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Enzymatic Synthesis of Dithiolopyrrolone Antibiotics Using Cell-Free Extract of *Saccharothrix algeriensis* NRRL B-24137 and Biochemical Characterization of Two Pyrrothine *N*-Acyltransferases in This Extract

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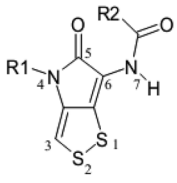
Saccharothrix algeriensis NRRL B-24137 produces naturally different dithiolopyrrolone derivatives. The enzymatic activity of pyrrothine *N*-acyltransferase was determined to be responsible for the transfer of an acyl group from acyl-CoA to pyrrothine core. This activity was also reported to be responsible for the diversity of the dithiolopyrrolone derivatives. Based on this fact, nine dithiolopyrrolone derivatives were produced *in vitro via* the crude extract of *Sa. algeriensis*. Three of them have never been obtained before by natural fermentation: acetoacetyl-pyrrothine, hydroxybutyryl-pyrrothine, and dimethyl thiolutin (holomycin). Two acyltransferase activities, acetyltransferase and benzoyltransferase catalyzing the incorporation of linear and cyclic acyl groups to the pyrrothine core, respectively, were biochemically characterized in this crude extract. The first one is responsible for formation of acetyl-pyrrothine and the second for benzoyl-pyrrothine. Both enzymes were sensitive to temperature changes: For example, the loss of acetyltransferase and benzoyltransferase activity was 53% and 80% respectively after pre-incubation of crude extract for 60 min at 20°C. The two enzymes were more active in neutral and basal media (pH 7–10) than in the acidic one (pH 3–6). The optimum temperature and pH of acetyltransferase were 40°C and 7, with a K_m value of 7.9 μM and a V_{max} of 0.63 μM/min when acetyl-CoA was used as limited substrate. Benzoyltransferase had a temperature and a pH optimum at 55°C and 9, a K_m value of 14.7 μM, and a V_{max} of 0.67 μM/min when benzoyl-CoA was used as limited substrate.

Keywords: Saccharothrix algeriensis, dithiolopyrrolone biosynthesis, acetyltransferase, benzoyltransferase, biochemical characterization

Introduction

Saccharothrix algeriensis NRRL B-24137 produces naturally at least five pyrrothine derivatives characterized by their different *N*-acyl groups, such as thiolutin (acetyl-pyrrothine), senecioyl-pyrrothine (SEP), tigloyl-pyrrothine (TIP), isobutyrylpyrrothine (ISP), and butanoyl-pyrrothine (BUP) (Fig. 1A) [18, 19]. Furthermore, the addition of precursors to the culture medium led to modified production levels of known dithiolopyrrolones [2, 3]. Precursor-directed biosynthesis (PDB) method also led to the production of new dithiolopyrrolone analogues. The addition of benzoic and valeric acids led to the production of unnatural dithiolopyrrolones valeryl-pyrrothine benzoyl-pyrrothine (BEP), demethyl-benzoyl-pyrrothine (benzoyl-holothin) [4]. Further exploitation of the PDB method revealed the remarkable flexibility of the dithiolopyrrolone biosynthetic pathway in *Sa. algeriensis*. The addition of 5 mM sorbic acid in the culture medium induced the production of new dithiolopyrrolone derivatives: crotonyl-pyrrothine, sorbyl-pyrrothine, 2-hexonyl-pyrrothine, and 2-methyl-3-pentenyl (Fig. 1B).

Dithiolopyrrolone antibiotics have strong activities against a variety of gram-positive and gram-negative bacteria,



A

R1=CH3	
R2	
CH_3	Acetyl-pyrrothine (thiolutin)
C_6H_5	Benzoyl-pyrrothine
$CH(CH_3)$	Iso-butyryl-pyrrothine
CH_2CH_2-CH_3	Butanoyl-pyrrothine
$CH=C(CH_3)_2$	Senecioyl-pyrrothine
$C(CH_3)=CH(CH3)$	Tigloyl-pyrrothine

B

H	Fomoyl-pyrrothine
CH_2-CH_3	Propionol-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 *Sa. algeriensis*.
(**A**) Natural products; (**B**) unnatural products using precursor-directed biosynthesis (PDB) method.

yeasts, filamentous fungi, and amoeboid parasites [6, 18, 31]. Thiolutin was also reported to inhibit in yeast all RNA polymerases [12, 16, 30] and the degradation of mRNA [26]. Recent study showed that thiolutin also potently inhibits developmental angiogenesis in zebrafish and vascular outgrowth from tissue explants in 3D cultures [15]. Biological activity of dithiolopyrrolones is strongly influenced by the nature of acyl groups [13, 18, 20, 25].

