A rhodanese-like protein is highly overrepresented in the mutant *S. clavuligerus oppA2::aph*: effect on holomycin and other secondary metabolites production

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Summary
A protein highly overrepresented in the proteome of *Streptomyces clavuligerus* oppA2::aph was characterized by MS/MS as a rhodanese-like enzyme. The rhlA gene, encoding this protein, was deleted from strains *S. clavuligerus* ATCC 27064 and *S. clavuligerus* oppA2::aph to characterized the RhlA enzyme activity, growth on different sulfur sources and antibiotic production by the mutants. Whereas total thiosulfate sulfurtransferase activity in cell extracts was not affected by the rhlA deletion, growth, cephapycin C and clavulanic acid production were impaired in the rhlA mutants. Holomycin production was drastically reduced (66–90%) in the rhlA mutants even when using *S. clavuligerus* DrhlA pregrown cells, suggesting that this enzyme might be involved in the formation of the cysteine precursor for this sulfur-containing antibiotic. While growth on thiosulfate as the sole sulfur source was particularly low the volumetric and specific antibiotic production of the three antibiotics increased in all the strains in the presence of thiosulfate. This stimulatory effect of thiosulfate on antibiotic production was confirmed by addition of thiosulfate to pre-grown cells and appears to be a general effect of thiosulfate on oxidative stress as was also evident in the production of staurosporin by *S. clavuligerus*.

Introduction
*Streptomyces clavuligerus* is industrially used for the production of clavulanic acid (Baggaley *et al.*, 1997) and therefore the biosynthesis of this enzyme inhibitor has received considerable attention. In addition, other antibiotics, some with a different β-lactam structure, as cephamycin C or the clavams, and other secondary metabolites, as the anti-tumoral holomycin, are produced by this strain, although most of the studies on biochemistry or genetics have been focused on β-lactam biosynthesis (Liras and Demain, 2009; Liras *et al.*, 2010).

Holomycin is a pyrrothinic compound formed by two fused heterocyclic rings, the first containing an internal amide group, while the second is formed by an internal disulfide bridge (Fig. 1). Several antibiotics of the same family, such as holothin, aureothricin or thiolulin, differ in the N-7 and N-4 substituents, and are produced by organisms as *Xenorhabdus* sp. (Li *et al.*, 1995), *Alteromonas rava* (Shiozawa *et al.*, 1993) and *Saccharothrix algeriensis* (Lamari *et al.*, 2002). Some derivatives may be obtained by semisynthesis (Chorin *et al.*, 2009). Holomycin possesses antibacterial activity against *Micrococcus luteus* and has been described to act as precursor of an RNA polymerase inhibitor (Oliva *et al.*, 2001). In addition, holomycin has anti-tumoral activity (Webster *et al.*, 2000).

The wild-type *S. clavuligerus* ATCC 27064 does not produce detectable amounts of holomycin. However, mutants blocked in the late steps of clavulanic acid formation produce large amounts of holomycin. This is the case in *S. clavuligerus* car::aph, blocked in the last step of the pathway, and of null mutants in oppA2 or cyp, genes probably involved in the last steps of clavulanic acid biosynthesis. The oppA2 gene encodes a putative oligopeptide permease (Mackenzie *et al.*, 2010), but its exact role is still unknown. Mutants in the regulatory genes ccaR or claR also showed holomycin production (de la Fuente *et al.*, 2002).

Holomycin production seems to be related to sulfur metabolism in *S. clavuligerus*, and has been reported to be stimulated by cysteine while it is inhibited by methionine and ethionine (Bouras *et al.*, 2006). In the course of a proteomic study to compare the wild-type strain *S.
S. clavuligerus ATCC 27064 and different clavulanic acid non-producer mutants we detected a strong overrepresentation of an intracellular protein characterized as a rhodanese-like (rhlA gene) in the holomycin overproducer strain S. clavuligerus oppA2::aph.

