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Genes involved in an antibiotic resistance system in *Streptomyces clavuligerus*

Genes implicados en un sistema de resistencia a antibióticos en *Streptomyces clavuligerus*

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Abstract

The cephamycin C gene cluster in *Streptomyces clavuligerus* contains the genes *cmcT*, *bla* and *pbp74*, which have been suggested to be involved in antibiotic resistance. To evaluate the role of these genes, mutants of *S. clavuligerus* ATCC 27064 deleted in *pbp74* and *cmcT* have been constructed. The resistance of these mutant strains as well as *S. clavuligerus* Δbla::aphII, a *bla*-deleted mutant, to cephalosporin and penicillin G have been analyzed. A 11% increase in resistance to penicillin G was detected when the *cmcT* gene was deleted compared to parental strain. Disruption of *bla* and *pbp74* increased penicillin G sensitivity. Cephamycin C production was 83% lower in the *cmcT*-deleted mutant, however, *bla*-deleted mutant, the production was enhanced 193% compared to the parental strain. Genes expression was measured by qRT-PCR. In addition, the *cmcT*, *bla* and *pbp74* proteins were modeled and their affinity for antibiotics was assessed. Based on our results, these three genes present in *S. clavuligerus* are suggested to be part of a self-defense system. Enhancement of cephamycin C production with the interruption of the *bla* gene was an interesting discovery which could be applied to improve the production of this antibiotic.

Keywords: Actinobacteria, *β*-lactam antibiotics resistance, Cephamycin, clavulanic acid, beta-lactam antibiotics.

Resumen

El grupo de genes de cefamicina C en *Streptomyces clavuligerus* contiene los genes *cmcT*, *bla* y *pbp74* y se ha sugerido que dichos genes están involucrados en la resistencia a antibióticos. Con el objetivo de evaluar la función de estos genes se obtuvieron mutantes de *S. clavuligerus* ATCC 27064 con los genes *pbp74* y *cmcT* delecionados. Se analizó la resistencia de estas mutantes y de *S. clavuligerus* Δbla::aphII, mutante con el gen *bla* delecionado, a cefalosporina y penicilina G. Se detectó un incremento del 11% en la resistencia a la penicilina G en la mutante *cmcT*-delecionada en comparación con la cepa parental. La interrupción de *bla* o *pbp74* provocó un aumento en la sensibilidad a la penicilina G. La producción de cefamicina C fue 83% menor en la mutante *cmcT*-delecionada; sin embargo, en la mutante *bla*-delecionada la producción aumentó un 193% en comparación con la cepa parental. La expresión de los tres genes se evaluó mediante qRT-PCR. Además, se modelaron las proteínas *cmcT*, *bla* y *pbp74* y se evaluó su afinidad por los antibióticos. Nuestros resultados sugieren que estos tres genes presentes en *S. clavuligerus* son parte de un sistema de autodefensa. Finalmente, el aumento de la producción de cefamicina C con la mutante *bla*-deleccionada fue un descubrimiento interesante que podría aplicarse para mejorar la producción de este antibiótico.

Palabras clave: Actinobacteria, resistencia a antibióticos betalactámicos, cefamicina, ácido clavulánico, antibióticos beta-lactámicos.

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1 Introduction

Antibiotics have been used to combat various bacterial infections for decades. Worldwide, infections caused by multi-resistant bacteria are on the rise, as they are acquiring resistance to antibiotics (Aslam *et al*., 2022). The production of antimicrobial substances by microorganisms, such as actinobacteria, is regulated by several environmental factors (López-Alcántara *et al*., 2022). This knowledge has led to microbial strains with higher production capacity. However, it is interesting to know the way in which an antibiotic-producing microorganism resists the toxicity of its own product implies the participation of several genes and is undoubtedly a key factor in the evolution and survival of these organisms. The most commonly found mechanism to control toxicity, in antibiotic-producing eukaryotes, consists in localizing the antibiotics in internal membrane vesicles that are released by exocytosis. Prokaryotes limit intracellular concentration through an external product transport system (Putman *et al*., 2000) or other alternative mechanisms (Severi and Thomas 2019). Antibiotic-producing actinomycetes frequently include in the biosynthetic gene cluster (BGC), genes putatively involved in detoxification of the antibiotic intracellular activity (Lv *et al*., 2016) and genes for exporter proteins, most of them of the ABC or MFS families or subfamilies, from which the exact role is frequently poorly understood (Severi and Thomas 2019). Some of these integral membrane proteins function as antibiotic efflux pumps (Folcher *et al*., 2001). In other cases, the intracellular concentration of the metabolite could also retro-regulate its biosynthesis (Tahlan *et al*., 2007; Wang *et al*., 2009).

