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Dissociation of cephamycin C and clavulanic acid biosynthesis by 1,3-diaminopropane in *Streptomyces clavuligerus*

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One sentence summary: The addition of 1,3-diaminopropane in the culture medium dissociates cephamycin C and clavulanic acid biosynthesis by *S. Clavuligerus*.

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ABSTRACT

Streptomyces clavuligerus produces simultaneously cephamycin C (CephC) and clavulanic acid (CA). Adding 1,3-diaminopropane to culture medium stimulates production of beta-lactam antibiotics. However, there are no studies on the influence of this diamine on coordinated production of CephC and CA. This study indicates that 1,3-diaminopropane can dissociate CephC and CA productions. Results indicated that low diamine concentrations (below 1.25 g l⁻¹) in culture medium increased CA production by 200%, but not that of CephC. Conversely, CephC production increased by 300% when 10 g l⁻¹ 1,3-diaminopropane was added to culture medium. Addition of just L-lysine (18.3 g l⁻¹) to culture medium increased both biocompounds. On the other hand, while L-lysine plus 7.5 g l⁻¹ 1,3-diaminopropane increased volumetric production of CephC by 1100%, its impact on CA production was insignificant. The combined results suggest that extracellular concentration of 1,3-diaminopropane may trigger the dissociation of CephC and CA biosynthesis in *S. clavuligerus*.

Keywords: *Streptomyces clavuligerus*; beta-lactam antibiotics; diamine; L-lysine; culture medium

INTRODUCTION

Streptomyces clavuligerus produces several β -lactam compounds, especially cephamycin C (CephC), a beta-lactam antibiotic highly resistant to beta-lactamases, and clavulanic acid (CA), a wide-range inhibitor of β -lactamases produced by bacteria resistant to penicillin and cephalosporin (Paradkar 2013). CephC and CA are produced at the same time during fermentation, albeit via entirely different biosynthetic pathways. These biosynthetic pathways are regulated by the same multifunctional element, which is encoded by the CCAR gene lo-

cated in the CephC biosynthesis gene cluster (Álvarez-Álvarez et al. 2014).

Several researchers have reported that the presence of biosynthesis inducers and precursors and carbon or nitrogen sources in the culture medium can favor the production of either biocompound. Adding soluble starch as carbon source or cottonseed extract as complex nitrogen source to the culture medium enhances CephC production (Antonio et al. 2012; Bellão et al. 2013). The use of glycerol and vegetable oils as carbon source or ornithine as nitrogen source increases CA production (Domingues et al. 2010; Teodoro et al. 2010; Bellão et al. 2013;

Salem-Bekhit, Alanazi and Alsarra 2013). Also, additions of diamines, L-lysine or alpha-aminoadipic acid promote the production of beta-lactam antibiotics in actinomycetes (Fang, Keables and Demain 1996; Leitão et al. 1999; Demain and Vaishnav 2006; Leite, Cavallieri and Araujo 2013). On the other hand, ammonium ions and inorganic phosphate can inhibit CephC and CA biosynthesis (Bibb 2005; Demain and Vaishnav 2006).

There are currently no available studies indicating full CA and CephC dissociation by manipulating the culture medium. However, this study has innovated by indicating that addition of appropriate concentrations of 1,3-diaminopropane promotes dissociated production of both bioactives. This data serve as a basis for conducting future transcriptional and/or proteomic analyses to improve the understanding of CephC and CA biosynthesis regulation. The results obtained herein may also have industrial applications by assisting in the formulation of media to produce either biocompound.

MATERIALS AND METHODS

Microorganism, culture medium and fermentation process conditions

The wild-type strain of *S. clavuligerus* ATCC 27064 was employed as the microorganism producing CephC and CA. Spores (ca. 10^8 spores ml^{-1}) were stored at -80°C in 2 ml cryotube vials (glycerol at 20% w v^{-1}) and allowed to germinate for 24 h in liquid medium containing (in g l^{-1}): tryptone (5), yeast extract (3), malt extract (10) and buffering agent 3-(N-morpholine) propanesulfonic acid (MOPS) (21), pH 6.8 ± 0.1 . A 10% (vol/vol) aliquot of this germinate was transferred to an inoculum medium composed of (g l^{-1}): soluble starch (10), undiluted cottonseed extract PROFLO® (8.5), yeast extract (1), K_2HPO_4 (0.8), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.75), MOPS (21) and 10 ml l^{-1} salt solution containing (in g l^{-1}) $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (1), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (1) and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (1.0), pH 6.8 ± 0.1 . After 24 h, 10% (vol/vol) of the inoculum was transferred to the main fermentation medium composed of (in g l^{-1}): starch (10), PROFLO (8.5) boiled down and filtered (using a vacuum pump), yeast extract (0.5), K_2HPO_4 (1.75), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.75), CaCl_2 (0.2), NaCl (2), MOPS (21), aforementioned salt solution (5 ml l^{-1}) and sodium thiosulfate (1) added at 30 h post-inoculation, pH 6.8 ± 0.1 . The percentage of nitrogen in the PROFLO filtrate corresponded to about 40% of gross PROFLO. All culture medium supplies were obtained from LabSynth (Diadema, Brazil) with the exception of PROFLO (Traders Protein, USA) and MOPS (Sigma-Aldrich Chem. Co, USA).

