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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, cephamycin 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* Δ*rhlA* 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*.

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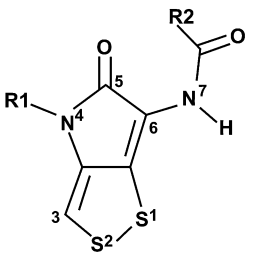
Introduction

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 thiolutin, 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.*



R1	R2	Compound
H	CH3	Holomycin
H	H	Holothin
CH3	CH2-CH3	Aureothricin

Fig. 1. Structure of the holomycin. The substituents R1 and R2 present in other members of the pyrrothine family are shown below.

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 ($S_2O_3^=$). During the reaction, one of the two sulfur atoms is oxidize 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 ALYTDEQVDLAK, 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 SSCG_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.

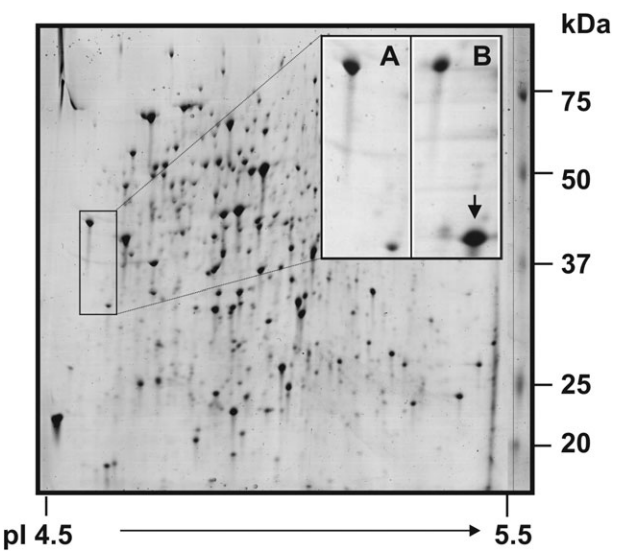


Fig. 2. 2D-gel of the proteome of *S. clavuligerus* ATCC 27064. The proteome corresponds to a culture grown for 36 h in SA medium. A fragment of the gel is amplified to show the rhodanese-like protein (indicated with an arrow) overrepresented in *S. clavuligerus oppA2::aph*.
A. *S. clavuligerus* ATCC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

Table 1. Growth of *S. clavuligerus* ATCC 27064 and their mutants in different sulfur sources.

Sulfur source	*S. clavuligerus* ATCC 27064	*S. clavuligerus* Δ*rhlA*	*S. clavuligerus oppA2::aph*	*S. clavuligerus oppA2::aph* Δ*rhlA*
Sulfate	++	++	++	++
Sulfite	++	++	++	++
Sulfur	++	++	++	++
Bisulfite	++	++	++	++
Thiosulfate	+	+	+	+
Persulfate	++	++	—	+
L-cysteine	++	++	+/–	+
L-methionine	++	++	+/–	+
Homocysteine	++	++	++	++
Homocystine	—	—	—	—
Cystathionine	++	++	+/–	+
Taurine	—	—	—	—
Thiocyanate	+/–	+/–	+/–	—
Glutathion	++	++	+/–	++
None	—	—	—	—

Deletion of the rhlA *gene in* S. clavuligerus

Plasmid pΔ*rhlA* 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* Δ*rhlA* and *S. clavuligerus oppA2::aph* Δ*rhlA* respectively.

Characterization of S. clavuligerus Δrhl*A and* S. clavuligerus oppA2::aph Δ*rhlA*

The *S. clavuligerus* Δ*rhlA* 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* Δ*rhlA* 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* Δ*rhlA* grow similarly on all sulfur sources. Both strains are unable to use homocystine or taurine, and grow poorly on thiocyanate or thiosulfate as sulfur source. Surprisingly, the Δ*rhlA* 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* Δ*rhlA* 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* Δ*rhlA* 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

