Expression of pyrroidine N-acyltransferase activities in *Saccharothrix algeriensis* NRRL B-24137: new insights into dithiolopyrroline antibiotic biosynthetic pathway

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Keywords
antibiotic synthases regulation, dithiolopyrroline biosynthesis, *Saccharothrix algeriensis*

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doi:10.1111/j.1365-2672.2009.04496.x

Abstract

Aims: The hypothetical dithiolopyrroline biosynthetic pathway includes a final step of pyrrothine nucleus acylation. The presence of an enzymatic activity catalysing this reaction was investigated in *Saccharothrix algeriensis* NRRL B 24137. To understand the effect exerted by organic acids on the level of dithiolopyrroline production, their influence on enzymatic expression was studied.

Methods and Results: The transfer of acetyl CoA or benzoyl CoA on pyrrothine was assayed in the cell free extract of *Sa. algeriensis* NRRL B 24137. This study reports the presence of an enzymatic activity catalysing this reaction that was identified as either pyrrothine N acetyltransferase or N benzoyltransferase. The stimulation of benzoyl pyrrothine (BEP) production by addition of benzoic acid at 1-25 mmol l⁻¹ into the culture medium was demonstrated, and results showed that under the same conditions of growth, pyrrothine N benzoyltransferase specific activity was doubled.

Conclusions: This study shows that BEP production is enhanced in the presence of benzoic acid partly because of an induction of pyrrothine N benzoyltransferase.

Significance and Impact of the Study: The antitumor and antibiotic properties of dithiolopyrrolines are related to their variable acyl groups. New insights into regulation of biosynthetic pathway, especially the step of pyrrothine acylation, could lead after further studies to yield improvement and to selective production of dithiolopyrrolines with new biological activities.

Introduction

*Saccharothrix algeriensis* NRRL B 24137 is a filamentous bacterium that produces bioactive metabolites belonging to the dithiolopyrroline class of antibiotics (Lamari et al. 2002a,b; Zitouni et al. 2005). Dithiolopyrrolines possess a common pyrroline monothiole nucleus linked to two variable groups R¹ and R² (Fig. 1a). *Saccharothrix algeriensis* NRRL B 24137 produces at least six pyrrothine derivatives characterized by their different N acyl groups R²: thiolutin (acetyl pyrrothine), senecioyl pyrrothine, tigloyl pyrrothine, isobutyryl pyrrothine, butanoyl pyrrothine and benzoyl pyrrothine (BEP) (Lamari et al. 2002a,b) (Fig. 1b). Pyrrothine derivatives differ from holothin derivatives by the presence of a methyl radical R¹ linked to the N₄ cyclic nitrogen.

Many bacteria have been identified as dithiolopyrrolines producers among the genera *Streptomyces* (Celmer et al. 1952; Bhide et al. 1960), *Xenorhabdus* (Li et al. 1995) and *Alteromonas* (Shiozawa et al. 1993); however, *Sa. algeriensis* NRRL B 24137 is the first producer identified within the *Saccharothrix* genus. In addition, naturally occurring and other dithiolopyrrolines can also be produced by multiple step chemical synthesis (Buch and Lukas 1964; Hagio and Yoneda 1974; Ellis et al. 1977; Hjelmaa et al. 2007; Li et al. 2007).
Dithiolopyrrolotones exhibit broad spectrum antibiotic activity against a variety of Gram negative and Gram positive bacteria, fungi (Lamari et al. 2002a) and even protozoa and insects (Mc Inerney et al. 1991). They also inhibit allergy (Stahl et al. 1988) and platelet aggregation (Ninomiya et al. 1980). Moreover, dithiolopyrrolotones generated new interests in the 1990s after the discovery of their antitumour properties. Thiolutin suppresses tumour cell induced angiogenesis (Minamigushi et al. 2001), and a variety of dithiolopyrrolotones show strong cytotoxic activities against human cancer cell lines, especially breast, colon and cervical ones (Webster et al. 2000). Guo et al. (2008) have recently described some new chemically synthesized dithiolopyrrolotones that promote production of white blood cells. They could be useful in the prevention and treatment of microbial infections such as HIV infections and for the treatment of blood disorders.