Rhodaneses are enzymes that catalyse the desproportionation of the thiosulfate anion (S$_2$O$_3^{2-}$). During the reaction, one of the two sulfur atoms is oxidized to sulfite whereas the other is reduced to sulfide. The only rhodanese studied in Actinomycetes is that of Saccharopolyspora erythraea (Donadio et al., 1990), encoded by a gene inappropriately named cysA. Disruption of cysA in S. erythraea resulted in an auxotroph mutant unable to grow on sulfite or sulfate as sole sulfur sources and which required the addition of methionine, cysteine or thiosulfate to grow. All the thiosulfate sulfurtransferases have one or two so-called ‘rhodanese’ domains, with the active site of the enzyme located in the C-terminal domain. Several functions have been ascribed to thiosulfate sulfurtransferases, including thiosulfate metabolism, cyanide detoxification (Sorbo, 1957), prosthetic groups formation in S-Fe proteins (Pagani et al., 1984) or thiamine biosynthesis (Palenchar et al., 2000). Recently, an important protection effect has been attributed to the rhodanese of Azotobacter vinelandii (Cereda et al., 2009) in that its absence renders the cells hypersensitive to oxidative stress.

To explore the possibility that the rhlA encoded activity was related to holomycin biosynthesis it was of great interest to study the effect of this gene and its disruption on holomycin production using the holomycin overproducer mutant, S. clavuligerus oppA2::aph.

**Results**

A protein overrepresented in S. clavuligerus oppA2::aph proteome corresponds to a rhodanese-like enzyme

The MALDI analysis of the protein highly overexpressed in the oppA2 mutant (Fig. 2) revealed fragments of 1227.558 (ms/ms spectra of M+2H+ 614.28) for a sequence DFIDQEGFEK, and 1365.691 (ms/ms spectra of M+2H+ 683.35) corresponding to the sequence ALYT-DEQVDLAK, 100% and 75% identical to internal peptides of S. avermitilis protein Q82G61, which corresponds to a rhodanese. Therefore, a 507 bp DNA fragment was amplified from S. clavuligerus genome using degenerated oligonucleotides; the nucleotide sequence of this fragment confirmed that it belongs to a gene encoding a putative rhodanese-like protein. The complete sequence of the gene, tentatively named rhlA, was provided by DSM (Delft, Holland) as SCLAV_3193 (which corresponds to the Broad Institute entry SSGC_01126.1). The rhlA encoded protein has 281 amino acids, is 69% identical to cysA (SACE_7106) of Saccharopolyspora erythraea (Donadio et al., 1990) and contains two rhodanese motifs at the C- and N-terminal ends (L7 to E117 and P148 to G268). The active site corresponds to the cysteine C235. Homologous proteins to SCLAV_3193 are SCO4164 in S. coelicolor and SAV_4037 in S. avermitilis.

Bioinformatic analysis of the S. clavuligerus genome allowed to detect an additional gene (SCLAV_4718) (Medema et al., 2010) encoding a different rhodanese-like protein. This also occurs in S. coelicolor and S. avermitilis genomes, which contain additional rhodanese-like genes, SCO5854 and SAV_2412 respectively. The percentage of identity between the two rhodanese-like proteins in each strain is in the order of 25%. The second putative rhodanese of S. clavuligerus is rather different from the first one and is not overrepresented in the oppA2 mutant proteome.

![Fig. 1. Structure of the holomycin. The substituents R1 and R2 present in other members of the pyrrothine family are shown below.](image)

![Fig. 2. 2D-gel of the proteome of S. clavuligerus ATCC 27064. The proteome corresponds to a culture grown for 36 h in SA medium. A fragment of the gel is amplified to show the rhodanese-like protein (indicated with an arrow) overrepresented in S. clavuligerus oppA2::aph.](image)

A. S. clavuligerus ATCC 27064.
B. S. clavuligerus oppA2::aph.
### Deletion of the rhlA gene in S. clavuligerus

Plasmid ΔrhlA was introduced by conjugation in *S. clavuligerus* oppA2::aph and in the parental strain *S. clavuligerus* ATCC 27064. Apramycin-resistant, hygromycin-sensitive exconjugants of both strains were analysed by PCR using oligonucleotides O7/O8 (annealing 239 bp upstream and 539 bp downstream of the rhlA-ORF respectively) to confirm the deletion. The rhlA-deleted exconjugants amplified the correct 2259 bp DNA fragment corresponding to the insertion of the apramycin-cassette in the rhlA-deleted region, while PCR of the wild-type and oppA2 control strains amplified a 1704 bp DNA fragment due to the presence of the intact rhlA gene. These fragments were partially sequenced for confirmation. The rhlA-deleted strains were named *S. clavuligerus* ΔrhlA and *S. clavuligerus* oppA2::aph ΔrhlA respectively.