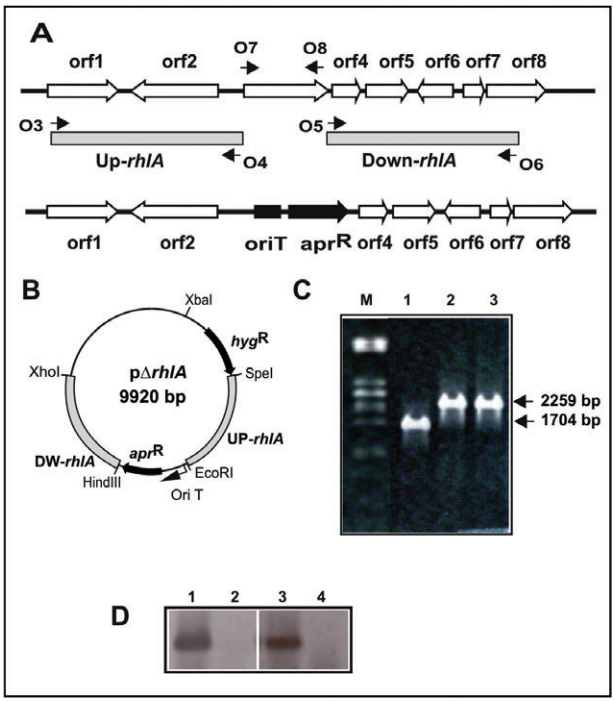


Fig. 3. Deletion of *rhlA*.
A. Map of the DNA region containing the *rhl*A gene in *S. clavuligerus* ATCC 27064 or *S. clavuligerus oppA2::aph* (above) and in the *rhlA*-deleted mutants (below).
B. Plasmid p∆*rhlA* used to delete the *rhlA* gene.
C. Fragments amplified by PCR using oligonucleotides O7/O8. M, molecular weight markers; 1, *S. clavuligerus* ATCC 27064; 2, *S. clavuligerus* ∆*rhlA*; 3, *S. clavuligerus oppA2::aph* ∆*rhlA*.
D. RT-PCR of RNA extracted from 32 h cultures of *S.clavuligerus* ATCC 27064 grown in SA medium and amplified with oligonucleotides O9/O10 to amplify *rhlA* (1,2) and O11/O12 to amplify SCLAV_4718 (3,4). Lanes 2 and 4 correspond to controls with only Platinum Taq polymerase.

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-mercaptopyruvate, 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, cephamycin 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).

Clavulanic acid and cephamycin C are produced by both *S. clavuligerus* ATCC 27064 and the *S. clavuligerus* ∆*rhlA* mutant. Production of clavulanic acid and cephamycin C by the wild-type strain reached maximum levels of 140 $\mu g\ mg^{-1}$ DNA and 2000 $\mu g\ mg^{-1}$ DNA, respectively, depending on the sulfur source. However, the production profile of both these antibiotics is in the order of 12–27% lower in the ∆*rhlA* mutants. Interestingly, the production level of these two antibiotics in sulfate was very small when compared with the levels reached by thiosulfate-grown cells (Fig. 4). This is specially surprising in the case of clavulanic acid which, in contrast with holomycin and cephamycin C, has no sulfur atoms in its molecule.

Holomycin production is undetectable in the wild-type strain (i.e. it may be considered a silent secondary metabolite) while it is produced by *S. clavuligerus oppA2::aph*. Interestingly, the lack of the *rhlA* gene in the double mutant *oppA2::aph* ∆*rhlA* resulted in drastically reduced holomycin levels (83% in sulfate, 91% in bisulfite and 66% reduction in thiosulfate at 70 h), indicating that *rhlA* gene might be involved in the formation of a cystine-like precursor of holomycin. Holomycin-specific production in *S. clavuligerus oppA2::aph* and *S. clavuligerus oppA2::aph* ∆*rhlA* growing in thiosulfate was five- to sevenfold higher than in sulfate. It is noteworthy that growth on thiosulfate was very poor, so it was not clear if thiosulfate was a good sulfur source to produce holomycin and cephamycin C or if the high specific production was due to the poor growth on thiosulfate observed in liquid medium. Therefore, the role of thiosulfate on the production of holomycin and the other secondary metabolites was studied in detail.

Production of antibiotics by pre-grown cells

To test whether the higher specific productivity of holomycin, cephamycin 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 $\mu g\ mg^{-1}$ DNA of holomycin (Fig. 5A, right panel) in spite of the low growth. The production of holomycin by pre-grown cells of the ∆*rhlA* 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 cephamycin C production by the wild-type strain or the *rhlA* mutant