Biological activity of dithiolopyrrolotones is strongly influenced by the nature of variable groups (Oliva et al. 2001; Chen et al. 2006; Li et al. 2007; Guo et al. 2008). As a consequence, little is known about the dithiolopyrrolocone biosynthetic pathway to date. A single putative pathway was described by Furumai et al. (1982) (Fig. 2). L. cystine has been identified as the potential precursor of pyrrothine nucleus. Amide bond formation between pyrrothine and an activated organic acid would lead to dithiolopyrrolocone synthesis. The presence of one enzymatic activity that catalyses amide bond formation between the holothine nucleus (deacetyl holomycin) and acetyl CoA was confirmed in cell free extracts of Streptomyces clavuligerus mutants, which are producers of holomycin (De la Fuente et al. 2002), but the enzyme responsible for this activity has not been purified yet. Additionally, the influence of medium composition has provided some information on the biosynthetic pathway in Sa. algeriensis NRRL B 24137. Addition of supposed precursors into the culture medium, such as organic and amino acids, led to modifications in dithiolopyrrolocone production levels (Bouras et al. 2006a,b, 2007) and to precursor directed biosynthesis of new dithiolopyrrolocone analogues (Bouras et al. 2008). However, little is known about enzymes involved in the biosynthesis of dithiolopyrrolotones by this strain.

To get a better understanding of dithiolopyrrolocone biosynthetic pathway in Sa. algeriensis NRRL B 24137, the enzymatic reaction of pyrrothine nucleus acylation (pyrrothine N acyltransferase activity) was investigated. For the first time, this study looks into the mechanism involved in the precursor directed biosynthesis of dithiolopyrrolotones by Sa. algeriensis NRRL B 24137. It leads to new insights into biosynthetic pathway regulation and thus could result in production yield improvement and to selective production of new and specific dithiolopyrrolotones.

**Materials and methods**

**Producing strain**

*Saccharothrix algeriensis* NRRL B 24137 (=DSM 44581) was used for this study. A stock of spores was prepared to maintain the strain. *Saccharothrix algeriensis* NRRL B 24137 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, MA), harvested and stored in 25% glycerol (Fisher) at −24°C.
Culture media

ISP2 was composed (per litre of distilled water) of 10 g d (+) glucose (Acros, Geel, Belgium), 10 g malt extract (Difco), 4 g yeast extract (Difco) and 18 g agar (Difco). The pH was adjusted to 7 with 2 N NaOH (Sigma) before autoclaving for 20 min at 121°C.

Semi synthetic (SS) medium (SSM) developed in our laboratory was used for growth and antibiotic production (Bouras et al. 2006a) with 15 g l⁻¹ of d (+) glucose (Acros). Organic acids were autoclaved separately and added aseptically to the culture medium, at the required concentration, before inoculation. The effect of organic acids on the time course of antibiotic production and enzymatic expression was investigated with 1·25 mmol l⁻¹ of benzoic acid (Acros) or 5 mmol l⁻¹ of acetic acid (Sigma). The influence of organic acid concentration on maximal enzymatic expression was studied by the addition of benzoic acid at concentrations of 0, 0·5, 2·5 and 5 mmol l⁻¹ and by the addition of acetic acid at concentrations of 0, 2·5, 5 and 7·5 mmol l⁻¹.

Culture conditions

ISP2 agar plates were inoculated with 50 µl of spore stock, i.e. 2 × 10⁵ CFU, and incubated for 7 days at 30°C for spore production. Precultures were inoculated with spores harvested from one ISP2 agar plate. They were grown in 250 ml Erlenmeyer flasks containing 50 ml of SSM and incubated during 48 h. Cultures were grown in 500 ml Erlenmeyer flasks containing 100 ml of SSM, which was, in some cases, supplemented with organic acids. They were inoculated with 5 ml of homogenized preculture. All liquid cultures were incubated at 30°C and 240 rev min⁻¹ on a rotary shaker (New Brunswick Scientific Company, New Brunswick, NJ, USA).

Dry cell weight measurement

Culture broth samples of 4 ml were taken every 12 h of fermentation and centrifuged at 16 000 g for 10 min in preweighed Eppendorf tubes (centrifuge Biofuge pico; Heraeus Instruments, Hanau, Germany). Dry cell weights (DCWs) were determined as described by Bouras et al. (2006a) and expressed in g l⁻¹.

Dithiopyrrolone extraction

A volume of 3·2 ml of supernatant fluid was extracted twice with 1·6 ml of dichloromethane. Organic phases containing antibiotics were pooled and dried under vacuum in a speed vac (Genevac, Ish, UK) at a temperature maintained under 40°C. Dry extracts were dissolved in 0·8 ml of methanol (HPLC grade; Fisher) for dithiopyrrolone quantification by high performance liquid chromatography (HPLC).