The cephamycin C biosynthesis gene (CBG) clusters, in *Nocardia lactamdurans* (Coque *et al*., 1993) and *Streptomyces clavuligerus* (Pérez-Llarena *et al*., 1998), contain genes possibly involved in cephamycin C resistance (*cmcT*, several pbp´s, bla), which are grouped together and located side by side to the pcd and cefE genes for cephamycin biosynthesis (Fig. 1A). The *cmcT* proteins of these actinomycetes have 14 transmembrane domains and belong to the 14-major facilitator superfamily (MFS) of proteins (Coque *et al*., 1993; Paulsen *et al*., 1996; Pérez-Llarena *et al*., 1998). The high similarity of the *cmcT* protein of *S. clavuligerus* with the homologous protein of *N. lactamdurans* (60% amino acids identity) and the lower identity with other transmembrane proteins for antibiotic export, as 33% with PurT of *S. alboniger* (Tercero *et al*., 1993) or 24% with TcmA of S. *glauscescens* (Guilfoile and Hutchinson 1992), suggests a specific role for *cmcT* in cephamycin production/resistance. In the fungus *Acremonium chrysogenum* a gene, *cefT*, located in the cephalosporin C gene cluster encodes a multidrug efflux pump of the MFS-type; this protein is involved in secretion of β-lactams containing the α-aminoadipic acid-derived chain, as isopenicillin N, penicillin N or deacetoxycephalosporin C (Ullán *et al*., 2002, 2008).

Genes encoding penicillin-binding proteins (PBPs), as *N. lactamdurans pbp40*, or *S. clavuligerus pcbR* and *pbp74*, are located in the CBG clusters of these strains. *S. clavuligerus* PcbR protein is membrane-anchored and confers penicillin G and cephalotin resistance (Paradkar *et al*., 1996); mutants in the pcbR gene could not be obtained, and the gene was suggested to be essential (Paradkar *et al*., 1996). The *pbp74* gene is located immediately downstream of *cmcT*, and opposite to bla; however, no studies on the *cmcT* or *pbp74* genes/proteins have been made.

In addition, genes encoding β-lactamases (bla) are present in *N. lactamdurans* (Coque *et al*., 1993) and *S. clavuligerus* (Pérez-Llarena *et al*., 1998) CBG clusters and in the cephabacin gene cluster of the producer strain, Lysobacter lactamgenus (Kimura *et al*., 1996). The *bla* gene of *S. clavuligerus* encodes a class A β-lactamase that was purified and found to have a weak penicillin-binding activity (Pérez-Llarena *et al*., 1997). This gene was deleted but the mutant has not been further characterized (Pérez-Llarena, 1997).

The three genes present in *S. clavuligerus* may be part of a self-defense system, where the first protein (*cmcT*) regulates the concentration of the intracellular antibiotic. The second protein (*pbp74*) acts as a 'bait' in order to be a "distracting" target of the accumulated antibiotic. Finally, the third line of defense involves a β-lactamase (Bla) which inactivates the antibiotic excess. The second possibility is that these proteins work independently, merely as a triple safety system, where if one fails, the remaining two will do the job. However, many questions remain about this resistance system.

In this study, we focus on the analysis of the *cmcT*, *pbp74* and *bla* genes located within the cephamycin-clavulanic acid gene cluster in *S. clavuligerus* (Pérez-Llarena *et al*., 1998), to increase our insight on the cephamycin C resistance system and eventually apply it to improve its production.

2 Materials and methods

2.1 Materials and chemicals

Antibiotics used in this work were purchased from Sigma Chemical Co. (St. Louis, Mo.). Media and individual components were from BD Bioxon and J.T. Baker Chemicals. Bactoagar was from Difco. Chemicals for molecular biology techniques were purchased from ©Qiagen and Promega Co. All other

fine chemicals were reagent grade and were purchased from Sigma Chemical Co.

2.2 Strains and culture conditions

The bacterial strains and plasmids used in this work are described in Table 1. *E. coli* strains and plasmid-containing transformants were grown at 37 °C in Luria-Bertani medium (LB); SOB medium (containing: tryptone 20 g/L, yeast extract 5 g/L, NaCl 0.584 g/L, KCl 0.186 g/L pH 7.5) and 1 mL sterile solution of $MgCl_2$ 1 M, $MgSO_4$ 1M per 100 mL SOB; SOC medium (SOB medium with 1 mL sterile glucose 1 M added per 100 mL SOB) or TB medium (10 mL of TB salts was added for every 90 mL of TB medium), as required. *S. clavuligerus* ATCC 27064 and the deleted mutants were grown at 28 °C in the following media: MS agar medium (mannitol 20 g/L, soybean flour and agar 2%), 2xTY (tryptone 20 g, yeast extract 10 g, pH at 7.2), TSB (trypticasein soy broth 30 g/L pH 7.2), TSA (TSB with agar 2% added) and ME medium (yeast extract 0.5 g/L, meat extract 0,5 g/L, NZ amine 1 g/L, glucose 5 g/L, MOPS 21 g/L, pH 7.0.) for sporulation. Media were supplemented with the following antibiotics as required: apramycin (50 μg/mL), ampicillin (100 μg/mL), kanamycin (50 μg/mL), nalidixic (12.5 μg/mL) hygromycin (80 μg/mL), chloramphenicol (25 μg/mL).

