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REVIEW

Regulatory mechanisms controlling antibiotic production in *Streptomyces clavuligerus*

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Abstract *Streptomyces clavuligerus* produces a large array of natural compounds with antibiotic, antitumor, β-lactamase inhibition or inmunomodulating activities. The production of cephamycin C, clavulanic acid and other compounds with a clavam structure has been studied for many years. A network of regulatory mechanisms is present in *S. clavuligerus* to control the formation of different compounds by pathway-specific regulators or pleiotropic regulators. The possible existence of a γ-butyrolactone signaling system in this streptomycete is emerging. In addition, *S. clavuligerus* possesses a stringent control mechanism somehow different from those previously reported in other *Streptomyces* species.

Keywords Clavulanic acid · Cephamycin C · Regulation · *Streptomyces clavuligerus* · ppGpp · Pleiotropic regulatory factors · γ-butyrolactone

Introduction

S. clavuligerus produces cephamycin C, a β-lactam antibiotic, the β-lactamase inhibitor clavulanic acid, and several compounds with a clavam structure and (3*S,* 5*S*) stereochemistry. All these compounds have the typical four-membered β-lactam ring. The 5*S*-clavam compounds, such as clavam-2-carboxylate, 2-formyloxymethylclavam, 2-hydroxymethylclavam, hydroxyethylclavam and alanylclavam, lack β-lactamase-inhibitory activity, which is apparently linked to the (3*R,* 5*R*) stereochemistry of clavulanic acid, but possess antifungal or antibacterial activities (for reviews see [4, 41]). In addition, *S. clavuligerus* produces the antibiotic holomycin with a pyrrothine structure [31], which has been described to have antitumor activity (Fig. 1). The formation of these compounds appears to be interrelated and controlled by a network of regulatory molecules. In this article, we consider some of the aspects that are known at present to be involved in these regulatory mechanisms. An antibiotic compound related to tunicamycin [31] and tacrolimus, an inmunomodulator [34], has also been reported to be produced by *S. clavuligerus*, but no studies have been carried out on these compounds and they will be not considered in this study.

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Antibiotic biosynthesis and genetics in *S. clavuligerus*

Cephamycin C and clavulanic acid biosynthesis

Production of cephamycin C in *S. clavuligerus* occurs in parallel with that of clavulanic acid. Biosynthetic genes for both compounds are adjacent in the genome forming a supercluster of about 60 kb. The cephamycin C gene cluster (Fig. 2a) contains biosynthetic enzyme-encoding genes such as *pcbAB, pcbC, cefD, cefE, cefF, cmcI, cmcJ, cmcH* [36, 40], genes for the biosynthesis of the α-aminoadipic precursor such as *lat* and *pcd*, genes for β-lactam resistance such as *bla, pcbR a*nd *pbp74*, the *cmcT* gene encoding a putative cephamycin transport protein, and the regulatory gene *ccaR*, encoding a SARP regulatory protein (*Streptomyces*-activator regulatory

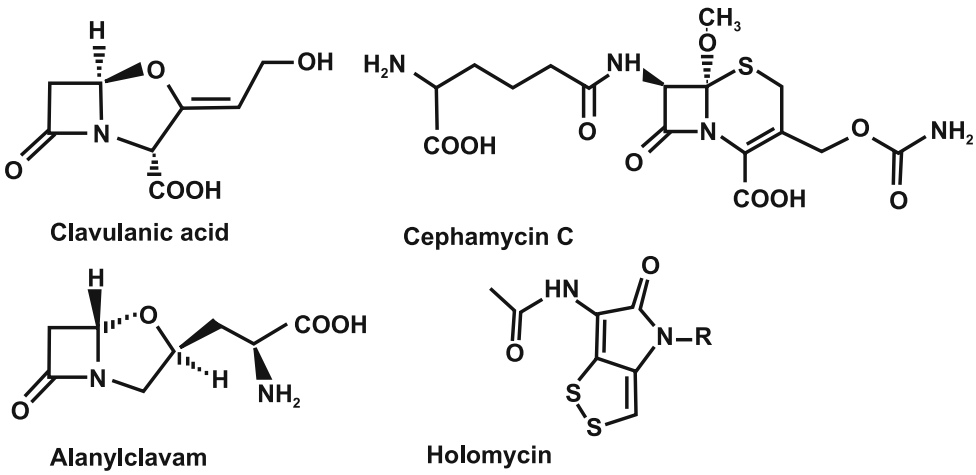

Fig. 1 Structure of some compounds with biological activity produced by *S. clavuligerus*. The structure of additional compounds with clavam structure and (3*S*, 5*S*) stereochemistry is shown at the bottom of Fig. 2

protein, see below; rewieved by [40]). The clavulanic acid cluster (Fig. 2b-I) carries biosynthetic enzyme-encoding genes *ceaS2, bls2, cas2, pah2, gcaS* and *car* [3, 4] (Figs. 2, 3), genes *pbpA* and *pbp2* encoding penicillin-binding proteins [25], genes of unknown function such as *cyp*, *oppA1* and *oppA2*, which are required for clavulanic acid biosynthesis, and gene *claR*, which encodes a LysR-type regulatory protein (see [41]).

