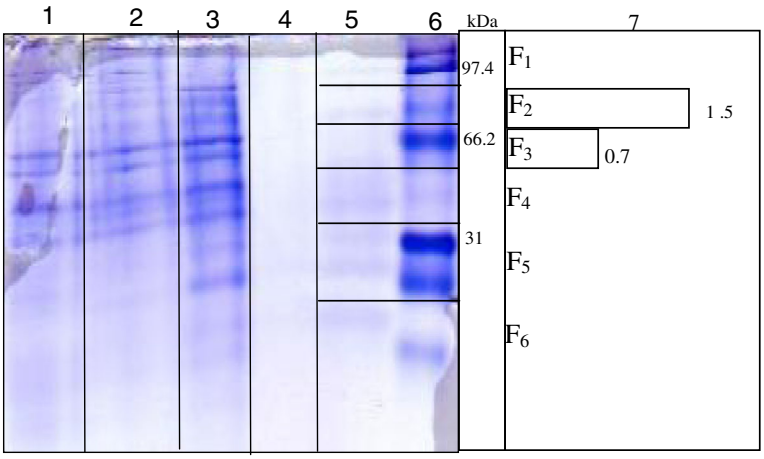


Fig. 4 Native PAGE of the enzyme preparations obtained at each step of the purification. *Lane 1*, cell-free extract; *lane 2*, 40–60 % ammonium sulfate fraction; *lane 3*, F5 + F6 (HIC); *lanes 4* and *5*, F19 and F20 (AEC); *lane 6*, molecular weight markers; *lane 7*, BEP appeared in micromoles per liter after 15 h of incubation of the band in the reaction medium at 30 °C

dithiolopyrrolone producers (i.e., 66 % identity to *N*-acetyltransferase in *Streptomyces griseus* subsp. *griseus* NPRC 13350, 62 % identity in *Streptomyces albus* J1074, and 60 % identity to *N*-acetyltransferase in *S. clavuligerus*) (Fig. 5). Furthermore, a protein-protein Blast search revealed that ActB has an acetyltransferase conserved domain with best specific hit of pfam00583, and this domain belongs to the *N*-acyltransferase superfamily. The sequences of the other fragments (five fragments) led to the identification of five ORFs in five different contigs. The products of these ORFs did not show any significant homology to any known acyltransferases. Finally, sequences of the remaining peptide fragments did not show any homology to known protein sequences.

Biomass, Thiolutin, and BEP Production

Neither thiolutin nor BEP was detected in the TSB medium, but was seen on SSM and SSM + BA (Fig. 6). Despite the absence of thiolutin and BEP in the TSB medium, *S. algeriensis* presented similar growth in the three studied media (Fig. 7).

The kinetic of antibiotic production showed that thiolutin production reached maximum values of 21 and 23.2 mg g^{-1} at 48 h of culture. The maximum-specific production rate of 0.94 and 0.78 mg g^{-1} h^{-1} was reached at 24 h on SSM and SSM + BA, respectively (Fig. 7a). The addition of benzoic acid to the SSM medium increased BEP production by 50-fold, and the maximum-specific production rate was 0.006 and 0.11 mg g^{-1} h^{-1} at 48 h on SSM and SSM + BA, respectively (Fig. 7b).

Pyrrothin-Acyltransferase Activities

No acyltransferase activities (neither acetyltransferase nor benzoyltransferase) were detected in the TSB medium. In the two other media, acetyltransferase-specific activity showed a peak at 24 h, followed by a sharp decrease. It is clear that this activity was not significantly modified