In *Sa. algeriensis*, a pyrrothine *N*-acyltransferase activity was reported to be responsible for the transfer of the acyl groups from the acyl-CoA to pyrrothine core, leading to production of different dithiolopyrrolone derivatives [7]. Early studies already reported the involvement of various acyltransferases in the antibiotics biosynthesis produced by the filamentous, gram-positive bacteria genus *Streptomyces*. In *S. clavuligerus*, an acetyltransferase activity, holomycin synthase, was reported to be responsible, *in vitro*, for holomycin formation [9]. Recently, the gene encoding this enzyme and its role in holomycin biosynthesis were identified as *N*-pyrrothine-acyltransferase. This enzyme catalyzed the transfer of acyl groups from acyl-CoA to the holothin core [21]. Another acyltransferase called ornithine acetyltransferase appeared to act as a modulator for clavulanic acid biosynthesis [10]. An arylamine *N*-acetyltransferase (NAT) responsible for the *N*-acetylation of exogenous 3-amino-hydroxybenzolic acid in *S. griseus* was mentioned to be involved in the metabolism of xenobiotic compounds [29]. An acyltransferase called *N*-benzoyltransferase had also been reported to be involved in the last step of biosynthesis of the anticancer drug Taxol in yew *Taxus* species [22].

The study of pyrrothine *N*-acyltransferase activities in the cell-free extract of *Sa. algeriensis* obtained on semi-synthetic medium supplemented or non-supplemented with benzoic acid was already investigated by Chorin *et al.* [7]. In particular, the transfer of acetyl and benzoyl groups on the pyrrothine core resulted in thiolutin and BEP, respectively. This study suggested that two different enzymes could support these enzymatic reactions. This hypothesis was emphasized by the fact that the addition of 1.25 mM of benzoic acid induced the pyrrothine *N*-benzoyltransferase activity specifically and did not affect the pyrrothine *N*-acetyltransferase activity. In addition, the ratio of both specific activities was not constant throughout the culture [7].

Here, we assayed the enzymatic synthesis of several dithiolopyrrolones using the crude extract of *Sa. algeriensis*. Different length acyl chains and cyclic acyl chains branched or not branched were tested using two substrates, holothin and pyrrothine. Next, we investigated the characterization of two intracellular acyltransferases present in this crude extract. This characterization could confirm the hypothesis of the presence of at least two *N*-acyltransferases in *Sa. algeriensis*.

Materials and Methods

Producing Strain and Culture Conditions

Saccharothrix algeriensis NRRLB-24137 (= DSM 44581) was used for this study. A stock of spores was prepared as follows. *Sa. algeriensis* was grown on International *Streptomyces* Project 2 (ISP2) agar plates for 7 days at 30°C. Spores were suspended in 0.1% Tween 80 (Fisher, Waltham, USA), harvested, and stored in 20% glycerol (Fisher) at −20°C. The spores were counted and represented as colony-forming units per milliliter (CFU/ml).

The semi-synthetic medium (SSM) [2] containing 15 g/l of D (+) glucose (Acros) was used. *Sa. algeriensis* precultures were prepared in 250 ml Erlenmeyer flasks containing 50 ml of culture medium inoculated with spores (2×10^7 CFU/ml). Cultures grown in 500 ml Erlenmeyer flasks containing 100 ml of SSM were inoculated with 5 ml of 48 h aged preculture. The culture was incubated at 30°C at 240 rpm for 72 h on a rotary shaker (New Brunswick Scientific Company, NJ, USA).

Cell-Free Extract Preparation (Crude Extract)

Sa. algeriensis was grown in SSM for 72 h, and then the biomass was separated by centrifugation at 5,000 ×*g* for 15 min at 4°C (4K15; Sigma, Germany). Then, the biomass was washed twice with physiological water (0.9% NaCl), and once with lysis buffer (50 mM Tris-HCl, pH 8). Wet cells were finally recovered by filtration using 0.2 µm membrane filters (Advantec, Ireland). Then 0.6 g of wet cells were resuspended in 1 ml of lysis buffer and transferred to a Fast Protein Blue tube (MP Biomedicals, USA). Two disruption cycles (30 sec, 5 m/sec) were carried out in a Fast Prep disruptor (MP Biomedicals, USA). The lysing matrix was discarded and then the sample was centrifuged at 10,000 ×*g* for 30 min (centrifuge 1-15K; Sigma, USA) to remove the cellular debris. The supernatant, which constitutes the soluble cell-free extract of *Sa. algeriensis* (crude extract), was used to synthesize the dithiolopyrrolones derivatives and to characterize both pyrrothine *N*-acyltransferase activities. The proteins were determined in the crude extract by the method of Bradford [5].

Assay of Enzymatic Activity

For enzymatic assay, the reaction mixture was created in the following order: 10 µl of pyrrothine solution in methanol (final concentration at 0.25 mM), 10 µl of acyl-CoA solution in deionized water (final concentration at 0.5 mM), and 80 µl of enzyme extract whose addition started the enzymatic reaction (T_0). The reaction mixture was incubated at 30°C for 10 min (standard assay conditions). The reaction was stopped by adding fresh trichloroacetic acid at 2.5% (w/v) (Fisher). The cell-free extract was properly diluted with Tris-HCl buffer (50 mM, pH 8) before the assay to obtain a linear product formation with time within 10 min. 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). A unit of enzyme was defined as the enzyme activity producing 1 µmol of thiolutin or BEP per minute. The specific enzymatic activity was expressed in µU/mg of protein.