In order to investigate the effect of diamine on the regulation of CephC and CA biosynthesis, the concentration of 1,3-diaminopropane (Sigma-Aldrich Chem. Co., $\geq 99\%$, CAS # 109-76-2) 0.5–20 g l^{-1} was added at the beginning of fermentation. Further experiments were carried out using diamine at the same concentrations plus L-lysine hydrochloride (LabSynth, CAS # 56-87-1), a promoter of biosynthesis of beta-lactam compounds in *S. clavuligerus*. Several concentrations of L-lysine were added to the culture medium (0–18.3 g l^{-1}) in order to evaluate CA production. The effect of L-lysine at the same concentration on CephC production was already described by Leite, Cavallieri and Araujo (2013). All submerged cultures were performed in triplicate using a shaker incubator (28°C , 260 rpm/5cm eccentricity) and Erlenmeyer flasks (500 ml) with total medium volume of 50 ml. Samples were collected at 48 and 72 cultivation hours and centrifuged at 15,550 g for 10 min at 4°C for further analyses.

Biomass

Biomass was washed twice with deionized water and dried at 105°C for 24 h before measuring dry weight of cells (gDWC l^{-1}).

Measurement of CA

CA was quantified by means of High-performance Liquid Chromatography (HPLC) as described in Foulstone and Reading (1982).

Measurement of CephC

CephC concentration was determined by agar diffusion bioassay as described in Liras and Martín (2005). In this method, cephalosporin C zinc salt (Sigma-Aldrich Chem. Co., CAS #59143-60-1) was used as standard and CephC concentration was expressed in terms of total cephalosporin (CephC, in mg l^{-1}). *Escherichia coli* ESS 2235, a supersensitive organism to beta-lactam antibiotics, was employed as test organism. This strain was allowed to grow for 24 h in culture medium containing (in g l^{-1}): peptone (5), beef extract (3) and agar (15) at 37°C . All samples were treated with BD Difco™ Penase® (Beckton Dickinson, cat. No.: 215331) - 20 μL per ml of sample, reacting at 25°C for 20 min to remove Penicillin N.

Analysis of L-lysine consumption

The analysis of residual L-lysine in the culture medium was done by HPLC. It employed a Shim-pack Amino-Na ion-exchange column (6 mm \times 100 mm), 0.6 ml minute^{-1} flow, oven at 60°C and a refractive index detector (Model RID-10A/Shimadzu). The mobile phase used was composed of borate 0.05 M l^{-1} as Solvent A and NaOH 0.2 M l^{-1} as Solvent B. The elution gradient was as follows: 100% A, linear for 25 min; 0% B \rightarrow 100% B over 5 min; 100% B, linear for 10 min; 0% A \rightarrow 100% A over 5 min; 100% A, linear for 15 min. The average retention time was 20.4 min.

Analysis of 1,3-diaminopropane consumption

Residual diamine was analyzed through HPLC using isocratic method. It employed a Shim-pack Amino-Na ion-exchange column (6 mm \times 100 mm), NaOH 0.2 M l^{-1} as mobile phase, 0.6 ml minute^{-1} flow, oven at 60°C and RID-10A refractive index detector. The average retention time was 3.3 min.

RESULTS

Effect of L-lysine on CA biosynthesis

Figure 1 shows that the addition of L-lysine to the culture medium stimulates CA production and the amount of this amino acid added to the broth was directly proportional to CA biosynthesis. The highest L-lysine concentration 18.3 g l^{-1} increased CA biosynthesis about 400% compared to data from the medium without L-lysine (Fig. 1).

Effect of 1,3-diaminopropane on CephC and CA biosynthesis

Figure 2 shows that maximum biomass yield occurred at 48 h by adding 2.5 g l^{-1} 1,3-diaminopropane. It represents an increase of 46% as compared to that of the control medium (without