2.3 Cephamycin C production

Streptomyces strains stock cultures were kept in 20% glycerol at -75 ºC and obtained from TSB (trypticasein soy broth 30 g/L, pH 7.2.) cultures grown at 28 °C and 220 rpm to an optical density $OD_{600\,nm}$ of 4-7. One mL of the stock culture was used to inoculate 100 mL of TSB medium and the pre-inoculum culture was grown for 24 h at the above indicated conditions. The $OD_{600\,nm}$ was adjusted to 5.0 and 5mL were used to inoculate triplicated Erlenmeyer flasks with 50 mL TSB medium. The production cultures were grown under the above indicated conditions for 72 h. Samples were taken at 11, 21, 36, 46, 60 and 70 h of culture. The growth was estimated by measuring the biomass. The culture samples were centrifuged, the cells were washed three times with saline solution and the final pellet was dried at 80 ºC until constant weight.

2.4 Cephamycin C production quantification

Cephamycin C quantification was performed by bioassay (Romero *et al*., 1984) using *Escherichia coli* Ess22-31, a β-lactam antibiotics supersensitive strain (Hu *et al*., 1984), which was cultivated in LB medium at 30 °C at 220 rpm to an $OD_{600\,nm}$ of 1 to 1.2. The inhibition halos were measured after 11- 14 h.

2.5 Construction of the *cmcT* and *pbp74* deleted strains

The *S. clavuligerus* ATCC27064 Δ*cmcT* and Δ*pbp74* null mutants were constructed by the PCR targeting procedure (Bertolt Gust *et al*., 2002).

2.6 Construction of *S. clavuligerus* Δ*cmcT::acc*

Plasmid pIJ774 was digested with EcoRI and HindIII enzymes to obtain an apramycin resistance-*oriT* cassette; this cassette was amplified by PCR using primers: 5'-ccg ata tct ctc ctg gcg ctc ggt gct gct ggt cat gtg att ccg gga tcc gtc gac c-3' and 5'-aca gcg ggc ggt ggg cgg acg gcg gat ggc gga cgg tca tgt agg ctg gag ctg ctt c-3'.

Table 1. Bacterial strains and plasmids used in this work.

	Characteristics	Reference (or source) source)
S. clavuligerus ATCC 27064	Produces cephamycin C and clavulanic acid	ATCC
S. clavuligerus Δ*cmcT*::acc	*cmcT* deleted mutant, apramycin resistant	This work
S. clavuligerus Δ*pbp74*::hyg	*pbp74* deleted mutant; hygromycin resistant	This work
S. clavuligerus Δbla::aphII	*bla* deleted mutant; kanamycin resistant	Perez Llarena (1997)
E. coli DH5 α	High efficiency transformation strain It contains the pIJ774 plasmid	Simon, *et al*. (1983)
E. coli BW25113	It contains the λ RED recombinant plasmid	Datsenko and Wanner (2000)
E. coli ET12567	Km^R Cm^R, Methylation-deficient, it contains pUZ8002 and cosmid G6-15	MacNeil, *et al*. (1992)
E. coli Ess22-31	Supersensitive to Cephamycin C	Hu, *et al*. (1984)
pIJ774	Apm^R, fragment HindIII/EcoRI containing *oriT*	Hopwood, *et al*. (2000)
pIJ790	Cf^R and a temperature sensitive origin of replication (requires 30°C for replication)	Hopwood, *et al*. (2000)
Cosmid G6-15	Km^R. Contains the cephamycin biosynthesis gene cluster.	Martínez-Burgo, *et al*. (2014)
pUZ8002	Km^R, Non-self-transmissible	Hopwood, *et al*. (2000)

E. coli BW25113 was transformed with the G6-15 cosmid. *E. coli* BW25113/pIJ790/G6-15 was electro-transformed with the extended resistance cassette in the presence of L-arabinose 10 mM and incubated to 37 °C overnight to facilitate the loss of pIJ790. Cosmid G6-15 was co-transformed with plasmid pUZ8002 in the methylation deficient *E. coli* ET12567 strain. The strains transformed with the cassette were selected by the kanamycin^R and apramycin^R. The presence of the vector carrying the cassette with the apramycin resistance gene (instead of the *cmcT* gene), named pULΔ*cmcT*::acc, was confirmed by plasmid extraction, restriction analyses and PCR. The PCR amplicon obtained was then sequenced.