### Characterization of *S. clavuligerus* ΔrhlA and *S. clavuligerus* oppA2::aph ΔrhlA

The *S. clavuligerus* ΔrhlA mutant, as well as its parental strain, was able to form aerial mycelium and spores in SA medium. *Streptomyces clavuligerus* oppA2::aph is a bald mutant (de la Fuente *et al.*, 2002); however, the double mutant *S. clavuligerus* oppA2::aph ΔrhlA was able to produce aerial mycelium but no spores.

Since the rhodanese-like (cysA) mutant of *S. erythraea* has been described to be a cysteine auxotroph, we characterized the phenotype of *S. clavuligerus* rhlA-deleted mutants in solid and liquid media using different sulfur sources.

### Solid medium cultures

The rhlA-deleted mutants and their parental strains were incubated for 36 h on the surface of plastic wrap on top of TSA medium plates (to start growth) and then transferred to SA medium plates (which contains sulfate) as positive control, SA-Sminus medium (negative control) and SA-Sminus medium supplemented with different sulfur sources at 3 mM concentration. The growth on the plates was followed for up to 10 days. Table 1 shows that wild-type strain and *S. clavuligerus* ΔrhlA grow similarly on all sulfur sources. Both strains are unable to use homocysteine or taurine, and grow poorly on thiocyanate or thiosulfate as sulfur source. Surprisingly, the ΔrhlA mutant strain grows well on sulfate, sulfite and sulfide, indicating that this mutant is not a cysteine auxotroph, unlike of what occurs in *S. erythraea* (see Discussion). The main differences in growth were found between *S. clavuligerus* ATCC 27064 and *S. clavuligerus* oppA2::aph. The mutation of oppA2 results in poor growth on L-methionine, L-cysteine and L-cystathionine and no growth on persulfate and glutathione; this effect was partially reverted in the double oppA2::aph ΔrhlA mutant.

### Liquid medium cultures

On SA-Sminus supplemented medium (Fig. 4, upper panels), sulfate was always the best sulfur source for all the strains, followed by bisulfite and sulfite (not shown), while thiosulfate was a poor sulfur source for all strains. The partial reversion effected by the rhlA deletion on the phenotype of the oppA2::aph mutant in solid medium was confirmed in liquid medium, where *S. clavuligerus* oppA2::aph ΔrhlA recovered almost wild-type growth levels on sulfate and bisulfite.

### Thiosulfate sulfurtransferase activity in the cultures

The rhlA gene is expressed in *S. clavuligerus* ATCC 27064 at 37 and 60 h of culture as detected by RT-PCR experiments (Fig. 3D). In order to know if rhlA encodes a rhodanese-like enzyme with thiosulfate sulfurtransferase activity, the enzyme was measured in *S. clavuligerus* ATCC 27064, *S. clavuligerus* oppA2::aph and

| Sulfur source | *S. clavuligerus* ATCC 27064 | *S. clavuligerus* ΔrhlA | *S. clavuligerus* oppA2::aph | *S. clavuligerus* oppA2::aph ΔrhlA |
|---------------|-----------------------------|-------------------------|-----------------------------|----------------------------------|
| Sulfate       | ++                          | ++                      | ++                          | ++                               |
| Sulfite       | ++                          | ++                      | ++                          | ++                               |
| Sulfur        | ++                          | ++                      | ++                          | ++                               |
| Bisulfite     | ++                          | ++                      | ++                          | ++                               |
| Thiosulfate   | +                           | +                       | +                           | +                                |
| Persulfate    | ++                          | ++                      | ++                          | ++                               |
| L-cysteine    | ++                          | ++                      | ++                          | ++                               |
| L-methionine  | ++                          | ++                      | ++                          | ++                               |
| Homocysteine  | ++                          | ++                      | ++                          | ++                               |
| Homocystine   | ++                          | ++                      | ++                          | ++                               |
| Cystathionine | ++                          | ++                      | ++                          | ++                               |
| Taurine       | --                          | --                      | --                          | --                               |
| Thiocyanate   | +/-                         | +/-                     | +/-                         | +/-                              |
| Glutathione   | ++                          | ++                      | ++                          | ++                               |
| None          | --                          | --                      | --                          | --                               |