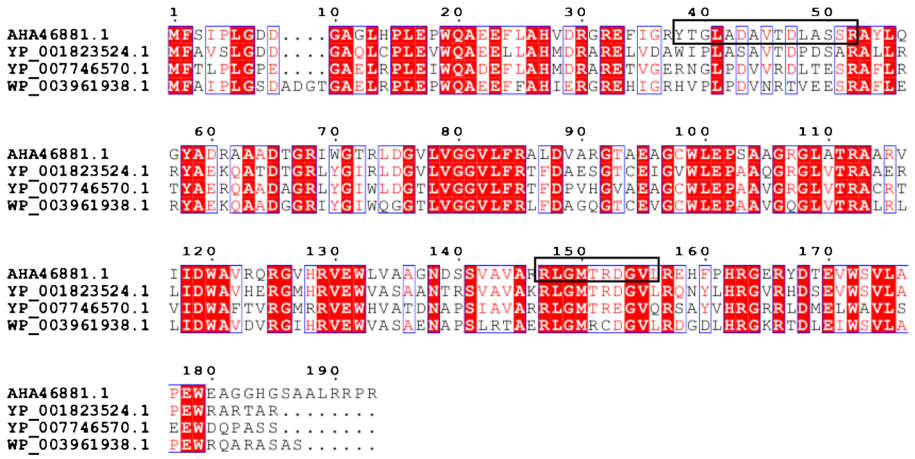


Fig. 5 Alignment of *S. algeriensis* ActB (NCBI reference sequence: AHA46881.1) with other acyltransferases in species already known as dithiolopyrrolone producers; *Streptomyces griseus* subsp. *griseus* NPRC 13350 (NCBI reference sequence: YP_001823524.1), *Streptomyces albus* J1074(NCBI reference sequence: YP_007746570.1), and *S. clavuligerus* (NCBI reference sequence: WP_003961938.1). Similarity significance value of 60 % are *colored in red* and *framed in blue*. The peptide sequences which were identified by microsequencing *are boxed in black*

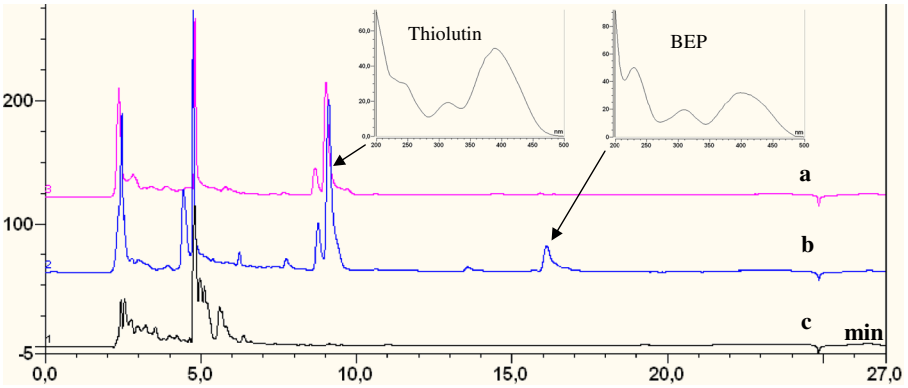


Fig. 6 Dithiolopyrrolone production: *a* under standard conditions (in SSM); *b* addition of 1.25 mM of benzoic acid (SSM + BA); and *c* TSB. HPLC analysis at 390 nm was done on supernatant of *S. algeriensis* culture

by the addition of benzoic acid to SSM (Fig. 8a). However, benzoyltransferase-specific activity was considerably modified by benzoic acid addition shown by an increase in activity on SSM + BA than on SSM. Benzoyltransferase-specific activity reached a maximum value of 20 μU mg^{-1} at 48 h, 1.6 times higher than without benzoic acid (Fig. 8b).

Transcriptional Analyses of *actA* and *actB* in *S. algeriensis*

The result from RT-PCR for *actA and actB* revealed that both genes were not expressed in TSB and were only expressed in SSM and SSM + BA medium. To verify the relationship between these expressions and acyltransferase activities, we analyzed the transcription of these genes in the two media. Total RNAs were extracted from the *S. algeriensis* at 16, 24, 48, 72, and 96 h of growth in SSM and SSM + BA and were used to perform RT-qPCR with the same previously described protocol. The results showed that *actA* and *actB* expressions started at 16 h and increased to its maximum at 24 h with relative values of 445 and 460 for *actA* and of 211 and 240 for *actB* for SSM and SSM + BA, respectively. Then, *actA* expression level decreased sharply while *actB* decreased slowly (Fig. 8a). The effect of