2.7 Construction of *S. clavuligerus* Δ*pbp74::hyg*

The strategy to construct this strain was the same but using a different resistance cassette. In this case, the pIJ10700 vector was digested with BamHI and HindIII to obtain a hygromycin resistance-*oriT* cassette. The cassette was obtained using the following primers: 5'-aca cgg cca cgt ggg cag aga gcg acg gcg cgg cgt atg att ccg gga tcc gtc gac c-3' and 5'-gct tga ggg ggt gct tcc gca gca gca cat agg ccg tca tgt agg ctg gag ctg ctt c-3'. Transformants were selected by kanamycin^R and hygromycin^R. The presence of the vector pULΔ*pbp74*::hyg, carrying the cassette with the hygromycin resistance gene (instead of the *pbp74* gene), was confirmed as indicated above. Vectors pULΔ*cmcT*::acc and pULΔ*pbp74*T::hyg were introduced in *S. clavuligerus* by conjugation. Double-crossover exconjugants were chosen by kanamycin^S and apramycin^R for the case of the strain with the deleted *cmcT* gene, and kanamycin^S and hygromycin^R for the strain with the *pbp74* gene deleted. The selected conjugants were confirmed by sequencing the PCR fragments.

2.8 Isolation of RNA and qRT-PCR

RNA was isolated using the RNeasy Kit (Qiagen) from 11, 21 and 36 h cultures grown in TSB medium. RNA was quantified in a Nano-Drop spectrophotometer (Thermo Scientific), and its integrity was confirmed, after a 10 min treatment at 50 °C, by electrophoresis in 2% agarose gels. cDNA was obtained with SuperScriptTM III retrotranscriptase using 85 ng RNA as template and Random Primers (Invitrogen) at 12.5 ng/μl final concentration. The oligonucleotides used for qRT-PCR of the different genes, were the same used in each construction indicated above. Gene expression analysis by reverse-transcriptase quantitative polymerase chain reaction (qRT-PCR) was performed as previously described (Kurt *et al*., 2013).

2.9 Modeling of proteins

cmcT, *pbp74* and *bla* ternary structures were predicted using iTasser web server (Zhang 2008; Roy *et al*., 2010). Hydrogen were added for the best iTasser models introducing them on charmm-gui (Jo *et al*., 2008) and energy minimized with 100 steepest descent using CHARMM c43b1 free version (Brooks *et al*., 1983, 2009) with charmm36 potential (Huang and MacKerell, 2013).

Cephalosporin, cephamycin, clavulanic acid and penicillin G were built using MarvinSketch (Marvin 19.15.0, 2019, ChemAxon, http://www.chemaxon.com). Binding sites were selected using those residues as noted on the original papers for the references structures (*cmcT*, 2GFP4 (Yin *et al*., 2006); *pbp74*, 3ITB (Chen *et al*., 2009); Bla, 3QHY (Brown *et al*., 2011)).

Protein-ligand docking was done using Autodock Vina (Trott and Olson 2010; Seeliger and Groot 2010). 1000 independent docking runs were done with every system here presented. A root-mean squared deviation (RMSD) of 2 Å was used to isolated cluster and only the most populated cluster was analyzed. Protein-ligand diagram were done using Maestro (Schrödinger, 2019). Chimera (Pettersen *et al*., 2004) and VMD (Humphrey *et al*., 1996) were used to analyzed and create the images on this work.

3 Results

3.1 Cephamycin C production by *S. clavuligerus* ATCC27064 and the deleted mutants

The deleted mutants Δ*cmcT* and Δ*pbp74* showed colonies morphology and sporulation on agar medium similar to those of *S. clavuligerus* ATCC27064. The kinetics of growth in TSB liquid cultures did not show statistically significant differences, as even the error bars overlapped (Fig. 1C). However, *S. clavuligerus* Δbla::aphII growth was slower. In addition, colonies of *S. clavuligerus* Δbla::aphII showed an earlier onset of sporulation in solid medium and slightly different morphology.

When cephamycin C was determined by bioassay the strain showing lower antibiotic production was *S. clavuligerus* Δ*cmcT*::acc which produced in the order of 17% of the cephamycin C produced by the parental strain (Fig. 1). These results suggest that the *cmcT* gene is strongly related to cephamycin C production. The effect of the mutation on cephamycin C production was lower in *S. clavuligerus* Δ*pbp74*::hyg that reached levels close to 82% of the production displayed by the parental strain at 36 h of culture. Surprisingly, the *S. clavuligerus* Δbla::aphII mutant

produced higher levels of cephamycin all along the fermentation, with a maximum level of 34.94 μg/mg of cell dry weight at 46 h (193% in relation to the parental strain), although slightly delayed in relation to the other strains. However, growth of this strain was lower, so the volumetric antibiotic production reached only 123 to 127% of the ones shown by the parental strain at different culture times. No substantial differences in clavulanic acid production were detected between the wild type strain and the Δ*bla* and Δ*pbp74* mutants.