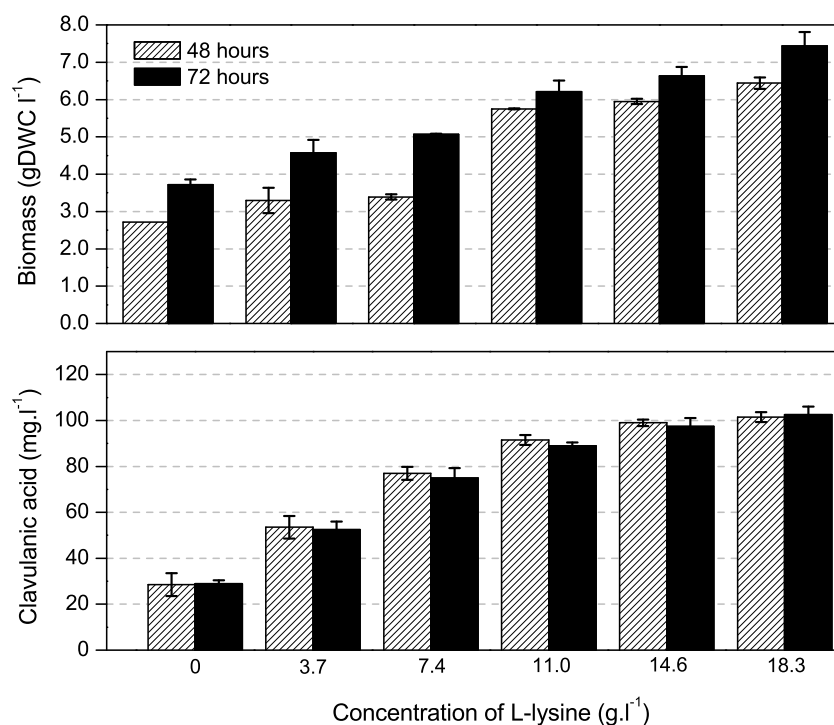


Figure 1. Biomass and CA obtained in *Streptomyces clavuligerus* culture in control medium without L-lysine (0) and with addition of L-lysine (3.7–18.3 g l⁻¹); all cultivations were done in triplicate.

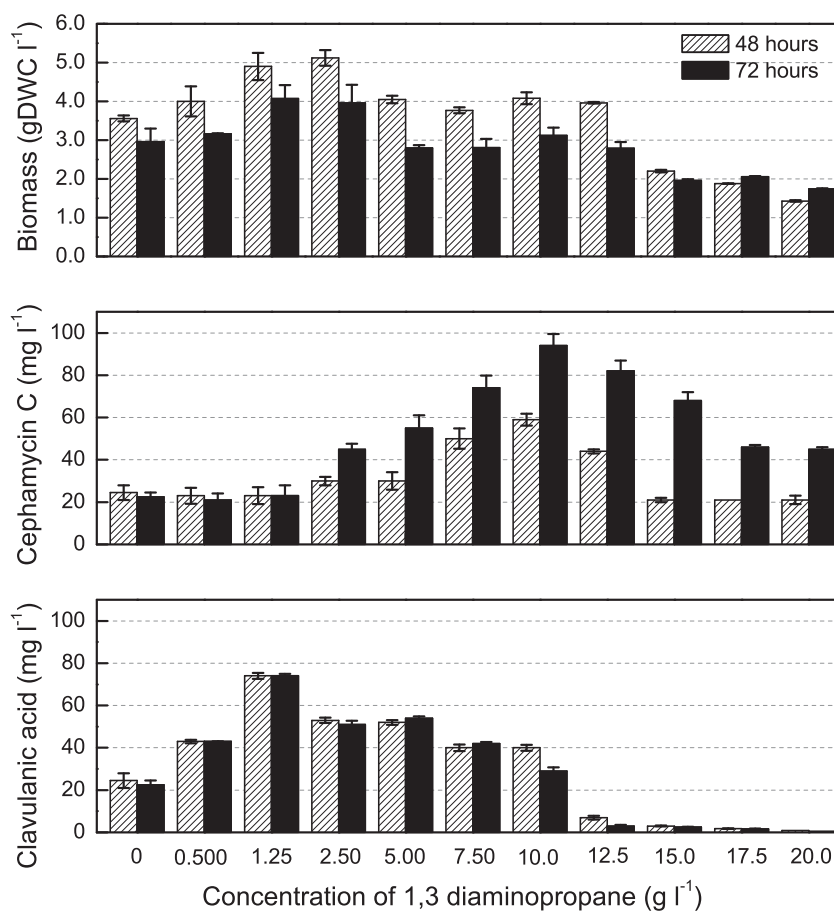


Figure 2. Biomass, CephC and CA obtained in *Streptomyces clavuligerus* culture in control medium without diamine (0) and with addition of 1,3-diaminopropane (0.5–20 g l⁻¹); all cultivations were done in triplicate.