Table 1. Growth of *S. clavuligerus* ATCC 27064 and their mutants in different sulfur sources.
their respective rhlA-deleted mutants. Cultures were grown in SA medium and the enzyme activity was measured in cell extracts of 37 and 60 h of culture. Two compounds, thiosulfate and 3-mercaptoppyruvate, were used as substrates for the enzyme, giving relatively similar specific activities (1.16 ± 0.33 mUnits versus 0.87 ± 0.20 mUnits µg⁻¹ protein respectively), with an activity slightly higher at 37 h than at 60 h. The results indicate that the rhlA deletion has no effect on the total thiosulfate sulfurtransferase activity (1.0 ± 0.24 in the wild-type strain versus 1.04 ± 0.37 mUnits µg⁻¹ protein in the rhlA-deleted mutant), suggesting that the rhlA gene does not encode a thiosulfate sulfurtransferase.

### Secondary metabolites production by rhlA-deleted strains

Production of holomycin, cephapycin C and clavulanic acid by the control and rhlA-deleted strains was analysed in liquid SA-Sminus medium containing sulfate, bisulfite and thiosulfate as sulfur sources (Fig. 4).

### Production of antibiotics by pre-grown cells

To test whether the higher specific productivity of holomycin, cephapycin C and clavulanic acid in thiosulfate grown cells was due to the low growth rate observed on this sulfur source, a system of cells pre-grown in SA medium was devised. Cells grown for 50 h in SA medium were washed twice and suspended in SA-Sminus medium supplemented with thiosulfate and also in SA medium as control. Regarding holomycin a very low holomycin production was observed in SA medium (Fig. 5A) while S. clavuligerus oppA2::aph cells suspended in SA-Sminus with thiosulfate produce holomycin linearly for more than 24 h, reaching a level of 720 µg mg⁻¹ DNA of holomycin (Fig. 5A, right panel) in spite of the low growth. The production of holomycin by pre-grown cells of the rhlA-deleted mutant was only 12% of that observed in the control strain, indicating that indeed the rhlA gene is required for holomycin biosynthesis.

Almost similar results were observed for the cephapycin C production by the wild-type strain or the rhlA mutant

![Image of deletion of rhlA](image-url)
Thiosulfate-suspended cells produced a very high level of cephamycin in relation to sulfate-suspended cells. Clavulanic acid was produced by cells suspended in either sulfate or thiosulfate (Fig. 5C). In the presence of thiosulfate clavulanic acid is produced linearly by the wild-type strain and reaches levels about fourfold higher. The production of the three antibiotics is always lower in the rhlA-deleted mutants, especially in the case of holomycin and cephamycin. All these results suggest that thiosulfate assimilation exert a general effect that leads to overproduction of secondary metabolites; the effect is exerted to a different extent depending on the biosynthetic pathways.

Addition of thiosulfate to sulfate growing cultures causes stress and leads to overproduction of four different secondary metabolites

Similar results were obtained when cultures growing for 39 h in SA medium were supplemented with either 3 or 5 mM thiosulfate. Under these conditions, in which sulfate is present in the culture medium, the three antibiotics production was stimulated by 3 mM and even more by 5 mM thiosulfate. This result was observed in the wild-type strain for cephamycin C and clavulanic acid, and for holomycin in the S. clavuligerus oppA2::aph and S. clavuligerus oppA2::aph ΔrhlA mutants.
Production of cephapemycin C and clavulanic acid by \textit{S. clavuligerus} \(\Delta rhlA\) mutant followed the same pattern but, as shown in previous experiments, the antibiotic production in this strain is 90\% lower for cephapemycin C and 60\% lower for clavulanic acid as compared with the parental strain (not shown).

The presence of genes for staurosporin production in \textit{S. clavuligerus} genome has been recently described (Medema \textit{et al.}, 2010). To test if this antibiotic, which has no sulfur in its structure, was also affected by the addition of thiosulfate, \textit{S. clavuligerus} ATCC 27064 was grown in TSB medium and 5 mM thiosulfate was added at 36 h of cultivation. The whole 72 h culture (100 ml) was extracted with ethylacetate (v/v), and staurosporin was determined by HPLC. It can be observed that the production of staurosporin increased about fourfold in the presence of 5 mM thiosulfate (Fig. 6). Since, again, staurosporin has no sulfur in the molecule, the effect of thiosulfate on the antibiotic production must be related to a general stimulatory effect, i.e. oxidative stress.