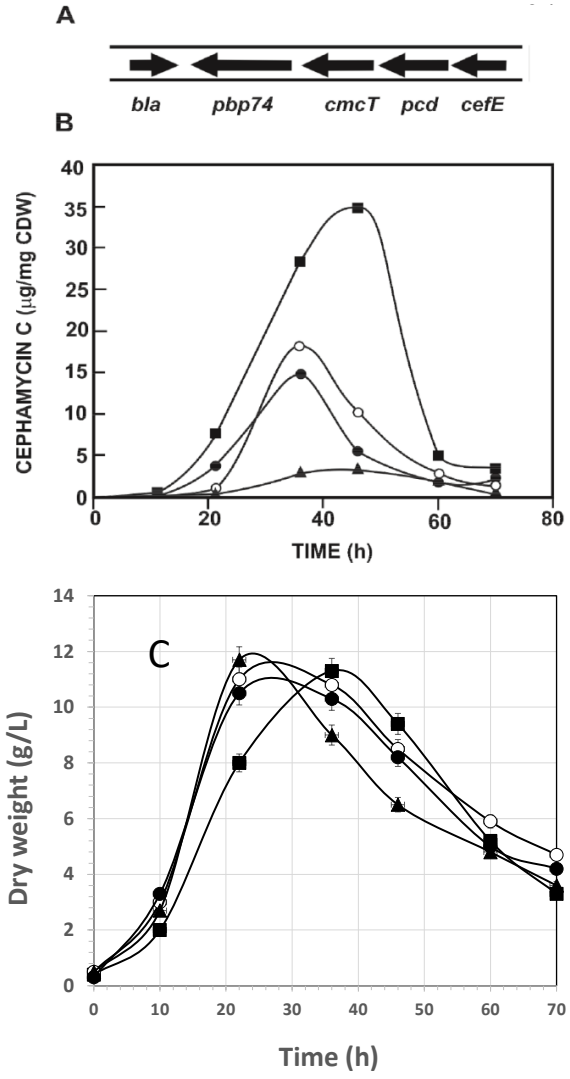

Figure 1. Location of the *cmcT*, *bla* and *pbp74* genes in the CBG cluster and specific production of cephamycin C in liquid cultures by the *S. clavuligerus* ATCC27064 and deleted mutants. A) Relative location of the *cmcT*, *bla* and *pbp74* genes next to the pcd and cefE structural genes, in the cephamycin C gene cluster of *S. clavuligerus*. B) Cephamycin C production in TSB medium by *S. clavuligerus* ATCC 27064 (white circles), *S. clavuligerus* Δ*cmcT*::acc (black triangles), *S. clavuligerus* Δ*pbp74*::hyg (black circles), *S. clavuligerus* Δbla::aph (black squares). Standard deviations are below the limits of detection of the graphic. C) Biomass concentration during cultivation on TSB medium.

Contrasting, the production of clavulanic acid in mutant Δ*cmcT* was about 50% lower than in the parental strain, suggesting that clavulanic acid might also be partially transported by *cmcT*.

3.2 *Expression of the cmcT, bla and pbp74 genes in S. clavuligerus ATCC 27064 and mutants were determined by RT-qPCR*

The expression of the genes *cmcT*, *bla* and *pbp74* was determined in TSB cultures. Samples were taken at 11h, which corresponds to the trophophase, before cephamycin C was formed; 21 h at the beginning of the idiophase, and 36 h in the middle of the idiophase, time at which the production of cephamycin C by the wild strain was maximal.

The expression observed in the mutants was compared with the one obtained in the parental strain *S. clavuligerus* ATCC 27064. The values of the studied genes were taken as reference in relative quantification of gene expression with respect to the values obtained for *S. clavuligerus* ATCC 27064 that were give the value one at time 11 h (Fig. 2A). In general, the expression of the genes in the deleted mutants was relatively similar to that of the wild type strain.

To understand whether the deletion of each of these genes affected the expression of the others, their expression was analyzed in the three mutants. The differences of expression in relation to that of the parental strain are shown in Fig. 2B. Given the relative position of these genes in the CBC (Fig. 1A) transcriptional polar effect of the mutations could not be observed. In each deletion mutant strain, the expression of the two remaining genes studied was practically unaffected, showing variations of +/- 3% in expression (Fig. 2B). This indicates that the three genes expression was independent from each other.

3.3 *β-Lactam antibiotic resistance in the parental strain and the deleted mutants*

Because of the *β*-lactam nature of cephalosporin and penicillin, resistance to these antibiotics was assessed. Furthermore, cephalosporin and cephamycin are both derived from *Streptomyces* species, having a similar mechanism of action to penicillins.