Clavam biosynthesis

Three clusters of genes for clavulanic acid–clavam biosynthesis have been isolated in *S. clavuligerus*. The clavulanic acid cluster (already mentioned) was initially isolated by hybridization with the *cas2* gene, encoding clavaminate synthase. A second cluster, named the *clavam gene cluster*, was isolated by using the *cas1* gene, a duplicate gene encoding a clavaminate synthase isoenzyme, as probe [46]. This gene cluster contains genes *cvm1* to *cvm13, cvmG* and *cvmP* (Fig. 2b-II). Their involvement in clavam biosynthesis was revealed by the disruption of certain genes (*cvm1, cvm2* and *cvm5*) that resulted in mutants impaired in the production of some of the 5*S* clavams but still able to synthesize clavulanic acid. Disruption of the *cvm2* gene resulted in a strain with low production of 2-hydroxymethylclavam and alanylclavam and completely unable to produce clavam-2-carboxylate, while disruption of *cvm5* showed that synthesis of both hydroxymethylclavam and

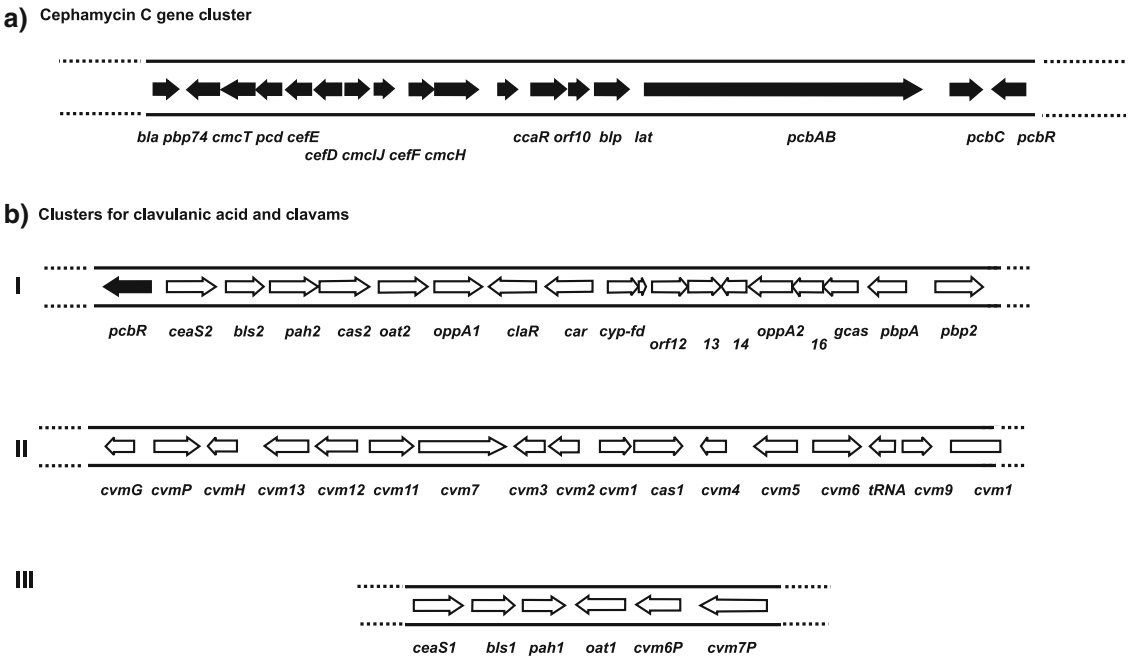

Fig. 2 Gene clusters encoding enzymes for cephamycin C (**a**), clavulanic acid (**b**-I) and clavams (**b**-II). A gene cluster containing genes paralogous to those of the clavulanic acid cluster is shown in (**b**-III). Notice, by the position of the *pcbR* gene, that the cephamycin C gene cluster is adjacent to the clavulanic acid gene cluster

clavam-2-carboxylate was impaired (Fig. 3). Mutants disrupted in *cvm1* produce undetectable levels of clavam-2-carboxylate, clavaminic acid or 2-hydroxymethylclavam [46]. The function of Cvm1, Cvm2 or Cvm5 in clavam biosynthesis is still unclear. No clear involvement of other genes of the clavam cluster in clavam or clavulanic acid formation has been found.

Hybridization of *S. clavuligerus* DNA with the *pah2* gene of the clavulanic acid gene cluster, encoding proclavaminic acid hydrolase, allowed the identification of a second copy of the *pah* gene (later named *pah1*). A third cluster of genes for clavulanic acid biosynthesis, named the *paralogous gene cluster*, has been located surrounding *pah1* [26, 27]. This cluster (Fig. 2b-III) contains genes duplicated from those of the first clavulanic acid cluster that were named *ceaS1, bls1, pah1* and *oat1* [61, 62]. Two additional genes named *cvm6P* and *cvm7P* have been located in this cluster. These last two genes are paralogous genes of *cvm6* and *cvm7* (located in the clavam gene cluster). Cvm7P is a regulatory protein of the LAL family and functions as a specific regulator for formation of 5*S* clavam structures. In spite of their similarity to *cvm7*, only the disruption of *cvm7P* (but not that of *cvm7*) results in complete loss of all the clavam structures without affecting clavulanic acid production [63].