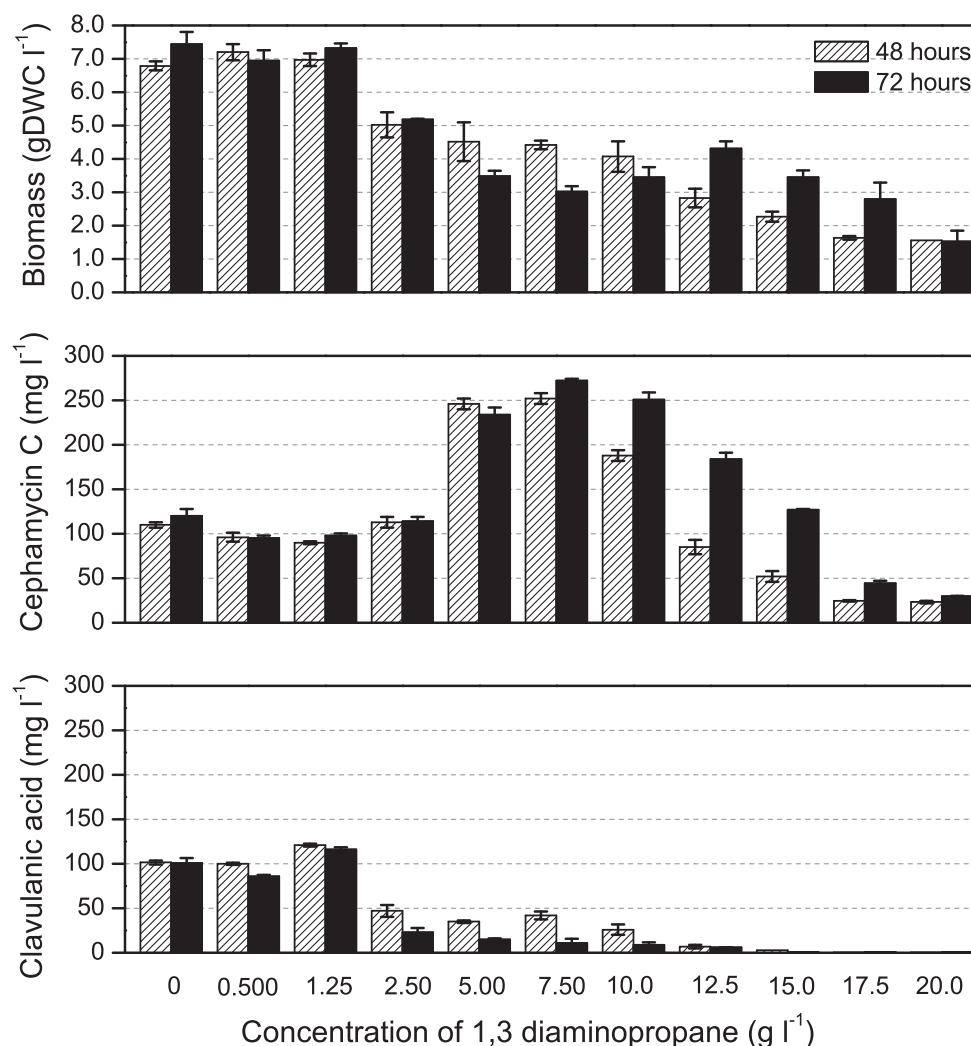


Figure 3. Biomass, CephC and CA obtained in *Streptomyces clavuligerus* culture in medium containing 18.3 g l⁻¹ L-lysine: control medium without diamine (0) and media with 1,3-diaminopropane (from 0.5 to 20 g l⁻¹); all cultivations were done in triplicate.

diamine). Concentrations above 15 g l⁻¹ of this diamine severely hindered cultivation.

Figure 2 also indicates that 1,3-diaminopropane concentrations above 2.5 g l⁻¹ had a positive effect on CephC biosynthesis. The highest volumetric production of this biocompound (94 mg g l⁻¹) was observed at 72 h of cultivation with 10 g l⁻¹ 1,3-diaminopropane. As compared to the CephC production obtained with the medium without diamine, this represents a 300% increase.

Positive effects were also observed for CA production, especially after adding 1.25 g l⁻¹ diamine, which resulted in a 200% production increase as compared to that obtained with the control medium. At concentrations above 1.25 g l⁻¹ 1,3-diaminopropane, CA biosynthesis decreased as diamine concentration increased in the culture medium.

Effect of 1,3-diaminopropane plus L-lysine on CephC and CA biosynthesis

In order to stimulate CephC and CA biosynthesis, the experiments were performed to assess the combined effect of

18.3 g l⁻¹ L-lysine with several 1,3-diaminopropane concentrations (Fig. 3).

Figure 3 indicates that addition of just L-lysine to the culture medium caused biomass increases by approximately 150% at 72 h of cultivation as compared to that obtained in control medium with no L-lysine nor diamine, as shown in Fig. 2. Addition of up to 1.25 g l⁻¹ 1,3-diaminopropane combined with L-lysine promoted no changes in cell concentration as compared to that in culture medium containing only L-lysine. However, biomass decreased at diamine concentrations above 2.5 g l⁻¹.