Results showed that *S. clavuligerus* ATCC 27064 was resistant to 9 mg/mL cephalosporin and the same occurs to the *S. clavuligerus* Δ*pbp74*::hyg and Δbla::aphII strain; however, the *cmcT*-deleted strain was slightly more resistant, being able to grow even at 10 mg/mL. In all cases, the amount of cephalosporin C required was very high, indicating that *S. clavuligerus* is "per se" highly resistant to cephalosporin.

Table 2. Minimum inhibitory concentration (MIC) of different strains of *Streptomyces clavuligerus* against penicillin G and cephalosporin C.

Strain	MIC Penicillin G	MIC Cephalosporin C
S. clavuligerus ATCC27064	180 μg/mL	9 mg/mL
S. clavuligerus Δ*cmcT*::acc	200 μg/mL	10 mg/mL
S. clavuligerus Δ*pbp74*::hyg	120 μg/mL	9 mg/mL
S. clavuligerus Δbla::aphII	120 μg/mL	9 mg/mL

Table 3. Docking of antibiotics to the proteins studied in this work.

PROTEIN	ANTIBIOTIC	Affinity energy (kcal/mol)	
		XRAY	MODEL
Bla	Cephalosporin C	-7.06 +/- 0.01 (617)	-6.89 +/- 0.04 (591)
Bla	Cephamycin C	-6.90 +/- 0.01 (775)	-7.28 +/- 0.10 (955)
Bla	Penicillin G -	7.10 +/- 0.00 (996)	-6.70 +/- 0.00 (1000)
pbp74	Cephalosporin C	-6.92 +/- 0.09 (557)	-6.56 +/- 0.12 (764)
pbp74	Cephamycin C	-7.70 +/- 0.00 (1000)	-7.00 +/- 0.00 (1000)
pbp74	Penicillin G	-7.10 +/- 0.01 (1000)	-6.93 +/- 0.05 (1000)
cmcT	Cephalosporin C	-8.34 +/- 0.06 (893)	-7.12 +/- 0.06 (440)
cmcT	Cephamycin C	-9.49 +/- 0.05 (550)	-9.10 +/- 0.04 (987)
cmcT	Penicillin G	-9.12 +/- 0.06 (894)	-8.50 +/- 0.01 (1000)

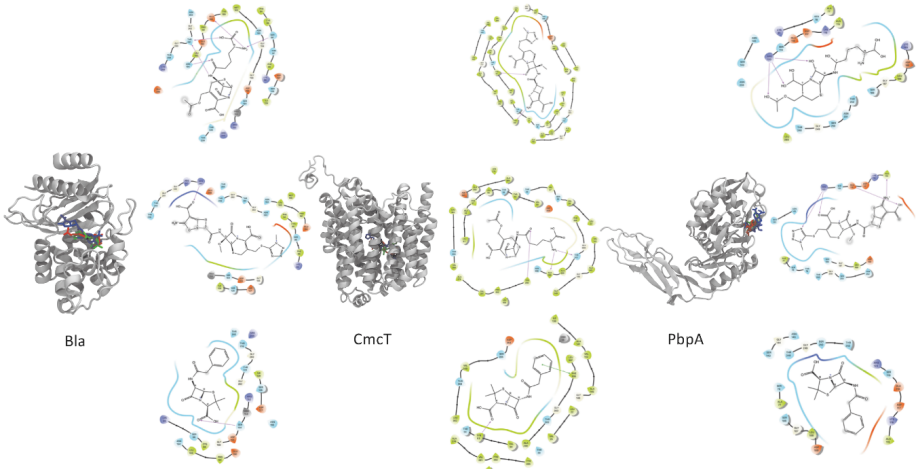

Figure 3. Docking of antibiotics to the proteins *cmcT*, *pbp74* and Bla.

In relation to penicillin G the parental strain was resistant to 180 μg/mL and both the *S. clavuligerus* Δ*pbp74*::hyg and Δbla::aphII strains were more sensible, growing only at 120 μg/mL, while the deleted *S. clavuligerus* Δ*cmcT*::acc mutant was slightly more resistant, growing at 200 μg/mL. The different sensitivity of these strains to cephalosporin versus penicillin G confirms the results described by Martínez-Burgo *et al*. (2014).

3.4 Ternary structures for cmcT, pbp74 and bla proteins

Structure prediction of the three proteins of *S. clavuligerus* were obtained by iTasser. The lowest C-Score models of each protein were used on this paper (-0.57, -0.44 and -1.71 for *cmcT*, *pbp74* and Bla, respectively).