Dithiolopyrrolones Detection and Quantification

For enzyme characterization, thiolutin and benzoyl-pyrrothine were quantified by HPLC (Bio-Tek Instruments, Italy). The analytical column was ProntoSIL 120-5-C18 SH, 150 × 4.6 mm (Bishoff chromatography, Germany) fitted with a pre-column of 10 × 4 mm, and detection was achieved with a diode array detector (UV-vis 545 V; Bio-Tek Instruments). The samples were analyzed by linear gradient elution using acetonitrile as solvent A and ultra pure water as solvent B. The separation gradient started with 0% solvent A and 100% solvent B, reached 30% solvent A and 70% solvent B in 5 min, continued from 30% to 100% solvent A in 25 min, using a flow rate of 0.8 ml/min. The injection volume was 80 µl. The detection of dithiolopyrrolones was carried out at 390 nm. Quantification of antibiotics was performed using a thiolutin standard calibration curve. The molar extinction coefficient (log e) of thiolutin is nearly the same for all fractions (log e390 = 3.92–3.97) as described by Lamari *et al*. [19].

The identity of the formed products (*i.e.,* dithiolopyrrolone derivatives) throughout the enzymatic synthesis was determined as follows: 10 µl of reaction mixture was injected after filtration using 0.2 µm membrane filters (Advantec, Ireland) in an LC-MS instrument (Agilent system 1100). All analyses were performed on a reverse phase column, Prontosil 120-5 C18-SH, 150 × 4.6 mm (Bischoff Chromatography, Germany). The ionization was performed by electrospray. Then the mass spectrum was determined with a Q TRAP device (Applied Biosystems) with a triple quadrupole.

Biochemical Characterization

Effect of temperature on acyltransferase activity (optimum temperature). The effect of temperature on acyltransferase activity was determined by realizing the enzymatic reaction described above at various temperatures (*i.e*., 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, and 60°C). Control reaction without the extract enzymatic was achieved in order to verify no transformation of pyrrothine to thiolutin or BEP whatever the chosen temperature.

Effect of temperature on acyltransferase stability. The thermal stability was studied by pre-incubating the crude extract at 20°C, 30°C, 40°C, and 50°C. Appropriate aliquots were withdrawn at different time intervals (*i.e*., 30, 40, and 60 min) and transferred rapidly on ice for 10 min. The activity of the enzyme before pre-incubation was taken as 100%. The temperature stability was studied by measuring the residual activities.

Effect of pH on acyltransferase activity and stability. For optimum pH determination, the crude extract was prepared in 50 mM Tris-HCl adjusted to different pH values (*i.e*., 4, 5, 6, 7, 8, 9, 10, 11, and 12), and then the acyltransferase activity was measured at 30°C using the protocol described above. Control reaction was performed for each value of pH buffer without the crude extract. For pH stability studies, the crude extracts prepared at different pH values were pre-incubated at 4°C for 24 h, and then the residual activity was determined as indicated above.

Determination of kinetic parameters for acetyltransferase and benzoyltransferase. In all kinetics studies, appropriate enzyme extract dilution and incubation times were chosen, so that the reaction velocity was linear during the incubation time period. To determine the K_m value for acyl-CoA as limited substrate, pyrrothine concentration was fixed at a saturated level and the concentration of the acyl-CoA was varied. A Lineweaver-Burk plot was used to report the apparent K_m value.

Results

Characterization of the *In Vitro* Enzymatically Synthesized Dithiolopyrrolones

Synthesis of pyrrothine and holothin derivatives was assessed *in vitro* using crude extract of *Sa. algeriensis* obtained as described above with different acyl group donors. From mass spectrometry and UV spectrometric analyses, as well as by comparison with all dithiolopyrrolone derivatives reported in the literature (data not show), we identified the structures of six dithiolopyrrolone derivatives naturally produced by *Sa. algeriensis*: thiolutin, benzoyl-pyrrothine, butyryl-pyrrothine, crotonoyl-pyrrothine, hexanoyl-pyrrothine, and acetoacetyl-pyrrothine (Table 1). Theses analyses also revealed three acylated pyrrothine and holothin products; their calculated (cald.) and observed (obsd) masses for $[M+H]^+$ are as follows: hydroxybutyryl-pyrrothine $C_{10}H_{12}N_2O_3S_2$, cald. 272.029, absd. 273; hexanoyl-pyrrothine $C_{12}H_{16}N_2O_2S_2$, cald. 270.013 absd. 271.00; and acetyl-holothin (holomycin) $C_7H_6N_2O_2S_2$, cald. 213.987, absd. 214.9 (Fig. 2). For the longer length acyl chain (lauroyl) as well as for branched cyclic acyl group (phenylacetyl), no acylated pyrrothine products were detected.