By adding just L-lysine to the culture medium, CephC and CA production increased by 430% as compared to that obtained with the control medium (without L-lysine or diamine). Volumetric production of CephC in media containing L-lysine plus 1,3-diaminopropane (from 5 to 10 g l⁻¹) increased by more than 110% as compared to that obtained for culture medium with only L-lysine. Compared to the control medium in Fig. 2, addition of L-lysine in conjunction with 7.5 g l⁻¹ 1,3-diaminopropane increased volumetric production of CephC by approximately 1100% (Fig. 3).

As to CA production, results show that additions of 1,3-diaminopropane plus L-lysine above 2.5 g l⁻¹ severely hindered the biosynthesis of this biocompound.

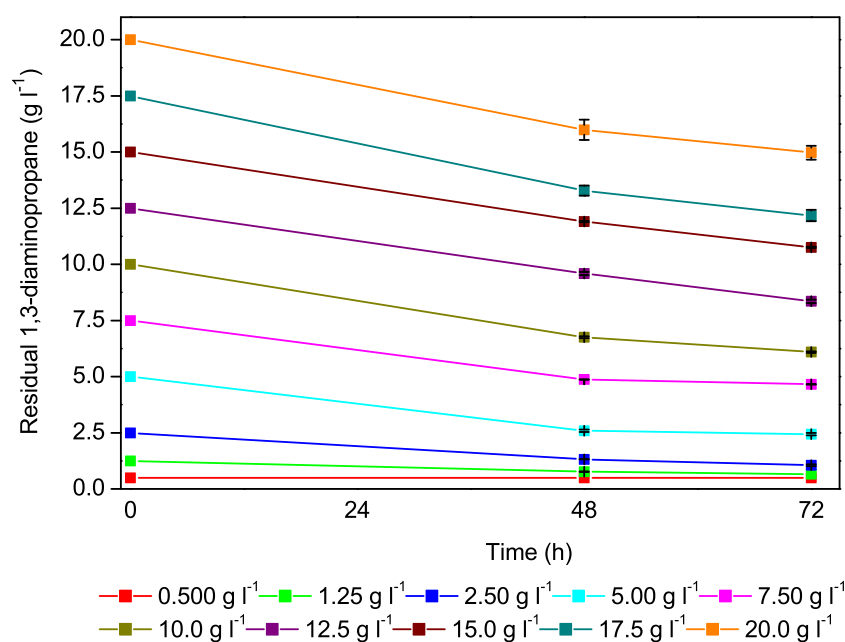


Figure 4. Residual 1,3-diaminopropane concentration in culture medium containing 18.3 g l^{-1} L-lysine.

Consumption of 1,3-diaminopropane and L-lysine

Figure 4 shows the 1,3-diaminopropane consumed throughout the fermentation. We observed that the proportion of 1,3-diaminopropane consumed depends of the initial diamine concentration added to the culture medium. Higher the initial diamine concentration in the culture medium, the smaller the percentage of this compound consumed at the end of fermentation. For instance, we observed a 50% consumption rate when 1.25 g l^{-1} diamine was added whereas addition of 20 g l^{-1} 1,3-diaminopropane resulted in 25% consumption rate ($\% = \text{total diamine consumed at the end of fermentation/initial diamine concentration in the culture medium} \times 100$). On the other hand, higher initial concentration of 1,3-diaminopropane in the culture medium leads to large quantity of global consumption of the diamine (in g l^{-1}). For example, when 1.25 g l^{-1} 1,3-diaminopropane was added in the culture medium, *S. clavuligerus* consumed 0.60 g l^{-1} , whereas when 20 g l^{-1} 1,3-diaminopropane was added in the culture medium, 5.00 g l^{-1} diamine was consumed by this actinomycete.

L-lysine consumption, in turn, was affected by 1,3-diaminopropane concentration in culture medium. Figure 5 shows that L-lysine consumption was approximately 75% in the culture medium with no 1,3-diaminopropane. However, in the culture medium containing 20 g l^{-1} 1,3-diaminopropane, there was observed an 18% L-lysine consumption.

DISCUSSION

Figure 1 results and the data obtained by Leite, Cavallieri and Araujo (2013) indicate that CA and CephC biosynthesis by *S. clavuligerus* is non-dissociable in the control medium (with neither diamine nor L-lysine) as well as in the culture medium supplemented with L-lysine. A similar production profile was observed both to CA (Fig. 1) as to CephC (Leite, Cavallieri and Araujo 2013), indicating that for this culture medium formulation, the addition of L-lysine equally affects CephC and CA biosynthesis. The positive effect of L-lysine on the biosynthesis of β -lactam

compounds in actinomycetes is widely known (Demain and Vaishnav 2006). Fang, Keables and Demain (1996), Rius, Maeda and Demain (1996), Antonio et al. (2012) and Leite, Cavallieri and Araujo (2013) reported enhancement on CephC production by *S. clavuligerus* when 18.3 g l^{-1} L-lysine was added to the culture medium. With relationship to CA production, Lynch and Yang (2004) demonstrated that culture medium supplemented with 20 g l^{-1} L-lysine also increases CA production by *S. clavuligerus*.