Chimera MatchMaker function was used to align the proposed best iTasser models against their corresponding crystallographic templates. To compare the resemble of the predicted models, we made 1000 independent molecular docking studies of cephamycin, penicillin G and cephalosporin against the predicted models and their corresponding templates. Those templates were: i) multidrug Transporter EmrD from *E. coli* was used by iTasser for *cmcT* model (RMSD model vs 2GFP_A: 0.849Å), ii) Penicillin-Binding Protein 6 (PBP6) from *E. coli* for *pbp74* (RMSD model vs 3ITB_B: 0.823 Å) and iii) β-lactamase from Bacillus anthracis for *bla* (RMSD model vs 3QHY_A: 1.042 Å), suggesting the same possible roles on *S. clavuligerus*. Number in parenthesis are members on the analyzed cluster docking results:

As noted, cephamycin had the best affinity energy

on all proteins (but one) tested no matter if it was docked to the predicted model or the crystallographic structure. The exception was for model of *BLA* and cephamycin, where the crystallographic one had a lower affinity energy value. Figure 3 shows antibiotics positions and protein-ligand interactions.

4 Discussion

Genes for antibiotic export are common in clusters of genes for antibiotic biosynthesis in *Streptomyces* and other Actinobacteria. These genes belong mostly to the groups of ATP-binding cassettes (ABC transporters) as is the case of DrrA-DrrB in the doxorubicin gene cluster of *Streptomyces peucetius* (Kaur, 1997; Srinivasan *et al*., 2010), or are members of the major facilitator superfamily (MFS), as occurs to the TcmA protein for tetracenomycin resistance in *Streptomyces glaucescens* (Guilfoile and Hutchinson, 1992), or to PurT for puromycin resistance in *Streptomyces alboniger* (Tercero *et al*., 1993). However, knowledge on how these proteins act is still poorly understood. In the actinorhodin biosynthesis gene cluster two genes, *actAB*, encode export pumps which are essential for the production of actinorhodin. Their expression is repressed by ActR, a TetR-type regulator. Binding of several intermediates of the actinorhodin pathway to ActR releases *actAB* expression (Tahlan *et al*., 2007). The subsequent export of the final product, actinorhodin, and its binding to ActR, additionally blast the expression of the actinorhodin biosynthesis genes in the whole cell population (Xu *et al*., 2012) increasing the yield of this compound.

The *cmcT* gene, located in the CBG cluster of *S. clavuligerus*, appears to be important to produce this antibiotic since the deleted *cmcT*-mutant produces only 17% of the cephamycin levels in relation to those of the parental strain. This residual cephamycin production might be related to cell lysis or to poorly specific, alternative systems for cephamycin C export, as occurs for the transport of nystatin and cephalosporin C (Ullán *et al*., 2002; Sletta *et al*., 2005). The *cmcT* gene expression increases from 11 to 36 h in the wild type strain, when cephamycin C production starts. As noted, *cmcT* gene encodes a protein that resemble to multidrug transporter EmrD from *E. coli*. The affinity energy results indicate that this transporter can be used by the cell to export cefamycin and other β-lactams. High intracellular cephamycin concentrations might be lowered by the exporting action of this *cmcT* transporter. The *cefT* protein is involved in cephalosporin C and isopenicillin N secretion in *A. chrysogenum* and in a recombinant *Penicillium chrysogenum* strain (Ullán *et al*., 2002; Nijland *et al*., 2008), and overexpression of this gene in *A. chrysogenum* results in a 100% increase in cephalosporin productions. The low yield of extracellular cephamycin by the *cmcT*-deleted strain may not trigger an additional expression of the CBG cluster resulting in low cephamycin formation, as is the case with actinorhodin in *Streptomyces coelicolor* (Xu *et al*., 2012). It will be of interest to determine whether overexpression of *cmcT*, results in higher cephamycin C yields, as occurs with *cefT* in *A. chrysogenum* (Ullán *et al*., 2002; Nijland *et al*., 2008).

The β-lactams action occurs in the outside of the cell, since their action on the integrity and reorganization of the cell walls occurs in the periplasmic space; therefore, the resistance to β-lactams may be due to its binding by PBP proteins and/or to inactivation by hydrolysis, as occur with β-lactamases. The small increase of resistance to β-lactams produced by *cmcT* deletion is therefore difficult to understand.

Computational results (RMSD against crystallographic and affinity energies) support the hypothesis that *cmcT* might be used by *S. clavuligerus* as transporter to export different types of antibiotics outside the cell. The very similar affinity energies showed in Table 3 indicate, that the antibiotics used on this work may interact with *cmcT* transporter on a comparable magnitude. Also, the *cmcT* model allows us to support the role of the *cmcT* transporter in the mutant one due to its low difference in affinity energy for the antibiotics studied (Table 3). These low differences in affinity energies might be attributable to a broader spectrum of *cmcT* antibiotics interaction. Therefore, this protein may be used by *S. clavuligerus* to export those molecules outside the cell.