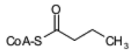
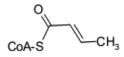
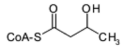
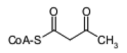
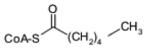
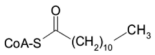
Biochemical Characterization

Optimum temperature. Acyltransferase activities in the crude extract of *Sa. algeriensis* were measured at temperatures ranging from 25°C to 60°C. The optimum acetyltransferase activity was observed at 40°C, and only about 3% of the activity was inactivated after exposure to 45°C for 10 min (Fig. 3). The optimum temperature of benzoyltransferase activity was 55°C, and up to this temperature the specific activity decreased sharply (*i.e.* at 60°C it was 42% lower than at 55°C).

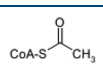
Thermal stability. The thermostability of acyltransferase activities was carried out at temperature ranging from 20°C

Table 1. Enzymatic synthesis of dithiolopyrrolone derivatives.

A

Group donors	Acyl-coA structure	Time retention of peak (min)	*m/z* for the formed ion $[M+H]^+$	Formula of the product formed (molar mass * g/mol)	Name of product formed
Acetyl-CoA		13.5	228.9	$C_8H_8N_2O_2S_2$ (228,002)	Thiolutin
Butyryl-CoA		24.8	257.0	$C_{10}H_{12}N_2O_2S_2$ (256,034)	Butyryl-pyrrothine
Crotonoyl-CoA		24.2	255.2	$C_{10}H_{10}N_2O_2S_2$ (254,018)	Crotonoyl-pyrrothine
β-Hydroxybutyryl-CoA		12.6	273	$C_{10}H_{12}N_2O_3S_2$ (272,029)	Hydroxybutyryl-pyrrothine
Acetoacetyl-CcoA		13.5	271.0	$C_{10}H_{10}N_2O_3S_2$ (270,013)	Acetoacetyl-pyrrothine
Hexanoyl-CoA		31.5	285.0	$C_{12}H_{16}N_2O_2S_2$ (284,065)	Hexanoyl-pyrrothine
Lauroyl-CoA		Not detected	Not detected	-	-
Benzoyl-CoA		29.5	291.2	$C_{13}H_{10}N_2O_2S_2$ (290,018)	Benzoyl-pyrrothine
Phenylacetyl-CoA		Not detected	Not detected	-	-

B

Acetyl-CoA		10	214.9	$C_7H_6N_2O_2S_2$ (213,987)	Holomycin
Benzoyl-CoA		29	277.0	$C1_2H_8N_2O_2S_2$ (276,003)	Benzoyl-holothin

(**A**) Pyrrothine derivatives; (**B**) holothin derivatives. Synthesis performed with a cell extract of *Sa. algeriensis* (obtained after 72 h of culture on SSM).