\textbf{Discussion}

\textit{Streptomyces}, as many other bacteria, are able to use sulfate, sulfide, methionine and cysteine, which contain the sulfur atom in different degrees of oxidation, as sulfur sources. Some bacteria also use thiosulfate or elemental sulfur. The thiosulfate molecule contains two sulfur atoms with lower degree of oxidation than sulfate. The enzyme known as rhodanese (2.8.1.1) is involved in the disproportionation of thiosulfate oxidizing one of the atoms to sulfate while reducing the other to sulfide. No clear function has been ascribed to rhodaneses (Cerletti, 1986), but the presence of rhodaneses has been well studied in

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{Antibiotic production by pre-grown cultures of the different strains. Production in sulfate supplemented cultures (left panels) or thiosulfate supplemented cultures (right panels).
A. Holomycin.
B. Cephamycin C.
C. Clavulanic acid production by \textit{S. clavuligerus} ATCC 27064 (white circles), \textit{S. clavuligerus} \(\Delta rhlA\) (black circles), \textit{S. clavuligerus oppA2::aph\textunderscore D\(rhlA\)} (white rectangles) and \textit{S. clavuligerus oppA2::aph\textunderscore D\(rhlA\)} (black rectangles).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6.png}
\caption{Production of staurosporin by \textit{S. clavuligerus}. HPLC profile of ethyl acetate extract of \textit{S. clavuligerus} cultures. \textit{Streptomyces clavuligerus} ATCC 27064 grown for 72 h in TSB medium (discontinuous lane). Cultures supplemented with 5 mM thiosulfate (continuous lane). Staurosporin retention time under the HPLC utilized conditions is 5.9 min and coincident with a sample of the pure compound.}
\end{figure}
Desulfovibrio and other sulfur chemolithotrophs (Aketagawa et al., 1985) and is general in plants, animal tissues and microorganisms.

During the proteomic studies to characterize the holomycin high producer S. clavuligerus oppA2::aph, a highly overrepresented protein was characterized by MS-MS spectroscopy as a rhodanese-like enzyme. Transcriptomic studies of this mutant confirmed the overexpression of the rhlA gene (R. Álvarez, personal communication). The rhodanese-like enzyme encoded by rhlA is not a standard rhodanese involved in thiosulfate utilization as sulfur source, as evidenced by the following observation: growth of S. clavuligerus strains on thiosulfate is poor and there are no differences in the utilization of thiosulfate or other sulfur nutrients between the control strains and the mutants deleted in the rhlA gene as opposed to what occurs in S. erythraea (Donadio et al., 1990). One difference observed between rhlA of S. clavuligerus and the homologous cysA in S. erythraea is that deletion of cysA in S. erythraea results in auxotrophy for cysteine (Donadio et al., 1990). Both in S. clavuligerus and S. erythraea there are sets of genes encoding enzymes for cysteine biosynthesis (encoding an authentic cysA gene) that could catalyse the condensation of serine and SH2 to form cysteine, or O-acetylserine and thiosulfate to form sulfocysteine and acetate respectively. The poor growth on thiosulfate suggests that formation of sulfocysteine and its subsequent reduction to cysteine in S. clavuligerus is not efficient, in contrast with what was reported for S. erythraea (Donadio et al., 1990).

Furthermore, no differences in thiosulfate sulfurtransferase activity were found in cell extracts of the control versus the rhlA-deleted strains, suggesting that the thiosulfate sulfurtransferase activity is not present in the RhlA protein. A different rhodanese-like gene in S. clavuligerus (SCLAV_4718) is expressed with similar intensity in the wild-type strain and rhlA-deleted mutants, as shown by RT-PCR using oligonucleotides specific for this second gene (Fig. 3D, lanes 3 and 4), and might be responsible for the thiosulfate sulfurtransferase activities assayed in this work, using thiosulfate and mercaptopyruvate as substrates. Similarly, an S. erythraea mutant disrupted in cysA showed the same thiosulfate sulfurtransferase activity as the parental strain (Donadio et al., 1990).