Pyrothine synthesis

6 amino 4 methyl [1,2]dithiolo[4,3 b]pyrrol 5 one hydro chloride, also called pyrothine, was synthesized by multiple step chemical synthesis using the protocol of Hjelmaa et al. (2007) with slight modifications as described in Fig. 3. Especially, the methyl group on cyclic nitrogen was introduced during the step (b) by substituting p methoxybenzylamine by methylamine. Finally, the amide moiety of the trifluoroacetyl pyrothine (5) was hydrolysed (f) in methanol in the presence of concentrated HCl to afford the pyrothine hydrochloride (6) that

![Figure 3 Pyrothine (6) synthesis adapted from Hjelmaa et al. (2007)](image-url)

Steps (a) sodium methoxide (NaOMe, 2 eq.), p methoxybenzylthiol (PMBSH, 2 eq.), ethanol, reflux, 100%, (b) methylamine (MeNH₂), Et₂N, THF, TiCl₄, 0°C to reflux (c) oxalyl chloride (2 eq.), Et₂N, THF, 31·4% (d) ammonium acetate, ethanol, reflux, 53·6% (e) m cresol (10 eq.), TFA, reflux, 42·3% (f) HCl(aq), MeOH, reflux, 49·6%.
was recovered by filtration. The $^3$H NMR spectrum, obtained on a Bruker DPX 300 MHz spectrometer, confirmed that the precipitate is pyrrothine hydrochloride: $\delta_{H}$ (DMSO, $d_6$): 7-41 (1H, s, C=CH) 3-9 5-3 (1H, br s, NH$_2$) 3-27 (3H, s, CH$_3$).

Cell free extract preparation

Saccharothrix algeriensis NRRL B 24137 was grown in SSM or SSM supplemented with organic acids as described in the section 'Culture conditions'. The biomass was recovered by centrifugation of culture broth at 5000 g for 15 min (4K15; Sigma), then washed twice with physiological water (0.9% NaCl) and once with lysis buffer (Tris HCl 50 mmol l$^{-1}$, pH 8). Wet cells were finally recovered by filtration on 0.2 μm membrane filters (Advantec, Dublin, Ireland). Wet cells (0.6 g) were resuspended in 1 ml of lysis buffer and transferred to a Fast Protein Blue tube (MP Biomedicals, Irvine, CA, USA). Two disruption cycles (30 s, 5 m s$^{-1}$) were carried out in a Fast Prep disruptor (MP Biomedicals). The lysing matrix was discarded, and then the sample was centrifuged at 10 000 g for 30 min (centrifuge 1 15K; Sigma) to remove the cell debris. The supernatant constituting the soluble cell free extract of Sa. algeriensis NRRL B 24137 was used immediately for the assay of pyrrothine N acyltransferase activity. A sample was frozen at −80°C for further protein assays. Samples were always kept in ice and centrifugations were carried out at 4°C.

Pyrrothine N acyltransferase assay

Pyrrothine N acyltransferase activity was assayed in a final volume of 100 μl. The reaction mixture contained 80 μl of cell free extract in Tris HCl buffer (50 mmol l$^{-1}$, pH 8), 10 μl of acetyl or benzoyl coenzyme A at 5 mmol l$^{-1}$ in bidistilled water and 10 μl of pyrrothine hydrochloride at 25 mmol l$^{-1}$ in methanol. The reaction mixture was incubated at 30°C for 5 and 10 min. The reaction was stopped by adding fresh 2.5% w/v trichloroacetic acid (Fisher). The cell free extract was properly diluted with Tris HCl buffer (50 mmol l$^{-1}$, pH 8) before assay to observe a product formation linear in 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 is defined as the enzyme activity producing 1 μmol of thiolutin (acyl pyrrothine) or BEP per minute. Specific enzymatic activity was expressed in μU mg$^{-1}$ of protein.

Enzymatic activity was also expressed in mU g$^{-1}$ of DCW. A value of 162 mg of proteins per gram of dry cells was used to achieve conversion from U mg$^{-1}$ of proteins to U g$^{-1}$ of DCW. Protein content in dry cells was determined as follow. DCW and intracellular protein concentration were determined every 24 h, for 7 days, in a culture of Sa. algeriensis NRRL B 24137 on SSM. The plot of proteins in mg l$^{-1}$ vs DCW in g l$^{-1}$ indicated a linear correlation between the two parameters with a slope of 162 mg of proteins per gram of DCW ($R^2 = 0.91$).