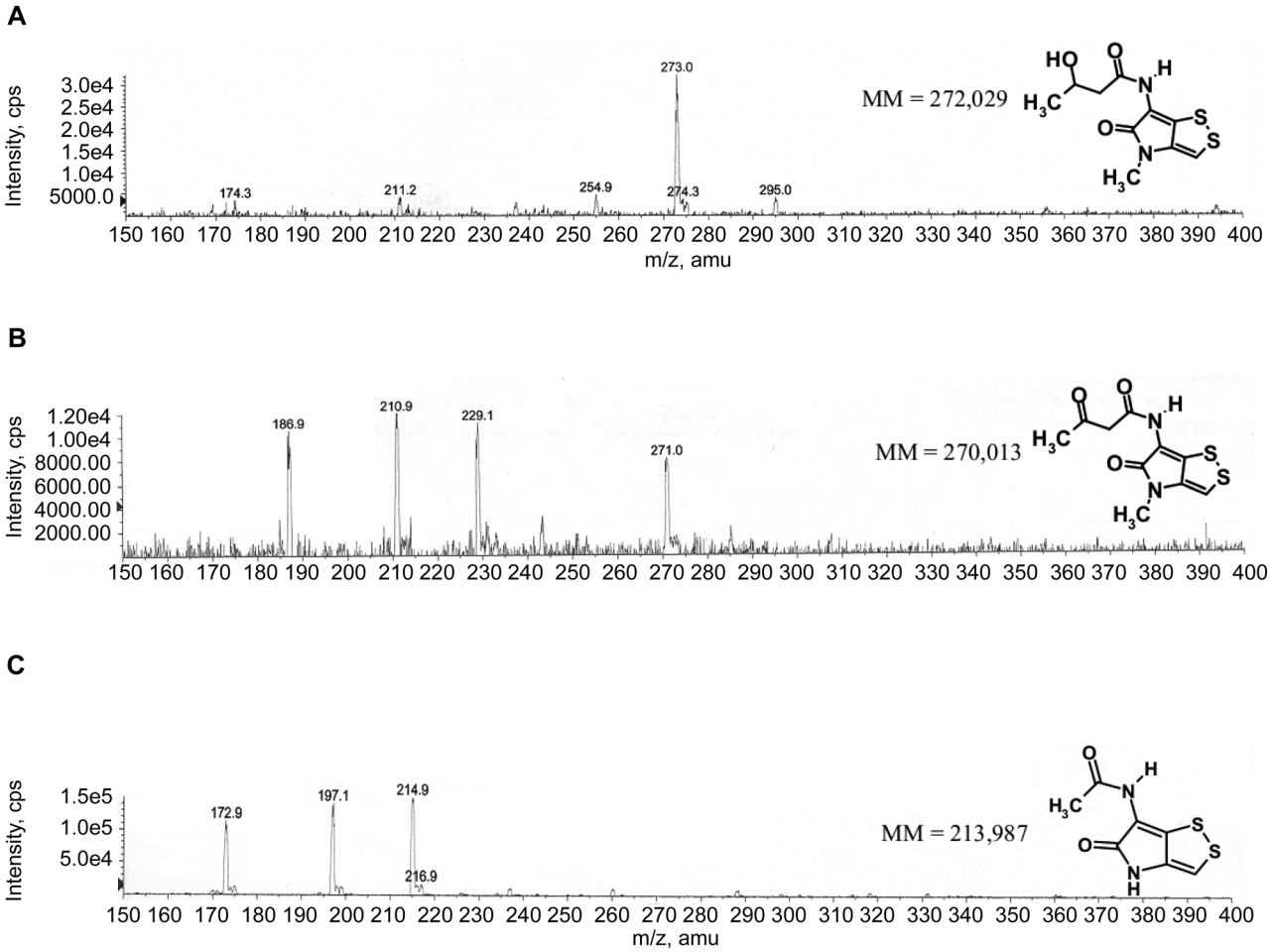


Fig. 2. Structures and mass spectra of dithiolopyrrolone derivatives synthesized with a free-cell extract of *Sa. algeriensis*. (**A**) Hydroxybutyryl-pyrrothine; (**B**) acetoacetyl-pyrrothine, and (**C**) acetyl-holothin (holomycin).

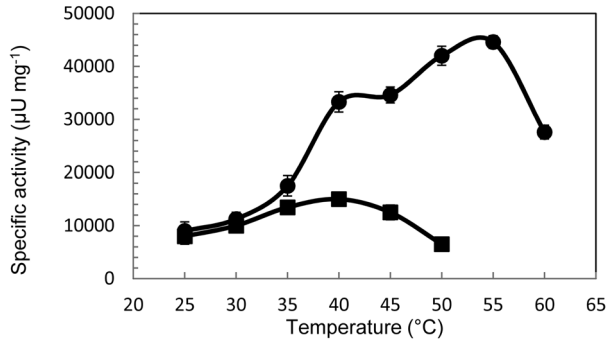


Fig. 3. Optimum temperatures of acyltransferase activities. Acetyltransferase (■), benzoyltransferase (●). Optimum temperatures were measured by realizing the enzymatic reaction at different temperature values at pH = 8, and then the specific activities were measured. The assays were carried out in triplicates. Error bars are represented in the graphs.

to 50°C. The thermal stability profile of acyltransferases showed that the two activities decreased after 60 min regardless of the incubation temperature (Fig. 4). The residual activities were 15% and 47% for acetyltransferase and benzoyltransferase, respectively, after 60 min at 20°C. The acetyltransferase activity decreaseed progressively up to 40°C–50°C with a residual activity of 47% and 20% after 30 min, respectively (Fig. 4A). In contrast, benzoyltransferase activity decreased dramatically with the increasing of temperature up to 30°C, with only 9% and 1% of residual activity at 40°C and 50°C after 30 min, respectively (Fig. 4B).

Long-term thermal stability. The thermostability at 4°C for 72 h was investigated for both activities. Fig. 5 shows that the maximum loss of activity accrued in the first day of incubation at 4°C. The residual activities were 54% and 71% after 8 h of incubation, and then they decreased progressively.

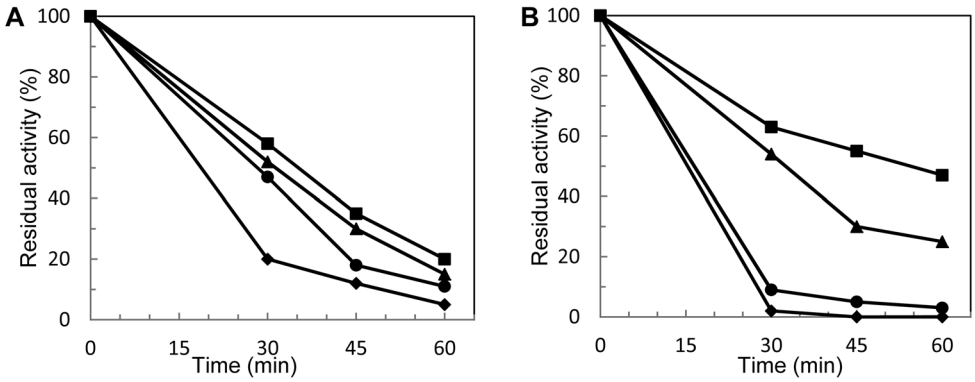


Fig. 4. Thermal stabilities of acyltransferases at 20°C (■), 30°C (▲), 40°C (●), and 50°C (◆).
(**A**) Acetyltransferase; (**B**) benzoyltransferase. The thermal stabilities were determined by pre-incubating the crude extract in different temperature values. Aliquots were withdrawn at regular time intervals after incubating, cooled rapidly in ice, and then the residual activities were measured at 30°C. The activity of the enzyme before pre-incubation was taken as 100%.