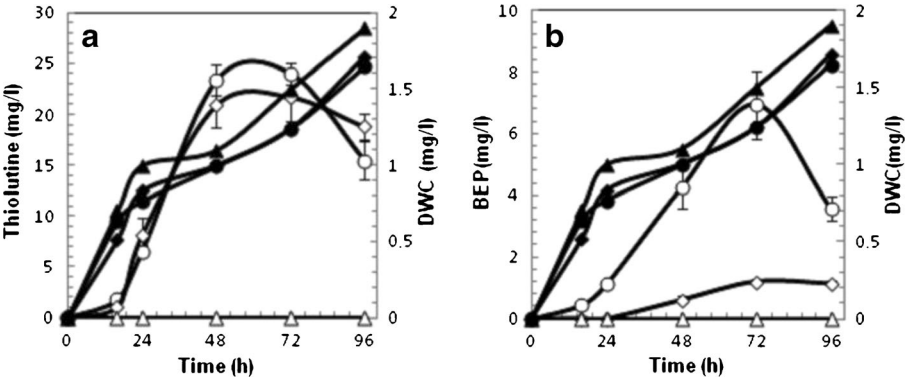


Fig. 7 Time course of dry cell weight (DCW) during cultures of *S. algeriensis* NRRL B-24137 on SSM (*black diamond*), SSM + BA(*black circle*), and TSB (*black triangle*), with the production of thiolutin (**a**) and BEP (**b**); SSM (*white diamond*), SSM + BA (*white circle*), and TSB (*white triangle*). Dry cell weight (DCW) is given in grams per liter; antibiotic production in milligrams per liter and the time in hours

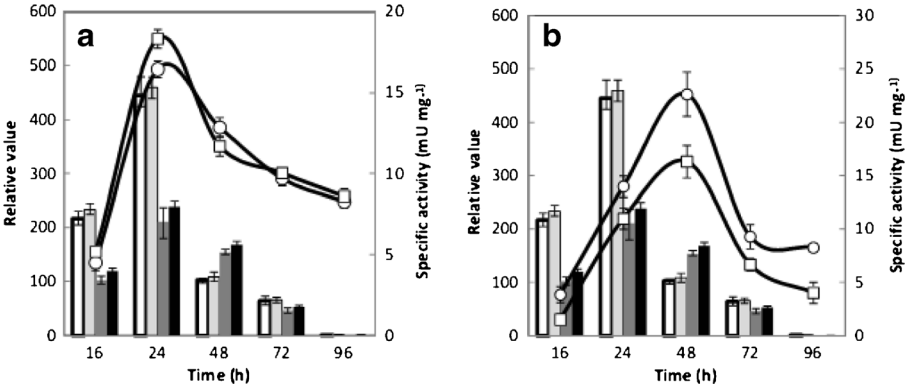


Fig. 8 Relationship of differential expressions with the specific activities of acetyltransferase (**a**) and benzoyltransferase (**b**) in two media: SSM (*white square with solid line*) and SSM + BA (*white circle with solid line*). Differential expressions were presented as follows: *actA* for SSM (*white bar*), *actA* for SSM + BA (*light gray bar*), *actB* for SSM (*dark gray bar*), and *actB* for SSM + BA (*black bar*). Specific activity presented in milliunits per milligram protein and the time in hours. Relative values are in comparison to *FtsZ*-like, which was used as a reference gene. The relative value for the 96-h sample was arbitrarily assigned as 1

benzoic acid addition was not significant and minimum p values done by t test were 0.16 and 0.052 for *actA* and *actB*, respectively, which confirm no significant difference between the two media throughout the time period. However, both *actA* and *actB* showed similar patterns of expression in both media. Maximum relative values of expression were reached at the same time of acetyltransferase activity (Fig. 8a) and 24 h before the benzoyltransferase activity (Fig. 8b).