In the present work, the *pbp74* gene was deleted from *S. clavuligerus*, and the mutant showed a normal phenotype, morphology and sporulation. This suggests that the main function of *pbp74* is not related to cell wall formation but to serve as a lure to trap beta-lactam molecules. The role of *pbp74* does not appears to be essential to antibiotic production (Fig. 1). It should be also noticed the lack of specificity for any of the three antibiotics used obtained by docking experiments. The resistance to cephalosporin in Δ*pbp74* mutant is identical to that of the parental strain, indicating that *pbp74* bind penicillins and, therefore, the deleted *pbp74* strain is more sensitive to penicillin G (MIC 120 μg/mL) when compared to the parental strain, suggesting that this protein is binding the external penicillin G, and probably other cephamycin intermediates of the pathway, like isopenicillin N or penicillin N, that might be released from the cells. In addition to *pbp74*, two genes for penicillin binding proteins (*pbpA* and *pbp2*) are located in the clavulanic acid gene cluster, at the other end of the cephamycin C-clavulanic acid supercluster. Their affinity for penicillin G is low and they appear

to have lower MIC for penicillin G than *pbp74*, but the experimental conditions used by Ishida *et al*. (2006) are different and comparison of the results is difficult.

The resistance of *S. clavuligerus* to cephalosporin is very high (MIC 9 mg/mL) as shown in Table 2. This *Streptomyces* specie, possess higher resistance to cefoxitin than that shown by cephamycin non-producer strains such as *S. coelicolor*, *Streptomyces albus* or *Streptomyces flavogriseus* (Martínez-Burgo *et al*., 2014). This resistance is probably not due to a single protein, i.e. *pbp74* or *cmcT*. It is possible that the cell-wall synthesizing machinery of *S. clavuligerus* has developed an intrinsic resistance to cephalosporin-type of β-lactams as a way to survive the production of cephamycin.

Strain *S. clavuligerus* Δbla::aphII shows the same behavior as the *pbp74*-deleted mutant, being the MIC values identical in both strains, both for penicillin G and for cephalosporin C. This is not surprising since the *bla* protein has penicillin binding activity *in vitro* (Pérez-Llarena *et al*., 1997). In fact, the Km values of this enzyme for penicillin G is 11 μM, and 250 μM for cephamycin C, showing Km values 2 to 4-fold higher for cephalosporin C and other semisynthetic cephalosporins, than for cephamycin (Pérez-Llarena *et al*., 1997). Since the MIC for cephalosporin *in vivo* is very high (Martínez-Burgo *et al*., 2014) (Table 2), although the *bla* protein is not very active against cephalosporin, we must conclude that either cephalosporin has not access to the cell-wall synthesizing enzymes or these enzymes have evolved to be naturally insensitive to cephalosporins The β-lactamase activity of *S. clavuligerus*, is not detectable *in vivo*, probably due to the β-lactamase inhibitory compounds (clavulanic acid, protein BLIP) produced by this strain, but somehow an intermediate molecule, perhaps penicillin N or isopenicillin N, may be accumulated intracellularly by the Δ*bla*-deleted strain and excreted to the broth affecting cell wall biosynthesis. In fact, growth of the *bla*-deleted strain was very poor, in relation to the parental strain. In addition, colonies of *S. clavuligerus* Δbla::aphII showed an earlier onset of sporulation in solid medium and slightly different morphology.

Otherwise, this intermediate may work as a second inducer of the cephamycin genes expression, since clearly the deleted mutant showed a 26% increase in volumetric cephamycin C production (in the order of 93% in specific production). Mutation of the *bla* gene located in the CBG cluster of *N. lactamdurans* resulted in a strain with higher sensitivity to penicillin G than the parental strain; this mutant produced more cephamycin C when cultured in solid medium (Kumar *et al*., 1996) revealing a behavior similar to that of the *S. clavuligerus* Δbla::aphII strain. Deletion of the *bla* gene in industrial strains may results in improving cephamycin C production at industrial levels.

The theoretical models and molecular docking studies allowed us to corroborate that the proteins *bla* and *pbp74* resemble those found in other organisms with similar structures and activities. The RMSD values lower than 2Å (Table 3), showed by computational modeling, resemble to those obtained experimentally; therefore, we could assume that the computational models, proposed in this work, could have similar functions to those obtained experimentally.

In summary, our results indicate that *cmcT* is a cephamycin exporter required for maximal cephamycin C production, and that *pbp74* supports the control and decrease of penicillin-type molecules in the periplasmic space. Finally, the *bla* gene has a role similar to *pbp74*, but in addition its deletion may result in higher concentration of an intracellular intermediate that trigger cephamycin production as occurs in the case of actinorhodin (Xu *et al*., 2012).

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