HPLC analysis for dithiopyrroline quantification

Dithiopyrrolones were quantified by HPLC (Bio tek Instruments, Milan, Italy). The analytical column was ProntoSIL 120 5 C$_{18}$ SH, 150 × 4.6 mm (Bishoff Chromatography, Leonberg, Germany) fitted with a guard column of 10 × 4 mm, and detection was achieved with a diode array detector (UV vis 545 V; Bio tek instruments).

For detection and quantification of thiolutin and BEP produced in the culture medium, analyses were performed as described by Bouras et al. (2006a) with an injection volume of 80 μl.

For detection and quantification of dithiopyrrolone formed during enzymatic assay, analyses were performed under the following chromatographic conditions. Samples were analysed by linear gradient elution using a mixture of methanol/bidistilled water (solvant A/solvent B) as mobile phase and a flow rate of 0.8 ml min$^{-1}$. Column temperature was maintained at 30°C and injection volume was 40 μl. UV detection of antibiotics was carried out at 390 nm. For the acetyltransferase assay, elution was carried out with a linear gradient from 30% A to 47% A in 17 min. Pyrrothine and thiolutin retention times ($R_t$) were 7 and 12.3 min, respectively. For the benzoyltransf erase assay, the mobile phase composition was 30% A, reached 45% A in 15 min, 100% A in 35 min and was finally kept at this value for 2 min. Pyrrothine and BEP retention times ($R_t$) were 8 and 29 min, respectively.

Both antibiotics were quantified using a thiolutin standard calibration curve. Indeed, the molar absorbivity values at 390 nm of pyrrothine derivatives are close. ε$^{390}$ is in the range of 8317 9333 mol$^{-1}$ l cm$^{-1}$ as described by Lamari et al. (2002b).

The BEP chemical structure was confirmed by comparison of the UV spectrum obtained with those mentioned by Bouras et al. (2008). The UV spectrum of BEP yielded $\lambda_{max}$ in nm (relative absorbance): 231 (1), 309 (0.45), 401 (0.68).

Replication of experiments and statistical analysis

Data of growth and dithiopyrroline production represent the average of triplicate flasks, and error bars denote standard errors. Values of acyltransferase specific activities
at 24 and 48 h on SSM, SSM with benzoic acid at 125 mmol l\(^{-1}\) and SSM with acetic acid at 5 mmol l\(^{-1}\) are the averages of four independent experiments, and error bars denote standard errors. Other pyrroline N acyltransferase specific activities are single values, and error bars denote a standard error of 15%, estimated from previous replications.

Statistical analyses were achieved using KALEIDAGRAPH 4.0 software (Synergy Software, Reading, PA, USA).

**Results**

**Involvement of pyrroline N acyltransferase activities in thiolutin and BEP production**

N acyltransferase enzymatic activity was assayed in the cell free extract of *Sa. algeriensis* NRRL B 24137 grown on SSM. A formation of thiolutin or BEP, linear in time within 10 min, was monitored by HPLC when acetyl CoA and benzoyl CoA were respectively used as acyl group donors (Fig. 4). No thiolutin or BEP was observed in the absence of one of the components of the reaction mixture, i.e., acyl CoA, cell free extract or pyrroline. Hence, it can be concluded that both pyrroline N acetyltransferase and pyrroline N benzoyltransferase enzymatic activities are present in the cell free extract of *Sa. algeriensis* NRRL B 24137.

Biomass, thiolutin and BEP productions on SSM

DCW and dithiolopyrrolone concentrations were quantified during growth on SSM (Fig. 5a). Time courses of specific growth rate, thiolutin specific production and BEP specific production on SSM are shown in Fig. 6a.

Two distinct periods of growth were observed. Each growth period is characterized by an increase in the specific growth rate followed by a decrease in the same. Between 0 and 48 h, specific growth rate reached the maximal value of 0.096 h\(^{-1}\) at 12 h and then decreased up to 0.010 h\(^{-1}\) at 48 h. At the end of this first period of growth, a DCW of 1.2 g l\(^{-1}\) was observed. Between 60 and 96 h, the specific growth rate increased again to the maximal value of 0.022 h\(^{-1}\) at 60 h and then decreased to zero at 96 h. The maximal specific growth rate during this second period was 4-4 times lower than the earlier one. The maximal level of DCW, 2.5 g l\(^{-1}\), was attained at 96 h. Biomass finally dropped after 108 h probably because of cell lysis.