Effect of Benzoyl-CoA Precursor Supply on Benzoyltransferase Activity

To study the effect of benzoyl-CoA precursors on BEP-specific production in *S. algeriensis*, we selected compounds that are already known to be involved in benzoyl-CoA biosynthesis and to be an inducer of BEP production.

We observed that the growth of *S. algeriensis* was influenced only by the addition of L-phenylalanine and shikimic acid, whereas no change was observed for cinnamic acid, benzoic acid, or benzoyl-CoA (Fig. 9a). L-Phenylalanine significantly stimulated the growth of *S. algeriensis* and the highest value of dry cell weight of 1.3 g $L^{-1}$was obtained in the presence of 5 mM of L-phenylalanine at 96 h. Shikimic acid, however, caused a reduction in growth throughout the culture and maximal dry cell weight was 0.8 g L^{-1} at 96 h compared with 1.1 g L^{-1} for the control (Fig. 9a).

Analysis of the results indicated that the addition of benzoic acid and cinnamic acid supported higher BEP production (6.2 and 5.6 mg g^{-1}, respectively) than benzoyl-CoA (4.1 mg g^{-1}) at 72 h. On the other hand, the production of BEP was higher in the presence of benzoyl-CoA than in the presence of L-phenylalanine (1.5 mg g^{-1}) and shikimic acid (0.6 mg g^{-1}) (Fig. 9b). The results of the effect of these supplementations on benzoyltransferase activity at 72 h showed that cinnamic acid considerably enhanced benzoyltransferase activity and increased this activity by 3.5-fold when compared to the control. However, benzoic acid increased benzoyltransferase activity only by 1.7-fold. Benzoyltransferase activity was slightly increased by L-phenylalanine. In the presence of shikimic acid, there was no significant impact on benzoyltransferase activity (Fig. 10).

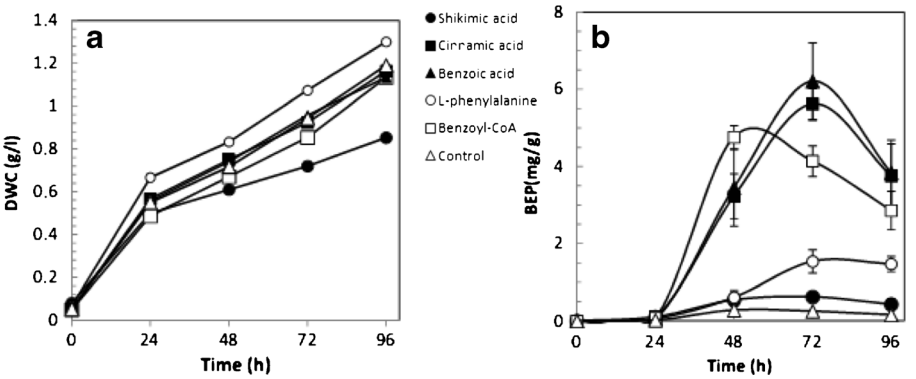


Fig. 9 Growth and benzoyl-pyrrothin-specific production of *S. algeriensis* during fermentation in SSM (*white triangle*) as control, SSM + shikimic acid (*black circle*), cinnamic acid (*black square*), benzoic acid (*black triangle*), L-phenylalanine (*white circle*), and benzoyl-CoA (*white square*). **a** Growth curves measured as dry weight. **b** BEP-specific production

Discussion

S. algeriensis has the ability to produce a wide range of pyrrothine derivatives with different radicals (R_2s) depending on the precursors added to the culture medium [3, 20–22]. Acyltransferase activities were previously reported to be responsible for the structural diversity of pyrrothine by transferring different acyl groups from acyl-CoA to the pyrrothine core [25]. In this study, two methods were followed to identify acyltransferase genes potentially involved in dithiolopyrrolone biosyntheses in *S. algeriensis*.