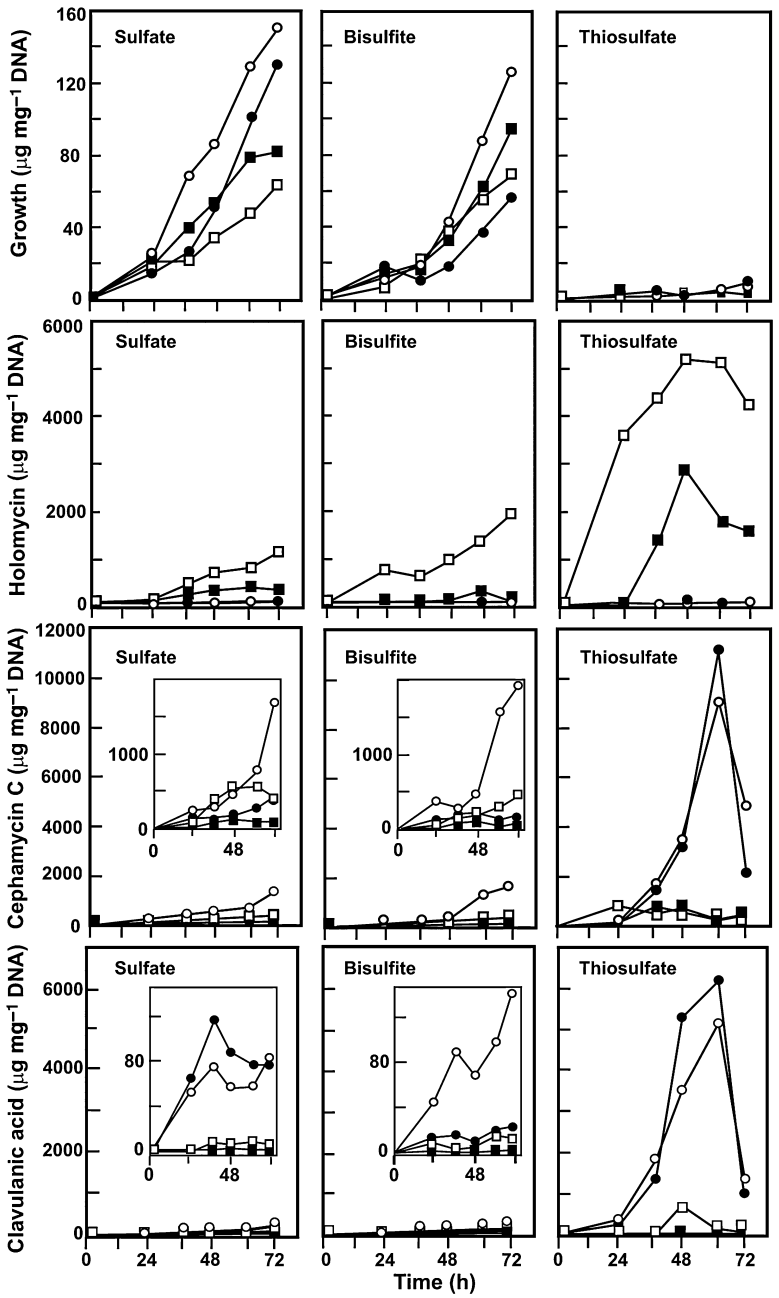


Fig. 4. Growth, holomycin, clavulanic acid and cephamycin C production in SA cultures. The panels correspond to cultures containing sulfate (left), bisulfite (centre) or thiosulfate (right) as sulfur source. The strains used are: *S. clavuligerus* ATCC27064 (white circles), *S. clavuligerus* Δ*rhlA* (black circles), *S. clavuligerus oppA2::aph (*white rectangles), *S. clavuligerus oppA2::aph* Δ*rhlA* (black rectangles). Insets show amplifications of the antibiotic produced in the same panel by the different strains.

(Fig. 5B). 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 over-production 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* Δ*rhlA* mutants.