Thiolutin and BEP production started at 24 and 36 h of culture, respectively. At 72 h of culture, the maximal level of thiolutin, 25.6 mg l\(^{-1}\), was observed. Then, the thiolutin concentration in the culture medium decreased. A BEP production of 0.15 mg l\(^{-1}\) was reached as early as 48 h, i.e. 80% of the maximal BEP concentration, 0.18 mg l\(^{-1}\), observed only at 144 h. Maximal specific productions of thiolutin and BEP were reached after 48 h of culture at the end of the first growth period, when the specific growth rate was at its lowest level, 0.010 h\(^{-1}\). Maximal specific productions of thiolutin and BEP were respectively observed for thiolutin and BEP. The maximal specific production rate of thiolutin, 0.70 mg g\(^{-1}\) h\(^{-1}\), was reached at 24 h and the maximal specific production rate of BEP, 0.006 mg g\(^{-1}\) h\(^{-1}\), was observed at 36 h when the specific growth rate was reduced to 26.5 and 12% of its maximal level, respectively.

Addition of benzoic acid and acetic acid to the culture medium

Time courses of growth, thiolutin production and BEP production on SSM completed with benzoic acid at

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**Figure 4** Conversion of pyrroline to dithiolopyrrolones. (a) Conversion of pyrroline (R\(_t\) = 7 min) to thiolutin (R\(_t\) = 12.3 min) and (b) Conversion of pyrroline (R\(_t\) = 8 min) to benzoyl pyrroline (R\(_t\) = 29 min). (1) Reaction scheme. (2-4) Dithiolopyrrolone and pyrroline present in the reaction at 0 (2), 5 (3) and 10 min (4), as measured by high performance liquid chromatography. Absorbance is given in mV min\(^{-1}\) and time in minutes.

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1:25 mmol l⁻¹ are shown in Fig. 5b. Time courses of specific growth rate, thiolutin specific production and BEP specific production on SSM supplemented with benzoic acid at 1:25 mmol l⁻¹ are shown in Fig. 6b. A maximal BEP production of 8:47 mg 1⁻¹ was observed at 72 h. This is 56 times more than on SSM. We observed a BEP maximal specific production of 4:77 mg g⁻¹ at 60 h, i.e. a value 39 times higher than without benzoic acid in culture medium. Additionally, the maximal specific production rate of BEP, 0:15 mg g⁻¹ h⁻¹, at 36 h, was 25 times higher than the one on SSM. By contrast, thiolutin production and growth were not significantly modified by benzoic acid addition.

DCW and dithiopyrrolone concentrations were also quantified in a Sa. algeriensis NRRL B 24137 culture on SSM completed with acetic acid at 5 mmol l⁻¹ (Fig. 5c). Specific growth rate, thiolutin specific production and BEP specific production are shown in Fig. 6c. A maximal thiolutin concentration of 9.68 mg l⁻¹ was observed at 72 h, which is 2.6 times less than on SSM. Data also showed that thiolutin specific production reached a maximal value of 6.53 mg g⁻¹ at 48 h, i.e. three times lower than that on SSM. The maximal specific production rate of thiolutin was also reduced by acetic acid addition. The value of 0.23 mg g⁻¹ h⁻¹, three times lower than the one on SSM, was reached at 24 h. On the contrary, acetic acid
addition did not significantly modify BEP production and growth.

Expression of pyrothine N acyltransferase activities on SSM

Time courses of acetyltransferase and benzoyltransferase specific activities during growth on SSM are shown in Fig. 7a. Acetyltransferase specific activity peaked at 24 h and the peak value was 18 823 μU mg⁻¹ of protein. Then, enzymatic specific activity decreased sharply. At 48 h, the expression was already seven times lower.

Benzoyltransferase activity was detected from 24 h and reached its maximal value of 9450 μU mg⁻¹ of protein after 48 h of culture, while acetyltransferase specific activity was already reduced to 40% of its maximal level. Then it decreased down to 1760 μU mg⁻¹ at 84 h. At 96 h of culture a new increase in specific activity was observed until the maximal value of 3690 μU mg⁻¹ of protein was reached. Then activity decreased down to 1800 μU mg⁻¹ at 144 h and remained stable until 168 h of culture.

From both profiles comparisons, it is particularly noteworthy that acetyltransferase and benzoyltransferase specific activities did not peak at the same time and that the ratio of both enzymatic specific activities was not constant throughout culture on SSM.