The comparison of known acetyltransferase, HlmA, which is mentioned to be involved in holomycin biosynthesis in *S. clavuligerus*, led to the identification a putative acyltransferase, ActA, within the genome of *S. algeriensis* with a percentage of identity/similarity of 51 %/60 %. This percentage is slightly higher than that found between the two acyltransferases, Hom3 and HolE (42 %/57 %), in marine Gram-negative bacteria *Y. ruckeri* and *Pseudoalteromonas*, which belong to same class of *Gammaproteobacteria*. We also compared ActA with Hom3 in *Y. ruckeri* and HolE in *Pseudoalteromonas*, which revealed a low

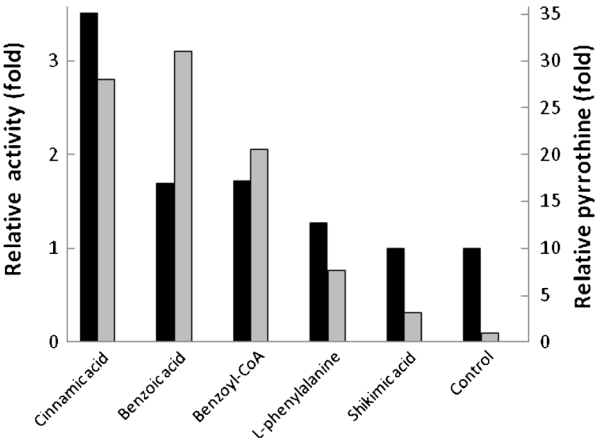


Fig. 10 Effect of medium supplementations on benzoyltransferase activity (*black bar*) and benzoyl-pyrrothin production (*gray bar*). SSM used as the control medium was supplemented by 5 mM of cinnamic acid, benzoic acid, L-phenylalanine, and shikimic acid and 0.05 mM of benzoyl-CoA. The activity and BEP production were expressed in relative values in comparison to the control medium values at 72 h of fermentation, which is the optimal time for BEP production

percentage of identity (about 30 %). Only HlmA was characterized biochemically, in vitro, and was able to transfer different acyl groups from acyl-CoA to the holothin core forming different holothin derivatives which have never been naturally produced by *S. clavuligerus*. No mutations of *hlmA* have shown as HlmA is the only acetyltransferase implicated in holomycin biosynthesis in *S. clavuligerus*.

In order to identify additional acyltransferase genes that may have a role in dithiolopyrrolone biosynthesis in *S. algeriensis*, the purification of two acyltransferases (acetyltransferase and benzoyltransferase) previously proposed by Chorin et al. [25] was investigated. An ammonium sulfate precipitation followed by HIC and AEC supplied only a semipurified benzoyltransferase with a purification factor of 12.23. The loss of acetyltransferase activity and the low purification factor of benzoyltransferase could be due to the instability of both these activities during the purification stages. In fact, these activities were exposed to different inactivation conditions (e.g., pH change, oxidizing environment, proteases, etc.). Furthermore, the stability of these activities may be influenced by the low concentration of protein. Some enzymes could be stabilized by a high concentration of proteins [36, 37]. As benzoyltransferase has not been purified to homogeneity, the meager increased in fold activity could also be due to contaminating residual activity. However, acetyltransferase and benzoyltransferase showed different ammonium sulfate precipitation profiles, and acetyltransferase activity was significantly less stable. The microsequencing of peptide sequences derived from F2 fraction of native PAGE (Fig. 3) provided eight peptide fragments, two of them located in the *S. algeriensis* genome and encoded by one gene *actB*. ActB was completely different from ActA and the other identified acyltransferases (HlmA, HolE, and Hom3). On the other hand, ActB was homologous to acetyltransferases in other bacteria, specifically *S. griseus*, *S. albus*, and *S. clavuligerus*, which are known holomycin producers [38].