These results suggest that exogenous L-lysine, regardless of the concentrations added to the culture medium, increases beta-lactam antibiotics production, but does not promote the dissociation of CephC and CA.

Several researchers have promoted production of beta-lactam antibiotics by adding 1,3-diaminopropane to the culture medium (Leitão et al. 1999; Martín et al. 2011; Leite, Cavallieri and Araujo 2013). Leitão et al. (1999) and Leite, Cavallieri and Araujo (2013) reported a 100% increase in CephC production by *S. clavuligerus* by adding 5 g l^{-1} 1,3-diaminopropane to the culture medium. Martín et al. (2011) added low quantities of 1,3-diaminopropane (0.35 and 0.75 g l^{-1}) to a *Penicillium chrysogenum* culture medium and observed, too, that penicillin production increased by approximately 100%. However, there is no data in the literature concerning the effect of 1,3-diaminopropane on CA production or on coordinated production of CephC and CA by *S. clavuligerus*.

Our results indicate that additions of low concentrations of 1,3-diaminopropane (0.5 and 1.25 g l^{-1}) without L-lysine do not promote CephC biosynthesis. On the other hand, it was observed that CA production increased by 200% when the same concentrations of diamine were added (Fig. 2). However, additions of high concentrations of 1,3-diaminopropane (10 g l^{-1}) to the culture medium increased CephC production by 300% and maintained CA production at the same level as that of the control medium without diamine nor L-lysine (Fig. 2).

The highest dissociation between CephC and CA biosynthesis occurred when we added 12.5 g l^{-1} 1,3-diaminopropane. Under this condition, CephC volumetric production was 30 times higher than that of CA. Yet, the maximum addition of

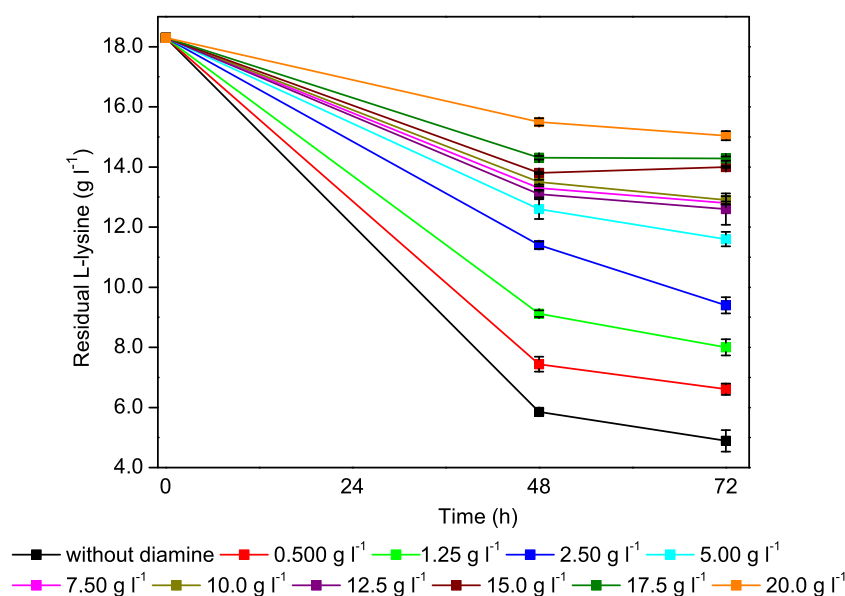


Figure 5. Residual L-lysine concentration in culture medium containing from 0.5–20 g l⁻¹ 1,3-diaminopropane.

1,3-diaminopropane (20 g l⁻¹) resulted in 100% increase in CephC production at the end of cultivation as compared to that of the control medium (with no diamine nor L-lysine) without producing CA (Fig. 2).

We also observed that adding L-lysine plus 1,3-diaminopropane had affected the dissociation between CephC and CA productions by *S. clavuligerus*. Compared to the control medium in Fig. 2, addition of L-lysine plus 12.5 g l⁻¹ 1,3-diaminopropane led CephC production to increase by 700% whereas the increase in CA production was insignificant (Fig. 3).