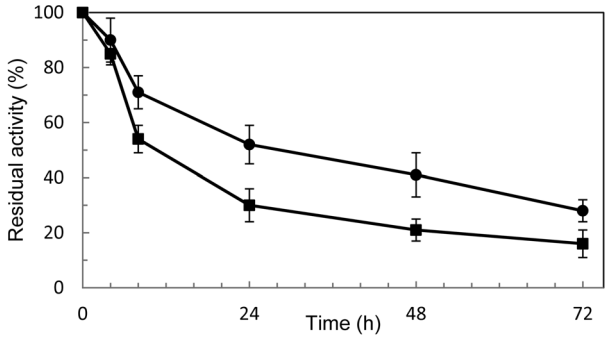


Fig. 5. Thermal stabilities of acyltransferase activities at 4°C.
Acetyltransferase (■), benzoyltransferase (●). The thermal stabilities were determined by pre-incubating the crude extract at 4°C. Aliquots were withdrawn at regular time intervals after incubating, and then the residual activities were measured at 30°C. The activity of the enzyme before pre-incubation was taken as 100%.

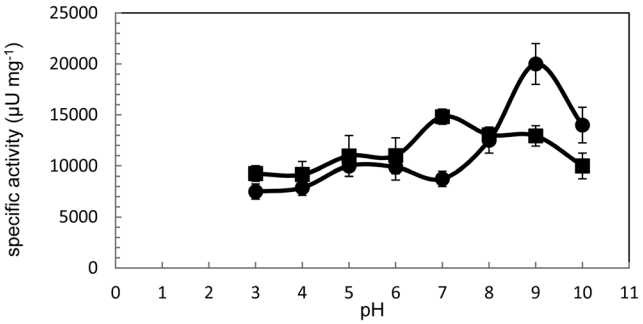


Fig. 6. pH optimums of acyltransferase activities (■ acetyltransferase, ● benzoyltransferase).
pH optimums were measured by realizing the enzymatic reaction at different pH values at 30°C, and then the specific activities were measured. Assays were carried out in triplicates with error bars shown in the graphs.

At 72 h, they were 16% and 28% for acetyltransferase and benzoyltransferase respectively.

Effect of pH on enzyme activity. Both acyltransferase activities were active in the pH range of 3–10, with an optimum at pH 7 and 9 for acetyltransferase and benzoyltransferase, respectively (Fig. 6). The specific activities at pH 7.0 and 9.0 were about 114% and 160%, respectively, when the activity at pH 8.0 was considered as reference.

Effect of pH on enzyme stability. The result indicates that although both enzymes were affected by the pre-incubation at 4°C for 24 h in the pH range of 3–10, they were still active in these conditions (Fig. 7). Acetyltransferase was more stable in the acidic and neutral media, with maximum residual activity of 44% at pH 7. In contrast, benzoyltransferase activity was more stable in the basic

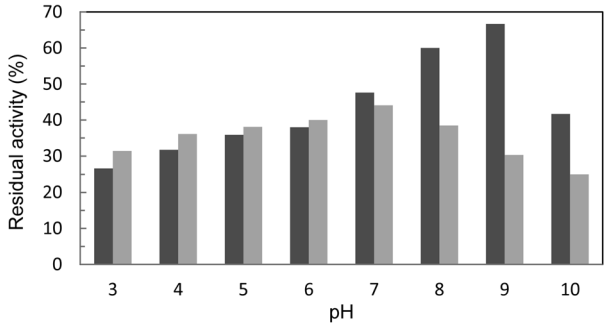


Fig. 7. Effect of pH on acyltransferase stabilities, (acetyltransferase activity, gray column; benzoyltransferase activity, black column).
The pH stabilities were determined by pre-incubating the enzymes in different pH values for 24 h at 4°C, and then the residual activities were measured at 30°C. The activities of the enzyme before pre-incubation were taken as 100%.