Holomycin biosynthesis

The structure of holomycin is completely unrelated to that of the β-lactam compounds produced by *S. clavuligerus.* Holomycin is a yellow-colored, organic solvent-extractable compound that contains a disulfur bond and shows antibiotic activity against Gram-positive and Gram-negative bacteria. The holomycin producing cultures also contain small amounts of holothin, the deacylated precursor of holomycin. The wild-type strain, *S. clavuligerus* ATCC 27064, produces only traces of both compounds, close to their detection limits. However, mutants blocked in the regulatory

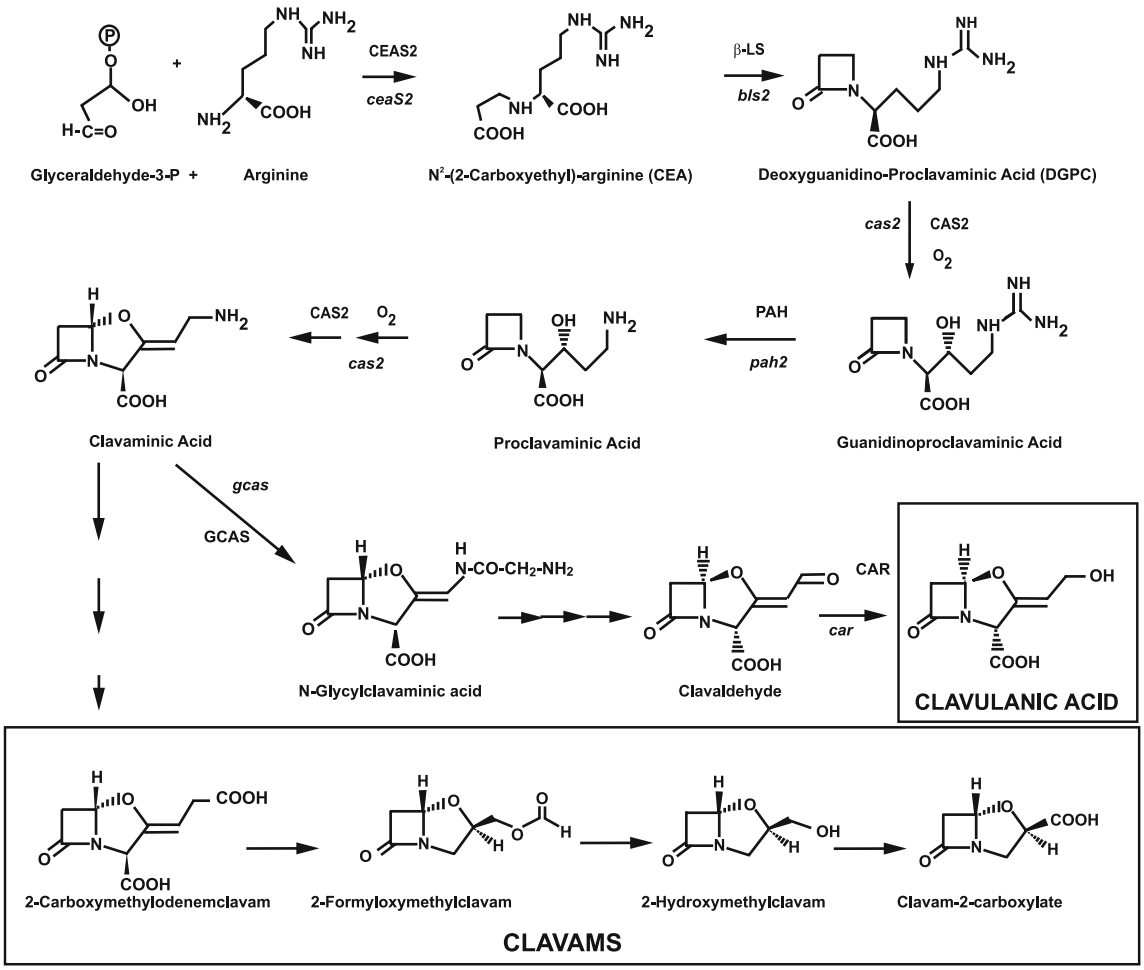

Fig. 3 Biosynthesis pathway for clavulanic acid and clavams. The steps between *N*-glycylclavaminic acid and clavaldehyde, or between clavaminic acid and 2-carboxymethylodenemclavam, are still unknown. The sequential formation of the different clavams has been deduced from the products formed by different mutants, but there is no knowledge of the enzymes involved