Another difference between S. erythraea and S. clavuligerus lies in the gene located immediately downstream of the cysA or rhlA genes, named SACE_7107 and SSCG_01125 respectively. Both of them encode 46% homologous small proteins (about 100 amino acids) of unknown function that have been proposed to be involved in sulfur metabolism (Donadio et al., 1990). Only three base pairs separate reading frame SACE_7107 and cysA, while there is a 57 nt intergenic region between rhlA and SSCG_01125. This different arrangement implies that deletion of cysA in S. erythraea could affect SACE_7107 gene transcription, while in S. clavuligerus ΔrhlA strains transcription of SSCG_01125 is probably not affected.

The role of the rhodanese-like protein encoded by rhlA gene is puzzling. Mutants defective in rhlA are severely impaired in holomycin production, and produce lower levels of cephamycin C and clavulanic acid than the parental strain. Although it is tempting to propose that the rhlA gene encodes a rhodanese involved in the formation of the S–S bridge in the molecule of holomycin (perhaps through a sulfocysteine intermediate), it is difficult to explain why its deletion decreases also clavulanic acid, since the last compound has no sulfur atom in its structure. Rather, we believe that the rhodanese-like enzyme might be involved in the oxidoreduction of disulfide bonds by a mechanism similar to that of thiosulfate sulfurtransferases and might exert a general effect in the cells and specifically on the formation of the disulfide containing antibiotic holomycin.

Thiosulfate accumulation appears to cause a metabolic stress in the cell causing reduction in growth as observed after its addition to sulfate-growing cultures; this might result in the volumetric and specific overproduction of the sulfur-containing antibiotics cephamycin C and holomycin but also of staurosporine and clavulanic acid, through a general stress rather than a sulfur-specific phenomenon, i.e. oxidative stress, as occurs in Azotobacter vinelandii (Cereda et al., 2009). Heat shock, pH, oxidative stress or environmental signals in other Streptomyces (Kim et al., 2008) result in overproduction of proteins as DnaK, GroEL2, catalase or the superoxide dismutase, and this might be the case for the rhlA-encoded protein. The study of the proteome of thiosulfate-grown cultures might shed more light on this phenomenon.

**Experimental procedures**

**Bacterial strains and cultures**

Streptomyces clavuligerus ATCC 27064 was used as control strain, producer of clavulanic acid and cephamycin C. S. clavuligerus oppA2::aph (Lorenzana et al., 2004) produces holomycin and cephamycin C but does not produce clavulanic acid. Mutants S. clavuligerus ATCC 27064 ΔrhlA and S. clavuligerus oppA2::aph ΔrhlA have been constructed in this work. SA medium (in g l⁻¹: asparagine, 2; starch, 10; MgSO₄·7H₂O 0.6; K₂HPO₄ 4.4; trace elements 1 ml, MOPS 21, pH 6.8; Trace elements in g l⁻¹: FeCl₃·6H₂O, 0.97; ZnCl₂ 0.474; MnCl₂·4H₂O 1; CaCl₂ 1) was used to grow S. clavuligerus strains. The effect of different sulfur sources was analysed in a modified SA medium named SA-Sminus, lacking sulfur sources, in which MgSO₄·7H₂O and the sulfate salts in the original trace elements were substituted by MgCl₂ and metallic chlorides, respectively, and MOPS buffer by Tris-HCl buffer. This medium was supplemented with the required sulfur sources at 3 mM concentration. Pre-grown cells were prepared from 50 h cultures of SA grown mycelia,
which were washed with NaCl 0.9% and suspended at optical density 4.0 in SA-Sminus medium supplemented with the required sulfur sources. To determine stauroporine production, the fermentations were carried out in TSB (trypticase soy broth) medium using as inoculum a 5% v/v of a 24 h culture grown in the same medium. TSA medium is TSB containing 2% agar.

**Antibiotics determination**

Cephamycin C was determined by bioassay using *E. coli* Ess 22-31 as tester strain (Hu et al., 1984). Clavulanic acid was determined both by bioassay using *Klebsiella pneumoniae* ATCC 29665 and by HPLC (Liras and Martin, 2005). Stauroporine was extracted from the cultures with an equal volume of ethylacetate and quantified by HPLC using the conditions described by Onaka and colleagues (2002) and a commercial standard of stauroporine (Roche).

**Nucleic acids manipulations**

General DNA manipulations were performed using standard techniques (Sambrook et al., 1989). *Streptomyces* genomic and plasmid DNA preparations, and *S. clavuligerus* conjugation with *E. coli* ET12567/pUZ8002 as donor strain was made following standard methods (Kieser et al., 2000). Oligonucleotides used in this work are described in Table 2.