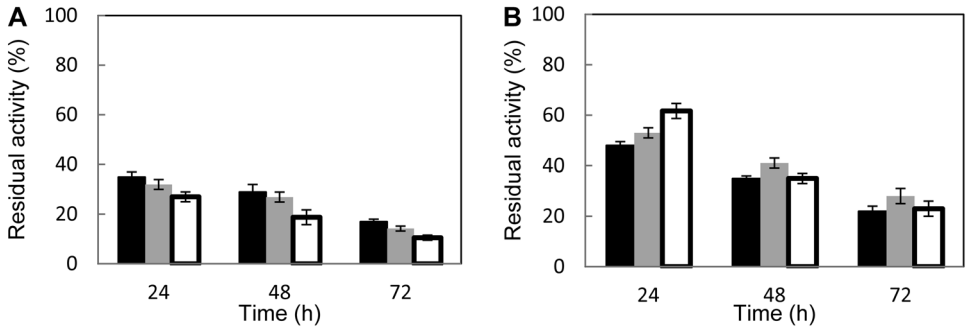


Fig. 8. Effect of stock time on acyltransferase stabilities according to different pH values.
(**A**) Acetyltransferase; (**B**) benzoyltransferase; pH = 7 black column; pH = 8 gray column; pH = 9 white column. The pH stabilities were determined by pre-incubating the enzymes in three different pH values for 24, 48, and 72 h at 4°C, and then the residual activities were measured at 30°C. The activities of the enzyme before pre-incubation were taken as 100%.

medium with maximum residual activity of 44% at pH 9.

However, the pH stability profile showed that the benzoyltransferase activity was more affected by the change of pH than acetyltransferase. The loss of activity between the maximal and the minimal benzoyltransferase activities was of 40%, whereas it was 19% for acetyltransferase activity.

Effect of stock time on acyltransferase stability. The stability of both acyltransferase activities were investigated for 3 days in three pH values, which represented the best stability values 7, 8, and 9 at +4°C. The result revealed that whatever the pH value, acetyltransferase lost more than 65% of its activity after 24 h of incubation. Then it seemed to be more stable, where the loss of the activity between 24 and 72 h was 50% (Fig. 8A). Concerning benzoyltransferase activity, the maximal loss of its activity, more than 40%, accrued after 24 h, then it decreased progressively, and at 72 h the residual activity was more than 20%. Acetyltransferase presented the best stability at pH 7 during the 3 days (Fig. 8A), whereas benzoyltransferase was more stable at pH 9 only for the first day, and then it was more stable at pH 8 (Fig. 8B).

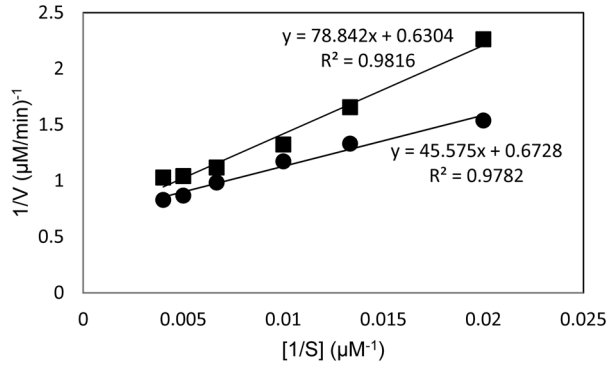


Fig. 9. Lineweaver-Burk plot for acyltransferase activities at 30°C and pH 8 (■ acetyltransferase; ● benzoyltransferase).

Enzymatic kinetic parameters. The kinetic model of the global enzymatic activity using acyl-CoA as a reaction limited substrate could be expressed using simple Michaelis-Menten kinetics. The resulted plot has a slope equal to K_m/V_{max} and an intercept equal to $1/V_{max}$. The values of V_{max} and K_m were 0.63 µmole/min and 7.9 µM for acetyltransferase; and 0.67 µmole/min and 14.7 µM for benzoyltransferase (Fig. 9).

Discussion

Sa. algeriensis produced either natural dithiolopyrrolone products or unnatural products, using the precursor-directed biosynthesis (PDB) method. Thus 14 dithiolopyrrolone derivatives belong to the pyrrothine and holothin families with different chain length of acyl groups identified [4, 18, 23, 24]. This extraordinary flexibility of the dithiolopyrrolone biosynthetic pathway in *Sa. algeriensis* may be rendered by the enzymatic system, which was reported to be responsible for this diversity by incorporating different acyl groups at pyrrothine or holothin cores [3, 7]. In *Sa. algeriensis*, two acyltransferase activities, pyrrothine *N*-acetyltransferase and pyrrothine *N*-benzoyltransferase, were proposed to be responsible for the last step of thiolutin and benzoyl-pyrrothine biosynthesis, respectively [7].

Herein, we investigated, *in vitro*, the enzymatic synthesis of different dithiolopyrrolone derivatives using the crude extract of *Sa. algeriensis* and the acyl-CoA as donor groups with different length of acyl moiety and two forms (linear and cyclic) of the acyl. Pyrrothine and holothin were used as substrates. Then, we investigated the characterization of

the two acyltransferases, acetyltransferase and benzoyltransferase, responsible for the incorporation of linear and cyclic acyl groups, respectively.