gene *ccaR* or *claR*, and especially mutants blocked in the late steps of the clavulanic acid biosynthesis pathway (*car, cyp, orf12, oppA2*), produce levels of holomycin that range between 5 and 1,200-fold higher than the wild-type strain. All these mutants are clavulanic acid non-producers; however, surprisingly, clavulanic acid non-producer mutants blocked in early steps of the pathway (*ceaS2, bls2*) did not overproduce holomycin [16]. The enzymatic activity holomycin synthase, forming holomycin from holothin, increases proportionally with the levels of holomycin produced by the different mutants. Addition of arginine stimulates the production of holomycin. It is possible that a late intermediate of the clavulanic acid pathway (e.g., clavaminic acid) might trigger the formation of holomycin. This intermediate will be accumulated by the cells blocked in the late steps of the pathway, and the intracellular levels will increase in the presence of arginine, a precursor of clavulanic acid, which also stimulates the formation of holomycin. The genes for holomycin production have not yet been localized in *S. clavuligerus* genome.

Regulation of antibiotic production in *S. clavuligerus*

The knowledge about the regulation of antibiotic production in *S. clavuligerus* is far from complete, but some insight has been obtained, both at the global regulatory level and at pathway-specific levels. These mechanisms are analyzed in the following sections (Fig. 4).

Pleiotropic regulatory factors

The activation of antibiotic biosynthesis is genetically controlled at several levels. The most fundamental level involves genes encoding pleiotropic regulators, which control both secondary metabolism and morphological differentiation. Many *bld* genes are known to control both sporulation and antibiotic production in *S. coelicolor*, but only *bldA*, *bldH*, *bldN* and *bldG* have been studied in other *Streptomyces* spp. The *bldA* gene encodes the $tRNA^{Leu}$ for translation of the rare UUA codon [38] and its absence in the *bldA*-null mutant render this strain unable to produce actinorhodin and undecylprodigiosin and yields a bald phenotype [12, 69]. Expression of almost 150 genes, involved in different aspects of *Streptomyces* biology, appears to be affected by *bldA* in *S. coelicolor*, of which only two contain a TTA codon, indicating an upper level of regulation by TTA-containing transcriptional factors on polycistronic operons [19]. Although CcaR, the pathway-specific activator for cephamycin C-clavulanic acid production in *S. clavuligerus*, also contains a TTA codon, the *S. clavuligerus bldA* mutant has its aerial mycelium formation blocked, but there is no effect on translation of the *ccaR* gene or on antibiotic production levels [67]. The TTA codon context appears to have an important role on efficient mistranslation (i.e., in the context of a TTAC or TTAT sequence; [7]). Therefore, the CcaR *bldA*-independent translation might be determined by an in-frame mistranslation or frame-shift due to the G 3′ after the *ccaR* TTA [67].

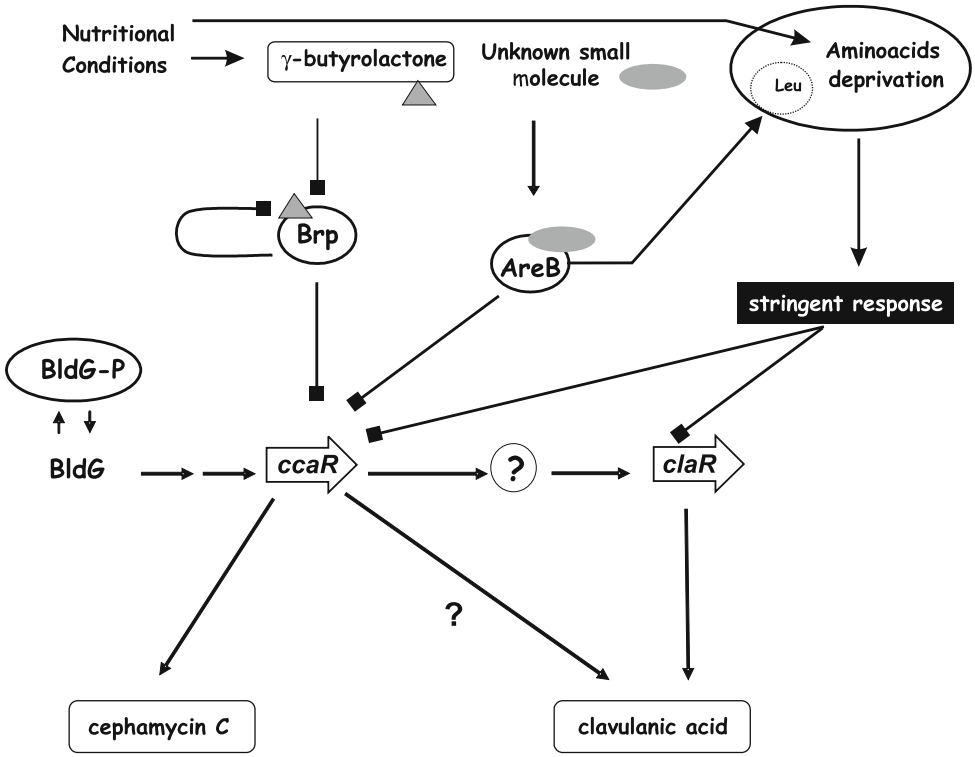

Fig. 4 Hypothetical scheme relating the different regulatory proteins involved in cephamycin C and clavulanic acid biosynthesis