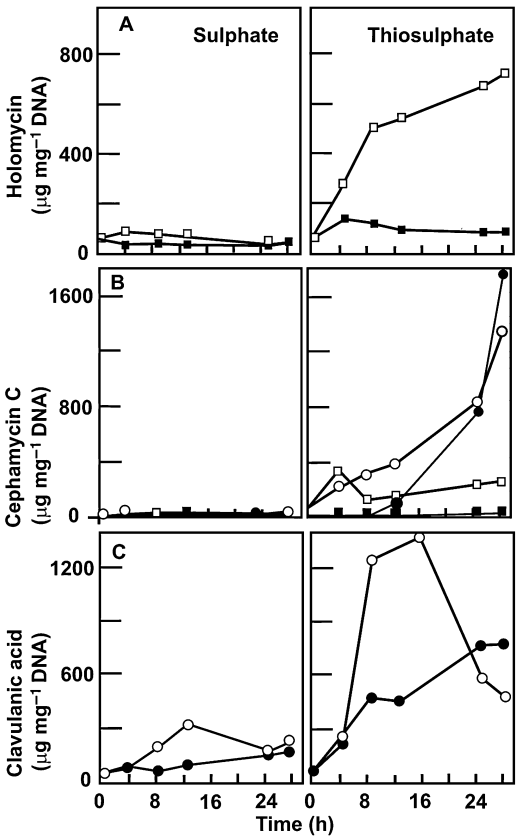


Fig. 5. Antibiotic production by pre-grown cultures of the different strains. Production in sulfate supplemented cultures (left panels) or thiosulfate supplemented cultures (rigth panels).
A. Holomycin.
B. Cephamycin C.
C. Clavulanic acid production by *S. clavuligerus* ATCC 27064 (white circles), *S. clavuligerus* Δ*rhlA* (black circles), *S. clavuligerus oppA2::aph (*white rectangles) and *S. clavuligerus oppA2::aph* Δ*rhlA* (black rectangles).

Production of cephamycin C and clavulanic acid by *S. clavuligerus* Δ*rhlA* mutant followed the same pattern but, as shown in previous experiments, the antibiotic production in this strain is 90% lower for cephamycin C and 60% lower for clavulanic acid as compared with the parental strain (not shown).

The presence of genes for staurosporin production in *S. clavuligerus* genome has been recently described (Medema *et al*., 2010). To test if this antibiotic, which has no sulfur in its structure, was also affected by the addition of thiosulfate, *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.

Discussion

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

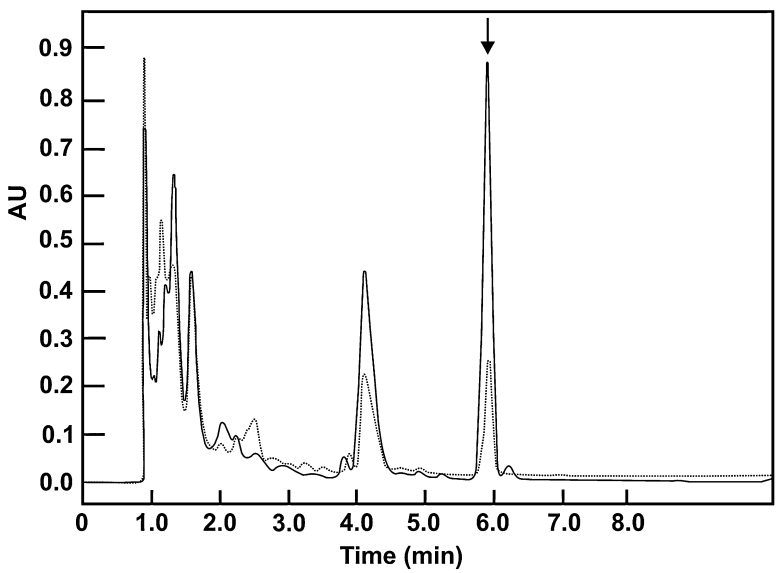


Fig. 6. Production of staurosporin by *S. clavuligerus*. HPLC profile of ethyl acetate extract of *S. clavuligerus* cultures. *Streptomyces clavuligerus* ATCC 27064 grown for 72 h in TSB medium (discontinuous lane). Cultures supplemented with 5 mM thiosulfate (continuous lane). Staurosporin retention time under the HPLC utilized conditions is 5.9 min and coincident with a sample of the pure compound.

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 SH_2 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* Δ*rhlA* 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 Δ*rhlA* and *S. clavuligerus oppA2::aph* Δ*rhlA* have been constructed in this work. SA medium (in g l^{-1}: asparagine, 2; starch, 10; $MgSO_4.7H_2O$ 0.6; K_2HPO_4 4.4; trace elements 1 ml, MOPS 21, pH 6.8; Trace elements in g l^{-1}: $FeCl_3{\cdot}6H_2O$, 0.97; $ZnCl_2$ 0.474; $MnCl_2{\cdot}4H_2O$ 1; $CaCl_2$ 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 $MgSO_4{\cdot}7H_2O$ and the sulfate salts in the original trace elements were substituted by $MgCl_2$ 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,

Table 2. Oligonucleotides used in this work.