First, the enzymatic synthesis of dithiolopyrrolone derivatives *via* the crude extract of *Sa. algeriensis* obtained after 72 h of growth on SSM led to the synthesis of nine dithiolopyrrolone derivatives, three of them that have never been naturally produced in *Sa. algeriensis* (acetoacetyl-pyrrothine, hydroxybutyryl-pyrrothine, and demetyl thiolutin (holomycin)). These data are consistent with fermentation reports on alternative acyl chain incorporations on the amino group of the holothin scaffold and the detection of longer acyl chain variants as natural products, notably the octanoyl-holomycin framework [21, 28]. Interestingly, when pyrrothine and holothin were used as substrates with benzoyl-CoA, benzoyl-pyrrothine and benzoyl-holothin were detected in the reaction medium. Furthermore, for longer-chain acyl (lauroyl group), as well as for branched cyclic groups (phenylacetyl group), no products were detected. Recombinant ORF3483 from *S. clavuligerus* produced very small amounts of benzoyl-holothin. However, this enzyme was able to use longer-chain acyl CoAs (hexanoyl-, octanoyl-, and palmitoyl-CoA) as substrates much less efficiently. For example, an apparent K_m of 30 μM was obtained for octanoyl-CoA in the presence of 20 μM holothin [21]. However, the holomycine biosynthetic gene cluster in two bacteria *S. clavuligerus* and *Yersinia ruckeri* revealed the presence of one gene to encode such enzyme, but these bacteria have never been reported to produce other dithiolopyrrolones apart the holomycin in all conditions [14, 21, 27].

Second, biochemical characterization of these two acyltransferase activities in the cell-free extract of *Sa. algeriensis* was investigated. The activities were measured in reaction mixtures of 50 mM Tris-HCl buffer with varying pH values. The optimum pH was found to be 7 and 9 for acetyltransferase and benzoyltransferase activity, respectively. However, at basic pH, both enzymes were more active than in acidic pH. Other acyltransferases seem to share this basic optimum pH, where spermidine/spermine acetyltransferase from *Streptomyces* sp. 139, ornithine acetyltransferase from *S. clavuligerus*, and streptothricin acetyltransferase from *S. lividans* were maximal at pH of 7.5, 7.8, and 8.5, respectively [1, 10, 17]. Furthermore, in *S. murayamaensis*, a kinamycin acetyltransferase I was more active in slightly acidic pH and its optimum pH was found to be 6.2 [11].

Our results for long-term stability at +4°C for both enzymes indicated that benzoyltransferase was more stable than the acetyltransferase at the three chosen values of pH 7, 8, and 9. Benzoyltransferase residual activity was maximal at pH 9 only at the first day, and this activity became more stable at pH 8 for the second and the third days. The acetyltransferase residual activity was maximal at pH 7 along the 3 days. Consequently, the long-term stability of the two enzymes appeared to be different in this pH range.

Benzoyltransferase activity was more thermostable than the acetyltransferase over 60 min until 30°C. Above this temperature, rapid inactivation occurred for benzoyltransferase, and at 50°C it seemed to be inactive, whereas acetyltransferase was still active. Similar stabilities were observed for other acetyltransferases under the same conditions, such as spermidine/spermine acetyltransferase, which was stable at 35°C and became inactive at 50°C [17]. BEBT, which catalyzes the formation of benzylbenzoate, was 100% stable for 30 min at 30°C and 80% stable for 30 min at 37°C. After incubation at 50°C for 30 min, the enzyme was completely inactivated [8].

Although benzoyltransferase appeared more sensitive to temperature (*i.e.*, at 50°C benzoyltransferase was inactive whereas acetyltransferase was still active), its optimum temperature was higher than the acetyltransferase; for the former it was 55°C, whereas it was 40°C for the latter. The optimum temperatures for known acetyltransferases were near to this and found for the pyrrothine *N*-acetyltransferase investigated. The optimum temperature measured in 0.1 mM Tris-HCl buffer (pH 7.5) was found to be 37°C for spermidine/spermine acetyltransferase and ornithine acetyltransferase [1, 10].

A Lineweaver-Burk representation of the acetyltransferase and benzoyltransferase activities on increasing concentrations of acetyl-CoA (50 to 200 μM) and benzoyl-CoA (50 to 200 mM) indicated that K_m and V_{max} were in the same range for both enzymes. These parameters were also in the same range as those found for holomycin synthase, which catalyzed the acylation of the holothin core in *S. clavuligerus* [21]. Spermidine/spermine acetyltransferase also had a K_m of 45.7 μM when acetyl CoA used as limited substrate [1]. The K_m value of BEBT for the substrate benzoyl-CoA was 20.5 μM [8].

As a consequence, this study provided new insights into dithiolopyrrolone synthesis. To date, the presence of more than one acyltransferase in *Sa. algeriensis* has never been proofed by purification of these enzymes. However, the temperature and pH profiles of these acyltransferases in the cell-free extract of *Sa. algeriensis* indicated clearly that the two enzymes have different kinetic constants and optimal pHs and temperatures. These results support the

hypothesis of the presence of at least two acyltransferases responsible for formation of dithiolopyrrolones in *Sa. algeriensis*.

The presence of such derivatives *in vitro* and *in vivo* in *Sa. algeriensis* reveals the importance of *Sa. algeriensis* as a source of dithiolopyrrolones production. This could be rendered by the enzymatic system flexibility and by the capacity of *Sa. algeriensis* to adapt to the culture medium composition to synthesize different acyl CoAs.

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