Acetyltransferase specific activity was also compared to the specific production rate of thiolutin (Fig. 8a). The time profile of acetyltransferase specific activity is tightly related to the time profile of thiolutin specific production rate. Both acetyltransferase specific activity and thiolutin specific production rate peaked at 24 h and decreased until 72 h.

**Figure 7** Time course of pyrothine N acyltransferase (■) and pyrothine N benzoyltransferase (□) specific activities during cultures of Saccharothrix algicola NRRL B 24137 on (a) SS medium (SSM), (b) SSM supplemented with benzoic acid at 1-25 mmol L⁻¹, (c) SSM supplemented with acetic acid at 5 mmol L⁻¹. Specific activities are given in μU mg⁻¹ of proteins and time in hours.

**Figure 8** Time course of pyrothine N acyltransferase specific activity (●) and rate of average dithiopyrrolone specific production (○) on SS medium. (a) Comparison of acetyltransferase specific activity and rate of average thiolutin specific production. (b) Comparison of benzoyltransferase specific activity and rate of average benzoyl pyrothine specific production. Pyrothine N acyltransferase specific activities are given in μU g⁻¹ of dry cell weight (DCW) and rates of average dithiopyrrolone specific production in mg h⁻¹ g⁻¹ of DCW. Time is given in hours.
Similarly, BEP specific production rate is tightly related to the level of benzoyltransferase specific activity (Fig. 8b). The peak of BEP specific production rate is associated with an increase in benzoyltransferase specific activity.

Expression of pyrothione N acyltransferase activities on SSM with organic acids

Acetyltransferase and benzoyltransferase specific activities were quantified during growth on SSM supplemented with benzoic acid at 1.25 mmol l⁻¹ (Fig. 7b) or SSM supplemented with acetic acid at 5 mmol l⁻¹ (Fig. 7c).

During growth on SSM with benzoic acid, benzoyltransferase specific activities were significantly higher than on SSM but the shape of the curve was not modified. Benzoyltransferase specific activity reached a maximal value of 20 881 μU mg⁻¹ of proteins at 48 h, i.e. 2.2 times higher than without benzoic acid. The second peak of benzoyltransferase specific activity observed at 96 h, like that on SSM, had a value of 15 920 μU mg⁻¹ of proteins, i.e. 1.8 times higher than without benzoic acid. On the contrary, acetyltransferase specific activities were not significantly modified by benzoic acid addition to culture medium.

Additionally, benzoyltransferase specific activity was also determined in cell free extracts obtained after 48 h of growth, on SSM with different concentrations of benzoic acid (Fig. 9a). Benzoyltransferase specific activity was two times higher with benzoic acid added to the culture medium than without, regardless of the benzoic acid concentration, which range from 0.5 to 2.5 mmol l⁻¹.

The time course of acetyltransferase specific activity was not significantly modified by acetic acid addition to SSM. Acetyltransferase specific activity reached a maximal value of 16 391 μU mg⁻¹ of protein at 24 h. Similarly, acetic acid had no effect on the time course of benzoyltransferase specific activity. The maximal benzyoltransferase specific activity, 9840 μU mg⁻¹ of protein, was reached at 48 h.

Acetyltransferase specific activity was also determined in cell free extracts obtained after 24 h of growth on SSM with different concentrations of acetic acid (Fig. 9b). Acetic acid had no effect on acetyltransferase specific activity regardless of the concentration of acetic acid added to the medium in the range from 2.5 up to 7.5 mmol l⁻¹.

Discussion

*Saccharothrix algeriensis* NRRL B 24137 has the ability to produce a wide range of dithiopyrrolones with different radicals (R2s) depending on the precursors added to the culture medium (Lamari et al. 2002a, b; Bouras et al. 2006a, 2008). To have such ability to produce many dithiopyrrolones with broad structural diversity, the microorganism must possess not only precursors but also a suitable enzymatic system with sufficient flexibility to attach a variety of R2 radicals on N₇. Data presented above provide evidence for the presence in the cell free extract of *Sa. algeriensis* NRRL B 24137 of an enzymatic activity that catalyses acylation of pyrothione nucleus on N₇ using acyl CoA as acyl group donors. Especially, acetyl CoA and benzoyl CoA are respectively substrates of a pyrothione N acetyltransferase and N benzoyltransferase enzymatic activity. These results suggest that the enzymatic reaction of pyrothione acylation takes part in the dithiopyrrolone biosynthetic pathway in *Sa. algeriensis* NRRL B 24137, which is able to use acyl CoA with very different structures (acytetyl CoA and benzoyl CoA), as substrates to produce corresponding dithiopyrrolones (thiolulin and BEP). The presence of a similar enzymatic activity, called holomycin synthase, catalysing the amide bond formation between holothin (deacetyl holomycin)