Name	Sequence 5′ to 3′	Use
O1	GACTTCATCGACCAGGARGG	To isolate a 507 bp DNA fragment internal to *rhlA*
O2	GGTCSACCTGYTCGTCSGTG	To isolate a 507 bp DNA fragment internal to *rhlA*
O3	GACTAGTTCGGCGGTCTCTTCGTCG	To amplify a 2031 bp DNA fragment upstream of *rhlA*
O4	GGAATTCATGCTGTTTCTCCTCCGG	To amplify a 2031 bp DNA fragment upstream of *rhlA*
O5	CCCAAGCTTAACACGTGACGTCCTGAG	To amplify a 2099 bp DNA fragment downstream of *rhlA*
O6	CCGCTCGAGTTCACATAGCCGTAGAGC	To amplify a 2099 bp DNA fragment downstream of *rhlA*
O7	TCGACATGGCGGTGACAT	To characterize the deletion of *rhlA*
O8	ATGTCTCCAGCGTACGACCG	To characterize the deletion of *rhlA*
O9	CTCGGCGTACGAGAAGAACCACA	To determine expression of SCLAV_3193 (513 pb) by RT-PCR
O10	TTGGACCAGGGGATGTTGCG	To determine expression of SCLAV_3193 (513 pb) by RT-PCR
O11	GAACGCCATCAGCCCGATCA	To determine expression of SCLAV_4718 (395 pb) by RT-PCR
O12	TCTCCAGCGGTCCGGTCCAG	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 (*Eco*RI), O4 (*Spe*I), O5 (*Hind*III) and O6 (*Xho*I).

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 staurosporine production the fermentations were carried out in TSB (trypticaseine 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 Martín, 2005). Staurosporine 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 staurosporine (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*Δ*rhlA to delete the* rhlA *gene. A* 2031 bp DNA fragment upstream of the *rhlA* ATG and a 2099 bp DNA fragment starting 8 pb upstream of the TGA codon were PCR amplified using oligonucleotides O3/O4 and O5/O6 respectively. The fragments, digested with EcoRI/SpeI and HindIII/XhoI respectively, were subcloned in pBluescript KS to give plasmids KS-U*rhlA* and KS-D*rhlA*. The 1375 bp cassette *acc(3)IV*-oriT was isolated from plasmid pIJ773 and subcloned in EcoRI-HindII digested KS-D*rhlA* to produce plasmid KS-*acc*Δ*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 pIJ10700 as a 1505 bp XbaI fragment, was inserted to give plasmid pΔ*rhlA* (9920 bp)

PCR amplification

i. Amplification of a DNA fragment internal to *rhlA*. In base to the amino acid sequences (DFIDQEGFEK and ALYTDEQVDLAK) provided by the MALDI-Toff analysis two degenerated oligonucleotides (O1/O2) were designed with the following degeneration % (R18: 80%G, 20%A; S5: 60%G, 40%C; Y11: 80%C, 20%T; and S17: 70%G, 30%C). The PCR conditions were as follows: oligonucleotides O1/O2 (0.2 μM, each) and Go-Taq polymerase (0.8 units), $MgCl_2$ 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.
ii. PCR's amplification for the construction of pΔ*rhlA*. The upstream and downstream fragments were amplified using total *S. clavuligerus* ATCC 27064 DNA as template (70 ng), Pfx DNA polymerase (0.6 units), oligonucleotides (0.3 μM each), $MgSO_4$ (1 mM), dNTP's (300 μM each) Pfx-enhancer solution and Pfx amplification buffer (invitrogen). The PCR conditions were as follows: 94°C/60″, 1 cycle; 94°C/30″, 1 cycle; 94°C/30″, 56°C/30″, 68°C/135″, 25 cycles; and one cycle of 10 min at 68°C.
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), $MgCl_2$ 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″, 56°C/30″, 72°C/2′30″, 25 cycles; 72°C/10′, 1 cycle.

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/2′, 1 cycle; 95°C/30″, 68°C/30″, 72°C/40″, 8 cycles; 95°C/30″, 60°C/30″, 72°C/40″, 40 cycles; 72°C/10′, 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 thiosulfate 10 mM and glycerol 10%. The cells were disrupted by sonication at 4°C for three pulses of 30″ at maximum speed in a Fast Prep FP120 (Thermo Savant). Thiosulfate sulfurtransferase activity was tested by the production of thiocyanate in the presence of thiosulfate 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 Ettan 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 Image MasterTM 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 Proteomic laboratory (CBM, Madrid).

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