It is known that polyamines have a fundamental role in microorganism physiology. These compounds are involved in cell proliferation, survival and apoptosis (Bassard et al. 2010). Several researchers have indicated that polyamines may interact with DNA stabilizing its conformation and structure that consequently affects the expression of certain genes (Tabor & Tabor 1985). Polyamines also stabilize macromolecules, proteins and membranes modulating electrostatic protein-protein interactions and affecting DNA-protein interactions (Bassard et al. 2010). Leitão et al. (1999) studied the effect of 1,3-diaminopropane on *Amycolatopsis lactamdurans* and found that this diamine induces gene expression and stabilizes messenger RNAs. These authors reported, also, high levels of LAT and 1-piperidine-6-carboxylate dehydrogenase (P6C dehydrogenase) enzymes and P7 protein. LAT and P6C dehydrogenase are responsible for converting L-lysine to α -amino adipic and P7 protein is responsible for CephC methoxylation. Proteomic analysis showed that this diamine is also capable of reprogramming *P. chrysogenum* metabolism to promote penicillin overproduction (García-Estrada et al. 2013). On the other hand, we observed that high diamine concentrations affect negatively CA production. *clara* gene encoding proteins involved in CA biosynthesis and $\Delta clara$ strains lost its ability to produce CA (Song, Jensen and Lee 2010) whereas mutant strains contained *clara* multicopy produces more CA and less CephC (Pérez-Redondo et al. 1998). A recent study shows that *S. clavuligerus* $\Delta clara::aac$ does not produce CA but CephC production was increased about 40% than wild type (Martínez-Burgo et al. 2015). Perhaps high concentration of 1,3-diaminopropane may act on *Clara* regulator or another similar regulator resulting in no CA production but high CephC title.

Unfortunately, there are no genetic studies that demonstrate the effect of 1,3-diaminopropane on coordinated CephC and CA production by *S. clavuligerus*.

According to Madduri, Stuttard and Vining (1989), L-lysine can be metabolized in *S. clavuligerus* via two pathways: L-lysine aminotransferase or cadaverine aminotransferase. In the former pathway, L-lysine is decarboxylated, producing α -amino adipic acid, a rare amino-acid precursor to beta-lactam antibiotics. In contrast, the latter pathway employs L-lysine as a source of nitrogen, carbon, and energy. Research on enzyme activity conducted by Antonio et al. (2012) with culture medium containing 18.3 g l⁻¹ L-lysine revealed that cadaverine aminotransferase activity was four times higher than that of L-lysine aminotransferase, favoring primary metabolism. With respect to diamine, it is known that 1,3-diaminopropane can be metabolized via beta-alanine (KEGG Database¹) by several *Streptomyces* species. Our results show that L-lysine consumption decreases proportionally to addition of 1,3-diaminopropane to the culture medium. It suggests that this diamine may be acting as a source of carbon and energy resulting in decreased L-lysine consumption via cadaverine aminotransferase. For this reason, surplus amino acid in culture medium is employed to synthesize antibiotics. It is important to remark that converting L-lysine to α -amino adipic acid constitutes a limiting step in CephC biosynthesis by both *S. clavuligerus* and *A. lactamdurans* (Malmberg, Hu and Sherman 1985; Chary et al. 2000).

In conclusion, our study shows that extracellular concentration of 1,3-diaminopropane may trigger dissociation of CephC and CA biosynthesis in *S. clavuligerus*. Genetic analyses should contribute to a better understanding of the regulation of beta-lactam compound biosynthesis by this organism.

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¹ <http://www.genome.jp/kegg/pathway/map/map00410.html>

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Conflict of Interest. All the authors of the submitted work (CAL, APC, ASB and MLGC) declare that there has been no financial relationship or support from any company in the past five years. We declare, too, that there are no competing interests, whether political, personal, religious, ideological, academic, intellectual or commercial, nor any other activities influencing the submitted work.