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**Figure 9** Influence of acid concentration on *N* pyrothione acyltransferase activities. (a) Influence of benzoic acid concentration in SS medium (SSM) on pyrothione *N* benzoyltransferase specific activity at 48 h. (b) Influence of acetic acid concentration in SSM on pyrothione *N* acetyltransferase specific activity at 24 h. Specfic enzymatic activities are given in μU mg⁻¹ of proteins and acid concentrations in mmol l⁻¹.
and acetyl CoA was already observed in the cell free extracts of *S. clavuligerus* mutants, which are producers of holomycin (De la Fuente et al. 2002). However, the enzyme responsible for this activity has not been purified yet. In contrast, the existence of *N* benzoyltransferase activity involved in dithiolopyrrolone synthesis has never been reported before.

To understand the regulatory mechanisms exerted on these new enzymatic activities, the time courses of pyrrothine *N* acetyltransferase and pyrrothine *N* benzoyltransferase specific activities were determined in the cell free extract of *Sa. algeriensis* NRRL B 24137 and compared to the time courses of formation of thiolutin, BEP and growth.

Data shown in this study indicate that the dithiolopyrrolone production phase is partly growth associated during culture of *Sa. algeriensis* NRRL B 24137 on SSM and occurs during a decrease in the specific growth rate. These results contrast with the traditional idea that the antibiotic production phase, called the idiophase, is separated from the growth phase, called the trophophase. However, several studies have shown that culture conditions supporting slow growth rates, e.g., nutritional limitations, can elicit an antibiotic production partly associated with growth (Martin and Demain 1980; McDermott et al. 1993; Fazeli et al. 1995; Untrau Taghian et al. 1995). Besides, studies on antibiotic production using chemostat cultures demonstrated that production can occur in association with growth although antibiotic production level is higher at low dilution rates (Vu Trong and Gray 1981; Lebrhi et al. 1988; McIntyre et al. 1996; Pamboukian and Facciotti 2004).

In addition, a noticeable decrease in thiolutin concentration in the culture medium is observed after 72 h of growth on SSM. It is associated with a decrease in antimicrobial activities against *Mucor ramannianus* and *Bacillus subtilis* (L. Lamari, unpublished data). Thiolutin could undergo a physical or a chemical transformation (Yoshioka and Stella 2007). This compound contains in particular a disulfide bond, which could be chemically broken with a relative ease (Parker and Kharasch 1959; Kice 1968). Thiolutin could also be converted enzymatically as already described in the literature for other antibiotics. Enzymatic conversion could be either extracellular or intracellular after reactivation of thiolutin by the cells (Argoudelis and Mason 1969; Perlman and Sebek 1971). However, no mechanism of thiolutin transformation has been identified yet.

Pyrrothine *N* acyltransferase activity was detected throughout the culture but was only overexpressed during a very short time that coincides with the peak of antibiotic productivity (i.e. specific production rate). Such expression profiles have been previously observed for different antibiotic syntheses (Ortmann et al. 1974; Nimi et al. 1981; Brana et al. 1985). It is an illustration, at the biochemical level, that antibiotic synthesis is very tightly regulated as it has been reported in the study of antibiotic gene clusters regulation in *Streptomyces* sp. (Cundliffe 2006; Rokem et al. 2007).

*Saccharothrix algeriensis* NRRL B 24137 showed a valuable capability to produce new dithiolopyrrolone analogues when adequate precursors were added into the culture medium (Bouras et al. 2008). Organic acid addition promoted the production of new dithiolopyrroloynes possessing a R2 radical either identical to or different from organic acid. Specifically, benzoic acid or cinnamic acid addition led to BEP production and valeric acid addition resulted in valeryl pyrrothine production (Bouras et al. 2008).

In our study, we showed that BEP was produced at a very low level on SSM and we confirmed that BEP production was strongly increased by benzoic acid addition. Additionally, analysis of pyrrothine *N* benzoyltransferase specific activity showed that specific activity was markedly enhanced during growth in the presence of benzoic acid. Benzoic acid had no activator effect on benzoyltransferase specific activity when added in the benzoyltransferase assay (data not shown). These results suggest that stimulation of BEP production by benzoic acid is not only because of an additional precursor supply but also includes the induction of a biosynthetic enzymatic activity, i.e. pyrrothine *N* benzoyltransferase, responsible for the transfer of benzoyl CoA to pyrrothine.