The first reported *bld* gene affecting cephamycin C and clavulanic acid production in *S. clavuligerus* was *bldG* [9]. These authors demonstrated that expression of *ccaR* is *bldG*-dependent, thus placing *bldG* above *ccaR* and *claR* in the regulatory cascade that controls antibiotic production in *S. clavuligerus*. The *bldG* gene encodes an anti-anti-sigma factor [8] and, although the function of these factors remains unclear, they normally act close to an anti-sigma factor regulating the activity of the target sigma factor [14]. A key aspect of this sigma factor regulation system is the activation by phosphorylation at a conserved serine residue, since only the un-phosphorylated form is able to bind to the anti-sigma factor [8, 13].

One of the main targets through which *bldA* influences morphological differentiation is *bldH*; this gene has been renamed *adpAc* (in *S. coelicolor).* Both *adpAc* and *adpAg* (from *S. griseus*) contain a TTA codon [32, 64]. However, the regulation of both genes is different: while the *S. griseus adpAg* gene is directly regulated by ArpA, the receptor protein for the γ-butyrolactone signaling molecule A-factor [48, 49], *adpAc* transcription is independent of the γ-butyrolactone signaling system [64]. Once genes homologous to those involved in the γ-butyrolactone signal transduction are detected in the *S. clavuligerus* genome (M. T. López-García and N. Nardiz, personal communication), additional studies will allow the elucidation of the complex antibiotic regulatory cascade in this microorganism.

Pathway-specific regulatory factors

The *ccaR* gene identified within the cephamycin C biosynthetic gene cluster [52] encodes the positive regulatory factor for both cephamycin C and clavulanic acid production in *S. clavuligerus*. Disruption of *ccaR* gene results in an inability to produce cephamycin C and clavulanic acid that is restored by *trans*-complementation. CcaR, as other known regulatory factors included in the SARP family of proteins [70] such as RedD, ActII-ORF4, AfsR and DnrI [see in 23], does not contain helix-turn-helix DNA binding motifs but does possess an N-terminal DNA binding domain similar to that in the C-terminal end of the OmpR-family of proteins. However, unlike other reported cross-complementation events [60], *ccaR* does not complement an *actII-ORF4* mutant of *S. coelicolor* [52].

Kyung et al. [37] identified the specific temporal-spatial expression profile of CcaR. Following the typical role of pathway-specific activators, CcaR controls antibiotic biosynthesis in *S. clavuligerus* by regulating the transcription of biosynthetic genes.

Several cephamycin C biosynthetic enzymes, such as lysine aminotransferase (LAT), isopenicillin-*N* synthase (IPNS), isopenicillin-*N* epimerase (IPNE) and desacetoxycephalosporin-*C* synthase (DAOCS), are clearly CcaR-dependent [1]. DNA-binding studies have demonstrated that CcaR interacts with the bidirectional *cefD-cmcI* promoter region of the cephamycin gene cluster [56]. Bearing in mind that the *cefD* promoter expresses a large transcript carrying transcriptionally coupled *cefDE-pcd* [53], CcaR could therefore simultaneously control early, middle and late genes of the pathway. Since it has been proposed that factors from SARP family recognize specific heptameric sequences sometimes overlapping with the -35 regions of structural genes [70], SARP boxes similar to those reported for genes controlled by ActII-ORF4 [2] and by DnrI [66] have been identified in the bidirectional region between the ATG start codon of *cefD* and *cmcI*. In contrast, CcaR does not show binding ability to other cephamycin C cluster promoter regions, such as those for *blp* and *lat* [56].

Binding sequences for CcaR within the clavulanic acid gene cluster remain to be identified; CcaR may act indirectly on the pathway-specific regulatory gene of this cluster, *claR*. This gene, located within the clavulanic acid gene cluster, encodes a protein similar to members of the LysR family of transcriptional activators and is specifically required for clavulanic acid production [54, 50]. Direct binding of CcaR to the *claR* promoter region has not been demonstrated and hypothetical targets, such as heptameric sequences SARP-like, are not evident.

Indeed, the ClaR transcriptional regulator is essential only for expression of the late genes of clavulanic acid biosynthesis, since a *claR*-null mutant accumulates the last intermediate of the pathway, clavaminic acid [50]. These authors identified the *claR*-dependent transcription of genes located immediately upstream and downstream of *claR* that have been later characterized as essential for clavulanic acid production. They encode an oligopeptide permease (*oppA1*) [42], the clavulanic-9-aldehyde reductase (*car*) [54] and a cytochrome P450 (*cyp*) [39, 43]. Expression of *claR* in *ccaR*-disrupted mutants is null as measured by Northern hybridization or S1-analysis. However PCR studies show that a *claR*-disrupted mutant expresses all the genes for clavulanic acid biosynthesis, although perhaps at lower levels (López-García, M.T., unpublished results).