REFERENCES

- Álvarez-Álvarez R, Rodríguez-García A, Santamarta I, et al. Transcriptomic analysis of *Streptomyces clavuligerus* Δ ccaR::tsr: effects of the cephamycin C-clavulanic acid cluster regulator CcaR on global regulation. *Microb Biotechnol* 2014;7:221–31.
- Antonio T, Bellão C, Corrêa T, et al. Evaluation of different media for the production of cephalosporins by *Streptomyces clavuligerus* ATCC 27064. *Braz Arch Biol Technol* 2012;55:819–25.
- Bassard J-E, Ullmann P, Bernier F, et al. Phenolamides: Bridging polyamines to the phenolic metabolism. *Phytochemistry* 2010;71:1808–24.
- Bellão C, Antonio, Araujo MLGC T, et al. Production of clavulanic acid and cephamycin C by *Streptomyces clavuligerus* under different fed-batch conditions. *Braz J Chem Eng* 2013;30:257–66.
- Bibb MJ. Regulation of secondary metabolism in Streptomycetes. *Curr Opin Microbiol* 2005;8:208–15.
- Chary VK, de la Fuente JL, Leitão AL, et al. Overexpression of the lat gene in *Nocardia lactamdurans* from strong heterologous promoters results in very high levels of lysine-6-aminotransferase and up to two-fold increase in cephamycin C production. *Appl Microbiol Biot* 2000;53:282–88.
- Demain AL, Vaishnav P. Involvement of nitrogen-containing compounds in β -lactam biosynthesis and its control. *Crit Rev Biotechnol* 2006;26:67–82.
- Domingues LC, Teodoro JC, Hokka CO, et al. Optimisation of the glycerol-to-ornithine molar ratio in the feed medium for the continuous production of clavulanic acid by *Streptomyces clavuligerus*. *Biochem Eng J* 2010;53:7–11.
- Fang A, Keables P, Demain AL. Unexpected enhancement of β -lactam antibiotic formation in *Streptomyces clavuligerus* by very high concentrations of exogenous lysine. *Appl Microbiol Biot* 1996;44:705–9.
- Foulstone M, Reading C. Assay of amoxicillin and clavulanic acid, the components of Augmentin, in biological fluids with high-performance liquid chromatography. *Antimicrob Agents Ch* 1982;22:753–62.
- García-Estrada C, Barreiro C, Jami MS, et al. The inducers 1,3-diaminopropane and spermidine cause the reprogramming of metabolism in *Penicillium chrysogenum*, leading to multiple vesicles and penicillin overproduction. *J Proteomics* 2013;85:129–159.
- Leitão AL, Enguita FJ, Fuente JL, et al. Inducing effect of diamines on transcription of the cephamycin C genes from the lat and pcbAB promoters in *Nocardia lactamdurans*. *J Bacteriol* 1999;181:2379–84.
- Leite CA, Cavallieri AP, Araujo MLGC. Enhancing effect of lysine combined with other compounds on cephamycin C production in *Streptomyces clavuligerus*. *BMC Microbiology* 2013;13:296–307.
- Liras P, Martín JF. Assay methods for detection and quantification of antimicrobial metabolites produced by *Streptomyces clavuligerus*. In: Barredo JL (ed.). *Microbial Processes and Products*. New Jersey: Humana Press, 2005, 149–64.
- Lynch HC, Yang Y. Degradation products of clavulanic acid promote clavulanic acid production in cultures of *Streptomyces clavuligerus*. *Enzyme Microb Tech* 2004;34:48–54.
- Madduri K, Stuttard C, Vining LC. Lysine catabolism in *Streptomyces* spp. Is primarily through cadaverine: β -lactam producers also make α -amino adipate. *J Bacteriol* 1989;171:299–302.
- Malmberg LH, Hu WS, Sherman DH. Effects of enhanced lysine ϵ -aminotransferase activity on cephamycin biosynthesis in *Streptomyces clavuligerus*. *Appl Microbiol Biot* 1985;44:198–205.
- Martín J, García-Estrada C, Rumero Á, et al. Characterization of an autoinducer of penicillin biosynthesis in *Penicillium chrysogenum*. *Appl Environ Microb* 2011;77:5688–96.
- Martínez-Burgo Y, Álvarez-Álvarez R, Rodríguez-García A, et al. The pathway-specific regulator ClaR of *Streptomyces clavuligerus* has a global effect on the expression of genes for secondary metabolism and differentiation. *Appl Environ Microb* 2015;81:6637–48.
- Paradkar A. Clavulanic acid production by *Streptomyces clavuligerus*: biogenesis, regulation and strain improvement. *J Antibiot* 2013;66:411–20.
- Pérez-Redondo R, Rodríguez-García A, Martín JF, et al. The claR gene of *Streptomyces clavuligerus*, encoding a LysR-type regulatory protein controlling clavulanic acid biosynthesis, is linked to the clavulanate-9-aldehyde reductase (car) gene. *Gene* 1998;211:311–21.
- Rius N, Maeda K, Demain AL. Induction of L-lysine & aminotransferase by L-lysine in *Streptomyces clavuligerus*, producer of cephalosporins. *FEMS Microbiol Lett* 1996;144:207–11.
- Salem-Bekhit MM, Alanazi FK, Alsarra IA. Improvement and enhancement of clavulanic acid production in *Streptomyces clavuligerus* using vegetable oils. *Afr J Biotechnol* 2013;9:6806–12.
- Song JY, Jensen SE, Lee KJ. Clavulanic acid biosynthesis and genetic manipulation for its overproduction. *Appl Microbiol Biot* 2010;88:659–69.
- Tabor CW, Tabor H. Polyamines in Microorganisms. *Microbiol Rev* 1985;49:81–99.
- Teodoro JC, Baptista-Neto, Araujo MLGC A, et al. Influence of glycerol and ornithine feeding on clavulanic acid production by *Streptomyces clavuligerus*. *Braz J Chem Eng* 2010;27:499–506.