Unfortunately, the genetic manipulations on *S. algeriensis* have not resulted in any mutants. Thus, to verify the involvement of *actA* and *actB* genes in DTP biosynthesis in *S. algeriensis*, another strategy was adopted. This strategy was based on the association of gene expression of both acyltransferase activities and antibiotic productions in two media, SSM, which promotes DTP production, and TSB medium, in which no DTPs have been detected.

The results showed that *actA* and *actB* are expressed only in SSM. In addition, this expression was accompanied with the acetyltransferase activity and DTP productions. This result allowed us to suggest that these genes may be involved in DTP biosyntheses. Thus, we associated *actA* and *actB* expressions with the previously reported *N*-pyrrothine acetyltransferase activity by realizing a kinetic on SSM. Analysis of acetyltransferase-specific activity and both *actA* and *actB* expressions showed that they presented the same profile throughout the culture with a maximum at 24 h. This similarity between acetyltransferase activity and gene expression profiles reinforces our hypothesis about the involvement of these genes in dithiolopyrrolone biosynthesis.

In order to identify the enzymes encoded by *actA* and *actB*, we based our research on the hypothesis proposed by Chorin et al. [25], in which benzoic acid increased BEP production by inducing benzoyltransferase gene. To verify this hypothesis, BEP production, benzoyltransferase activity, and *actA* and *actB* expressions were followed and compared on SSM and SSM supplemented by 1.25 mM of benzoic acid. The results showed that benzoic acid addition strongly increased BEP production (50-fold at 48 h); in contrast, this addition did not influence *actA* or *actB* expressions. According to the previous hypothesis, neither *actA* nor *actB* encodes for benzoyltransferase. But the poor increase in benzoyltransferase activity compared to BEP production (1.6- and 50-fold, respectively, at 48 h) weakens this hypothesis.

In *S. clavuligerus*, the acetyltransferase activity was proportional to the level of holomycin produced by different mutants. For example, in *S. clavuligerus oppA2::aph*, the increase of

hlmA transcription level (*hlmA* encodes for acetyltransferase) of 61.5-fold was accompanied by an increase of acetyltransferase activity of 172-fold and holomycin production of 120-fold compared with the wild-type strain [39, 40]. We suggest that benzoic acid can play other roles as a direct supplier of benzoyl-CoA precursor. To confirm our suggestion, the effect of benzoyl-CoA precursor supply on BEP production and benzoyltransferase activity was investigated. Our results showed that BEP production is not only dependent on benzoyltransferase activity, but also on benzoyl-CoA availability. The addition of cinnamic acid produced a greater increase in benzoyltransferase activity than seen with the addition of benzoic acid, while benzoic acid produced more of BEP (26- and 32-fold, respectively). The addition of L-phenylalanine also considerably increased BEP production by 8-fold and increased slightly benzoyltransferase activity, but less than cinnamic and benzoic acid. The difference in BEP production between these three compounds is strongly consistent with previous biosynthetic feeding studies on the conversion of these compounds to benzoyl-CoA. The marine bacterium *Streptomyces maritimus* is able to convert L-phenylalanine to cinnamic acid and then to benzoyl-CoA [41]. Furthermore, a biosynthetic pathway of benzoyl-CoA from L-phenylalanine and cinnamic acid was recently proposed in this bacterium [42]. We suggest that *S. algeriensis* could convert cinnamic acid and benzoic acid to benzoyl-CoA. We also observed that the addition of shikimic acid increased BEP production by 3-fold, with no effect on benzoyltransferase activity. It was reported that 4-hydroxybenzoic acid can be formed directly by early intermediates in the shikimate pathway, e.g., 3-dehydroshikimic acid. In addition, a direct conversion of shikimic acid to benzoic acid was already seen in *Streptomyces* [43].

In conclusion, this study resulted in the first identification of two putative acyltransferases, ActA and ActB, in *S. algeriensis*. Transcriptional analyses support our hypothesis of the involvement of *actA* and a*ctB* in dithiolopyrrolone biosynthesis in this bacterium. Our results also suggest that the role of benzoic acid as well as similar compounds is more important to benzoyl-CoA precursor supply than their role as gene inducer.

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