It has been shown that CcaR controls expression from the *ceaS2* promoter, since transcription of *ceaS2*, encoding the precursor-forming carboxyethyl-arginine-synthase, is almost eliminated in the *ccaR* mutant [61]. The *ceaS2* promoter leads to a polycistronic transcript encompassing the genes *ceaS2-bls2-pah2-cas2* encoding early enzymatic activities for clavulanic acid biosynthesis and is not controlled by ClaR [50, 51].

Therefore, while CcaR directly controls the production of cephamycin C by binding to promoter regions within the cephamycin C gene cluster, clavulanic acid regulation may be exerted in at least two ways: an indirect one mediated by

a specific role of ClaR, and a second way that may involve CcaR directly regulating *ceaS2* promoter activity.

The γ-butyrolactone signaling system

The existence of γ-butyrolactone type autoregulators acting as "microbial hormones" for the regulation of the onset of antibiotic production appears to be widespread in actinomycetes [15, 24]. Most of these autoregulators share a characteristic 2,3-disubstituted-γ-butyrolactone skeleton and are chemically classified into three types according to differences in the C2 side chain: (1) VB-type, which contains a 6-α-hydroxy group [35]; (2) the IM-2 type that contains a 6-β-hydroxy group [59, 65] and (3) the A-factor type, which contains a 6-keto group [45]. Additional autoinducer molecules different from the classical butyrolactone type have also been identified [55].

A gene encoding a butyrolactone receptor has been cloned from *S. clavuligerus*. Kim et al. [33] named this gene *scaR* and described studies supporting a dimeric form of the receptor as well as major binding affinity to IM-2 type molecules. Almost simultaneously, Santamarta et al. [57] characterized the function of the same *orf*, named *brp*, on clavulanic acid and cephamycin C production through gene disruption and DNA-protein mobility shift assays. Brp of *S. clavuligerus* has a repressor role, shown by a clear overproduction of both cephamycin C and clavulanic acid in the *brp* null mutant *S. clavuligerus* Δ*brp*. This is in agreement with the negative model of butyrolactone receptor proteins proposed in other *Streptomyces* species; in fact, the *brp* gene is similar to *scbR* from *S. coelicolor* [65], although the genomic environment in both genes is very different.

Specific sequences for binding of butyrolactone receptor proteins, named ARE boxes and consisting of palindromic inverted repeats [22, 24], have been described in several *Streptomyces* species, although the binding of receptors to those sequences has been demonstrated in only a few cases. Two different ARE sequences as targets for Brp have been identified in *S. clavuligerus* [57]. Brp directly binds an ARE target located in its own promoter region suggesting autoregulation as reported in ScbR in *S. coelicolor*, BarA in *S. virginiae*, and FarA in *S. lavendulae* [65]. The second ARE target sequence for Brp is located 815 nt upstream of the *ccaR* transcription start point, which is 75 nt upstream of the *ccaR* ATG codon [68]. In spite of being a great distance, clear additional open reading frames have not been identified between *cmcH* and *ccaR*; thus Brp might exert its repressor role through *ccaR* expression levels.

Very recently, the first example of multiple proteins binding to a γ-butyrolactone-receptor ARE target sequence was reported and characterized [58]. The AreB protein was isolated from *S. clavuligerus* through DNA-affinity chromatography, at the ARE sequence located upstream of the *ccaR* gene; it was identified as a member of the IclR family of regulatory proteins [44]. The *areB* gene is expressed from a divergent promoter region in an opposite orientation to the *leuCD* cluster for leucine biosynthesis. As reported in several back-to-back promoters [6], AreB plays a regulatory role in its own gene expression and also in the transcriptional regulation of the *leuCD* gene cluster. However, unlike the repressor role of the orthologous LtbR regulator in *Corynebacterium glutamicum* [10], AreB in *S. clavuligerus* is required for effective leucine assimilation and biosynthesis; in addition, AreB is required for fatty acid utilization as carbon source.

Cephamycin C and clavulanic acid levels are slightly increased in the absence of *areB*, as shown by antibiotic production analysis of the mutant strain *S. clavuligerus* Δ*areB*. Only a very small increase in *ccaR* transcription level, in contrast to a clear downregulation of *brp* expression, has been shown in the Δ*areB* mutant, and an indirect modulation role of AreB through *brp* has been proposed. Also, the characterization of the Δ*areB* mutant strain indicates a role for AreB in connecting primary and secondary metabolism. Underexpression of leucine biosynthetic genes in the Δ*areB* mutant leads to increased pools of valine precursor available for cephamycin C biosynthesis.