RNA samples from *S. clavuligerus* strains were prepared using RNeasy mini-spin columns as previously described by Santamarta and colleagues (2005) and treated with DNase I (Qiagen) and Turbo DNase (Ambion) to eliminate chromosomal DNA contamination. PCR and RT-PCR were performed in a T-gradient (Biometra) thermocycler.

**Construction of p.rsrlA to delete the rhlA gene.** A 2031 bp DNA fragment upstream of the rhlA ATG and a 2099 bp DNA fragment starting 8 bp upstream of the TGA codon were PCR amplified using oligonucleotides O3/O4 and O5/O6 respectively. The fragments, digested with EcoRI/SpeI and HindIII/I-Xhol separately, were subcloned in pBluescript KS to give plasmids KS-UrhlA and KS-DrhlA. The 1375 bp cassette acc(3)/IV-orl was isolated from plasmid pJ1773 and subcloned in EcoRI/HindIII digested KS-DrhlA to produce plasmid KS-acc>rhlA (6408 bp). After EcoRI/SpeI digestion, the amplified rhl-upstream DNA fragment was inserted. The plasmid was then digested with SpeI and the hygromycin resistance gene, isolated from plJ10700 as a 1505 bp XbaI fragment, was inserted to give plasmid p.rsrlA (9920 bp)

**PCR amplification**

i. Amplification of a DNA fragment internal to rhlA. In base to the amino acid sequences (DFFDQEGFEK and ALYTDSEQVLAK) provided by the MALDI-Toff analysis two degenerated oligonucleotides (O1/O2) were designed with the following degeneration % (R18: 80%; G, 20%; S5: 60%; G, 40%; Y11: 80%; C, 20%; and S17: 70%; G, 30%). The PCR conditions were as follows: oligonucleotides O1/O2 (0.2 μM, each) and Go-Taq polymerase (0.8 units) MgCl2 1.5 mM, DMSO 5%, dNTP’s 100 μM each, and *S. clavuligerus* ATCC 27064 DNA template (70 ng). The PCR conditions were as follows: 94°C/1’, 1 cycle; 94°C/30”, 58°C/30”, 72°C/45”, 25 cycles; 72°C/10’, 1 cycle.

ii. PCR’s amplification for the construction of p.rsrlA. The upstream and downstream fragments were amplified using total *S. clavuligerus* ATCC 27064 DNA as template (70 ng). PfX DNA polymerase (0.6 units), MgCl2 1.5 mM, DMSO 5%, dNTP’s 40 μM each, and *S. clavuligerus* ATCC 27064 DNA template (70 ng). The PCR conditions were as follows: 94°C/1’, 1 cycle; 94°C/30”, 58°C/30”, 72°C/45”, 25 cycles; 72°C/10’, 1 cycle.

iii. PCR amplification to confirm the deletion of rhlA. It was done using oligonucleotides O7/O8 (0.5 μM each) and Go-Taq polymerase (0.8 units) (Promega), MgCl2 4 mM, DMSO 1.5%, dNTP’s 100 μM and DNA template (200 ng). The PCR conditions were as follows: 95°C/5’, 1 cycle; 95°C/30”, 58°C/30”, 72°C/2’30”, 25 cycles; 72°C/10’, 1 cycle.

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**Table 2. Oligonucleotides used in this work.**

| Name | Sequence 5’ to 3’ | Use |
|------|------------------|-----|
| O1   | GACTTCATCGACCAGGAR | To isolate a 507 bp DNA fragment internal to rhlA |
| O2   | GGTCSACCCTGTTCGCTG | To isolate a 507 bp DNA fragment internal to rhlA |
| O3   | O1GAGATCAGGCCGCTGGCTG | To amplify a 2031 bp DNA fragment upstream of rhlA |
| O4   | GGAATTCCATGCTTGGTCTCCGG | To amplify a 2031 bp DNA fragment upstream of rhlA |
| O5   | CCAAGGTAAAACGACAATGCTGAG | To amplify a 2099 bp DNA fragment downstream of rhlA |
| O6   | CGCGCTAGTTTCACTAGCCGTAGACG | To amplify a 2099 bp DNA fragment downstream of rhlA |
| O7   | TCAGATGGCCGTTGAC | To characterize the deletion of rhlA |
| O8   | ATGTCTCCAGGCGACCG | To characterize the deletion of rhlA |
| O9   | CTCGCCGTACGAGAAGAACACA | To determine expression of SCLAV_4718 (395 pb) by RT-PCR |
| O10  | TTGACACCGGGAGAGTGTCGCG | To determine expression of SCLAV_3193 (513 pb) by RT-PCR |
| O11  | GAACGCCATCGACGCACGTCACA | To determine expression of SCLAV_4718 (395 pb) by RT-PCR |
| O12  | TCTCCACCGGTCGTCGTCAG | To determine expression of SCLAV_4718 (395 pb) by RT-PCR |