Other antibiotic precursors that stimulate antibiotic production by inducing biosynthetic enzymes are known. A typical example is the stimulation of ergot alkaloid production by tryptophan. Krupinski et al. (1976) showed that tryptophan enhanced the alkaloid production of ergot by inducing dimethylallyltryptophan synthase, the first enzyme in the ergot alkaloid biosynthetic pathway.

In addition, earlier studies provided evidence that methionine, a precursor of cephalosporin C in *Cephalosporium acremonium*, induces enzymes of the cephalosporin C biosynthetic pathway such as δ (L, α-aminoacidyl) L cysteinyld proline synthetase, isopenicillin N synthase and deacetoxycephalosporin C synthase (Demain and Zhang 1998). Regulation seems to occur at the transcriptional level because the transcription of genes encoding the above enzymes was strongly increased by culture on methionine (Demain and Zhang 1998). Lastly, it has been shown that some enzymes involved in pathways supplying antibiotic precursors, especially amino acid catabolism pathways, are induced by their substrate. Riis et al. (1996) observed that lysine induced the L-Lysine ε amino transferase, an enzyme involved in the conversion of
lysine to \( \alpha \) amino adipic acid, a direct precursor of cephalosporins in *S. clavuligerus*. Besides, valine deshydrogenase, the first enzyme of valine catabolism that provides \( N \) butyrate, propionate and methyl malonate for macrolide biosynthesis is induced by valine in *Streptomyces aureofaciens*, *Streptomyces fradiae* and *Streptomyces avermitilis* (Nguyen et al. 1995a,b).

By contrast, acetic acid addition to the culture medium depressed thiolutin production despite the fact that it is a potential precursor for thiolutin synthesis. Our assays of the pyrrothine \( N \) acetyltransferase activity showed that acetic acid had no effect on its level of activity, supporting the idea that the decrease in thiolutin production was not because of repression of this enzymatic activity. Acetic acid had no inhibitor effect on acetyltransferase specific activity when added in the acetyltransferase assay (data not shown). As a consequence, the negative effect of acetate addition on thiolutin specific production remains to be elucidated but some hypothetical mechanisms can be put forth. Possible explanations include an inhibition of the acetyltransferase activity by a change in intracellular pH, a negative effect of acetate on another enzyme of the biosynthetic pathway or on enzymes involved in a pathway supplying antibiotic precursors. Modifications of transport systems or redirection of thiolutin precursors to primary metabolism might also account for a decrease in thiolutin production in the presence of acetate but it is unlikely because growth was not affected by acetate addition.

Lastly, some of the results presented in this study support the idea that the transfer reactions of acetyl CoA and benzyol CoA to pyrrothine are not catalysed by the same enzyme. The existence of a unique enzyme could not account for the variability in the ratio between both specific activities throughout the culture. Additionally, this idea is strengthened by the finding that the benzyol transferase activity level is specifically increased during culture with benzoic acid, whereas acetyltransferase activity is not modified.

The purification of enzymes catalysing the transfer reaction of acetyl CoA and benzyol CoA to pyrrothine is in progress in our laboratory in order to confirm the hypothesis that two different enzymes are involved. Additionally, the amino acid sequence determination of enzyme(s) would be an essential starting point for gene identification and further studies on biosynthetic pathway regulation. Further studies should also be carried out on *Sa. algeriensis* NRRL B 24137 nutritional requirements and on its behaviour in a controlled environment within a bioreactor to better understand the conditions of anti-biotic onset.

This study provides evidence for the presence of a pyrrothine \( N \) acetyltransferase activity in the cell free extract of *Sa. algeriensis* NRRL B 24137 involved in the dithiopyrrolone biosynthetic pathway. Our work also suggests that enhancement of BEP production by *Sa. algeriensis* NRRL B 24137 in the presence of benzoic acid is partly because of an increase in pyrrothine \( N \) benzyol transferase specific activity. As a consequence, this study provides new insights into dithiopyrrolone synthesis, especially its regulation and also gives valuable experimental tools to further our understanding of the dithiopyrrolone biosynthetic pathway.

**Acknowledgement**

Authors thank Professor Arnold L. Demain (Drew University, Madison, NJ, USA) for his critical reading of the manuscript and valuable suggestions.

**References**