The most interesting finding about the role of this AreB additional protein in secondary metabolism regulation is the specific requirement of a small molecule for the binding of AreB to the ARE sequence, but not for the interaction with the *areB-leuCD* intergenic region. These findings suggest a differential effect of AreB on modulating antibiotic production, once the critical level of the small molecule is reached. Thus, AreB is identified as a member of a novel IclR-like subfamily specialized in connecting primary and secondary metabolism in *S. clavuligerus*.

Stringent response in *S. clavuligerus*

An important system in prokaryotes for sensing nutritional starvation and adapting to new environmental conditions is the "stringent response". Under amino acid starvation conditions, the presence of uncharged tRNAs in the A-site of the ribosome activates the activity of the ribosome-associated protein RelA, which produces a burst in the level of the polyphosphorylated guanine nucleotide ppGpp with a concomitant decrease in the intracellular level of GTP. In *E. coli*, ppGpp binds the beta-subunit of RNA polymerase and provokes a global reduction of transcription, while genes for amino acid biosynthesis and response to stress are induced [11].

In *Streptomyces*, the decrease in intracellular GTP level is required for the onset of morphological differentiation,

while the burst of ppGpp has been linked to the elicitation of secondary metabolism [47]. The first evidence of the link between ppGpp and secondary metabolism arose from the study of mutants lacking the stringent response in different *Streptomyces* species. These "relaxed" mutants are defective either in the RelA protein (i.e., *relA* mutants lacking ppGpp synthetase activity) or in the L11 ribosomal protein (mutants named either *rplK* or *relC*). Relaxed mutants are typically impaired in both antibiotic production and sporulation. Many of these mutants were initially obtained as thiopeptine-(a thiostrepton analogue) resistant clones [30]. In *S. coelicolor*, ppGpp synthesis switches the transcription from rapid-growth genes to stationary phase associated genes [20], including the induction of the actinorhodin pathway specific activator, but not that of undecylprodigiosin biosynthesis [21].

The stringent response in *S. clavuligerus* was initially studied by Bascarán et al. [5]. Fermentation studies did not establish a clear correlation between the onset of antibiotic production (either cephamycin C or clavulanic acid), and ppGpp intracellular levels [5, 29]. This lack of correlation between bursts of ppGpp and onset of antibiotic formation was later confirmed using the expression of structural genes (*ceaS2, cefD*) or regulatory genes (*ccaR, claR*) [17, 18]. It was shown that in typical batch fermentation in SA medium, *S. clavuligerus* wild-type strain produced antibiotic during the rapid growth phase, when the ppGpp level was still low, and stopped producing antibiotic after entering the stationary phase, concomitant with the highest level of ppGpp and a reduction in the mRNA abundance of antibiotic biosynthesis genes.

The *rplK* gene of *S. clavuligerus* encodes a 15.2 kDa protein with 93.7% identity to the homologous protein of *S. coelicolor*. Using this gene, a well-characterized *relC* mutant was obtained by deletion of the sequence encoding amino acids ^{29}PALG^{32} in the RplK (L11) protein [17]. The mutant, *S. clavuligerus rplK* ^{29PALG32}, shows a growth pattern only slightly delayed in relation to the wild-type strain and is more resistant to thiostrepton. As is usual in *relC* mutants, this strain produces very low levels of ppGpp in relation to the wild-type strain, and is unable to sporulate but does produce aerial mycelium. Cephamycin C and clavulanic acid production is impaired in *S. clavuligerus rplK* ^{29PALG32}, which correlates well with the poor expression of the *ccaR* and *claR* regulatory genes, *ceaS2* (for clavulanic acid biosynthesis) and *cefD* (for cephamycin biosynthesis). The impaired antibiotic production and sporulation of the *relC* mutant can be restored by complementation with the wild-type gene. These results agree with those found for other *relC* relaxed mutants of different antibiotic-producing *Streptomyces* [30, 47].

The *relA* gene of *S. clavuligerus*, that encodes an 843 amino acid protein, has been cloned and deleted or disrupted by two independent research groups. Jin et al. [28] disrupted *relA* by insertion of the hygromycin resistance gene to produce *S. clavuligerus relA::hyg*. Gomez-Escribano et al. [18] disrupted and completely deleted *relA*. Every *relA*-null mutant, *S. clavuligerus relA::hyg* [28], *S. clavuligerus relA::neo* or *S. clavuligerus ΔrelA* [18] is completely impaired in the formation of ppGpp, unable to sporulate and severely reduced in aerial mycelium formation. These phenotypes are reversed by reconstruction of the genetic organization of the *relA* region [28], or by complementation with the wild-type *relA* gene, or with a 804 bp DNA fragment encoding amino acids 228–495 of the RelA protein, which are expected to have ribosome-independent ppGpp-synthetase activity [18]. Surprisingly, although the *S. clavuligerus relA::hyg* mutant was unable to produce either cephamycin C or clavulanic acid in defined production medium (DP medium) containing glycerol as carbon source and arginine as nitrogen source, strains *S. clavuligerus relA::neo* and *S. clavuligerus* Δ*relA* overproduce cephamycin C (sixfold) and clavulanic acid (fourfold) in defined SA medium, containing starch and asparagine as carbon and nitrogen sources. Similar results were also found in TSB complex medium. The data on higher levels of cephamycin C and clavulanic acid production by *S. clavuligerus* Δ*relA* are supported by high resolution S1 nuclease protection analysis. Transcripts of *cefD*, and especially of *ceaS2*, are at much higher level in the *relA*-deleted mutant than in the wild type (Fig. 5). This was the first report of a *relA*-null mutant that overproduces antibiotics, but *S. clavuligerus* relaxed mutants, impaired in ppGpp synthesis, with higher cephamycin C production than the wild type had been previously found among spontaneous thiostrepton-resistant clones [5].