The underlined sequences have been added to digest with restriction enzymes, as follows: O3 (EcoRI), O4 (SpeI), O5 (HindIII) and O6 (XhoI).
RT-PCR. In RT-PCR experiments oligonucleotides O9 to O12 were used, as required. The 20 µl RT-PCR was performed using a one-step RT-PCR Platinum kit (invitrogen), containing in a final 20 µl volume RM buffer Mix, RT/Platinum Taq mix, oligonucleotides 0.2 µM each and RNA template 200 ng. The RT-PCR conditions were as follows: 50°C/30 min, 94°C/1 cycle, 95°C/30 s, 68°C/30 s, 72°C/40 s, 8 cycles; 95°C/30 s, 60°C/30 s, 72°C/40 s, 40 cycles; 72°C/10 min, 1 cycle. Control reactions to determine possible DNA contamination and the specificity of the oligonucleotides used were made by using Platinum Taq polymerase on RNA template and DNA templates.

Enzymatic assays

Cell-free extracts were prepared from mycelium of a 36 h and 60 h SA-grown culture. The mycelium was washed with 0.1 M phosphate buffer pH 7.2 containing EDTA 1 mM, sodium thiocyanate 10 mM and glycerol 10%. The cells were disrupted by sonication at 4°C for three pulses of 30 s at maximum speed in a Fast Prep FP120 (Thermo Savant). Thiocyanate sulfurrtransferase activity was tested by the production of thiocyanate in the presence of thiocyanate or mercaptopyruvate as sulfur donors (Westley, 1981). One unit is defined as the amount of enzyme that produces 1 µmol of thiocyanate per minute at 25°C.

Bidimensional electrophoresis

Mycelium was washed in 50 mM Tris-HCl pH 7.2 buffer (buffer A), then pelleted by centrifugation (5 min, 6000 r.p.m.) at 4°C. Washed cells were resuspended in buffer A supplemented with a Mini Protease Inhibitor Cocktail tablet (Roche) and disrupted in a FastPrep FP120 Homogenizer (Thermo Savant) (3 × 30 s bursts at amplitude 6.5 and 1 min intervals of rest). Cellular debris were removed by centrifugation (15 min, 14 000 r.p.m.) at 4°C and nucleic acids were eliminated by Benzonase (Roche) treatment (30 min at 37°C) and centrifuged (10 min, 14 000 r.p.m.) at 4°C. The supernatant was precipitated using the GE Healthcare 2D Clean-Up kit and the protein (300 µg) was resuspended in 350 µl of Rehydration Buffer (RB) (8M urea, 2% p/v CHAPS, 0.01% v/v Bromophenol Blue). Protein samples (80 µg), supplemented with 1% carrier ampholytes, were applied to 18 cm precast IPG strips of linear gradients (pH 4.5–5.5) using a previous 20 h rehydration step into an IPGphor IEG Unit (GE Healthcare). Separation was performed for 60,000 V h with a maximum voltage of 8000 V. After isoelectric focusing, IPG strips were equilibrated for second dimension during 15 min in IPG Equilibration buffer (6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS) plus 80 mM DTT. Electrophoresis was performed while cooling at 20,000 mW per gel constant power and a maximum voltage of 500 V in an Etan Dalt system (GE Healthcare). Gels were then stained with silver nitrate following a MS-compatible method. Gels from biological triplicates were scanned in an ImageScanner (GE Healthcare) imaging system and image analyses were performed by using the ImageMaster TM 2D Platinum v5.0 software (GE Healthcare). Triptic digestion of the proteins and MALDI-ToF and LC-ESI-IT of the proteins was performed at the Proteinomic laboratory (CBM, Madrid).

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