The different behavior of the *relA*-null mutants obtained by genetic engineering from the same parental strain, *S. clavuligerus* ATCC 27064, is surprising. However, sequencing of the intergenic region upstream of *relA* (147nt) shows marked differences between the parental strains used by both research groups. The nucleotide sequence of the intergenic region in *S. clavuligerus* ATCC 27064 used by Gomez-Escribano et al. [18] is identical to that of *S. clavuligerus* ATCC 27064 obtained independently in DSM (The Netherlands), while the sequence upstream of *relA* published for *S. clavuligerus* ATCC 27064 by Jin et al. [28] has an apparent deletion that affects the promoter region [18]. However, more differences between the strains, due to subculturing and differences in conservation, cannot be ruled out.

It is clear that the stringent response in *S. clavuligerus* plays a negative role in the control of antibiotic production. It is generally accepted that ppGpp is necessary for secondary metabolite production, which usually occurs during stationary growth phase. However, antibiotic production in

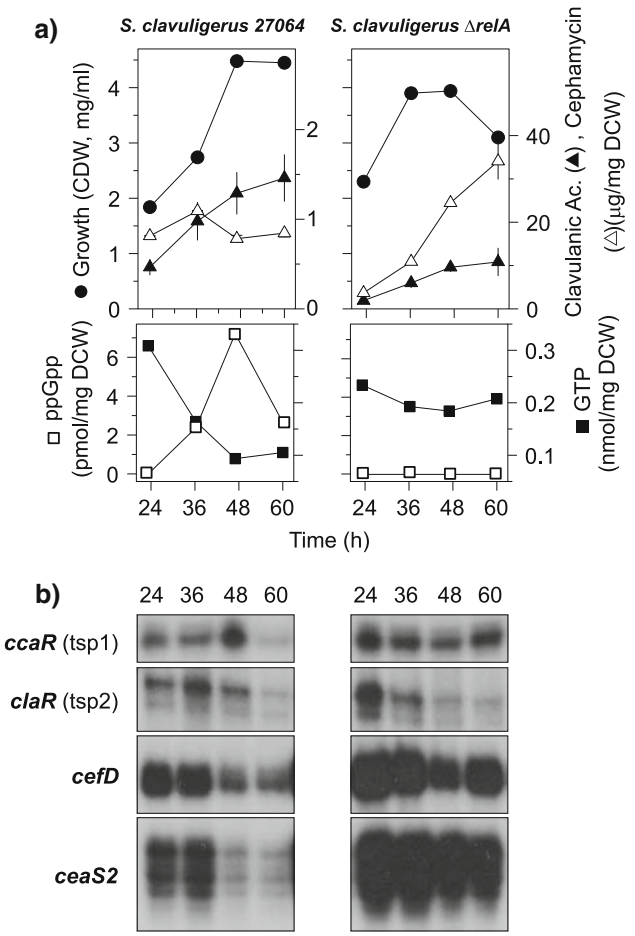

Fig. 5 Comparison of *S. clavuligerus* ATCC 27064 and *S. clavuligerus* Δ*relA* cultures grown in defined SA medium. **a** Growth (*black circles*), clavulanic acid production (*black triangles*), cephamycin C production (*white triangles*), intracellular ppGpp (*white squares*), intracellular GTP (*black squares*). **b** S1 nuclease protection analysis of the expression of *ccaR* (from tsp1), *claR* (from tsp2), *cefD* and *ceaS2* in the wild type (*left panel*) and the Δ*relA::neo* mutant (*right panel*) at 24, 36, 48 and 60 h of culture in SA medium. Notice the stronger expression and longer time of expression of the genes in the Δ*relA::neo* mutant, concomitant with the higher antibiotic production shown in panel (**a**) for this strain

S. clavuligerus takes place during rapid growth phase. Thus, the lack of correlation between ppGpp levels and antibiotic production is not surprising. More intriguing is the antibiotic overproduction by *relA*-null mutants, and it reveals the possibility of directed strain improvement by manipulating ppGpp metabolism in *S. clavuligerus*.

In summary, antibiotic production is subject to a complex network of interactions between small molecules, regulatory proteins and promoters of key genes. These molecules transmit the physiological and nutritional state of the cell to the antibiotic biosynthesis genes. At present, we know only little about these complex networks, which might be general and/or specific for every antibiotic-producing strain.

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