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REVIEW

Biosynthesis of clavam metabolites

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Abstract Naturally occurring clavam metabolites include the valuable β-lactamase inhibitor, clavulanic acid, as well as stereochemical variants with side-chain modifications, called the 5*S* clavams. Because of the clinical importance of clavulanic acid, most studies of clavam biosynthesis are based on the industrial producer species *Streptomyces clavuligerus*. Well-characterized early steps in clavam biosynthesis are outlined, and less well understood late steps in 5*S* clavam biosynthesis are proposed. The complex genetic organization of the clavam biosynthetic genes in *S. clavuligerus* is described and, where possible, comparisons with other producer species are presented.

Keywords Clavulanic acid · β-Lactam · Alanylclavam · Valclavam · Clavamycin · *Streptomyces clavuligerus*

Abbreviations

ARE	Autoregulatory element
BLIP	Beta-lactamase inhibitor protein
BLS	β-lactam synthetase
CAD	Clavaldehyde dehydrogenase
CAS	Clavaminic acid synthase
CcaR	Cephamycin and clavulanic acid regulator
CEAS	Carboxyethylarginine synthase
CYP	Cytochrome P450
FD	Ferredoxin
GCAS	Glycylclavaminic acid synthase
OAT	Ornithine acetyltransferase
OPP	Oligopeptide permease
PAH	Proclavaminic acid amidinohydrolase
PBP	Penicillin-binding protein
SARP	*Streptomyces* antibiotic regulatory protein

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Introduction

Clavam metabolites fall into two main categories, clavulanic acid, and the 5*S* clavams (Fig. 1), and all are products of fermentations by *Streptomyces* spp. Clavulanic acid is a weak but broad-spectrum antibiotic with strong β-lactamase inhibitory activity [10]. Compounds capable of inhibiting β-lactamases, such as clavulanic acid, render β-lactamase-producing pathogens sensitive to β-lactam antibiotics, and so they have considerable clinical importance. Clavulanic acid is normally marketed mixed with other β-lactam antibiotics such as amoxicillin. The resulting combination drug, Augmentin, was the second-best selling antibacterial in 1995 with sales of US $1.3 billion [14, 16], and remains widely prescribed today although revenues have dropped with the expiration of patents. Because of the clinical and commercial importance of clavulanic acid, the production of this class of compounds has been the subject of intensive research in recent years, and numerous reviews have been published [6, 29, 37, 38, 67]. This current review provides an overview of the subject with attention focused on recent developments not covered in previous reviews.

Clavulanic acid was initially discovered as a product of the organism *Streptomyces clavuligerus* in 1976 [10], and since that time, a handful of other *Streptomyces* spp. capable of producing the metabolite have been identified, including *S. jumonjinensis*, *S. katsurahamanus*, and *Streptomyces* sp., an organism identified only to the genus level [29]. More recently, genome-sequencing projects have resulted in deposits of DNA sequence, which suggest that clavulanic acid biosynthetic capability exists in a wider

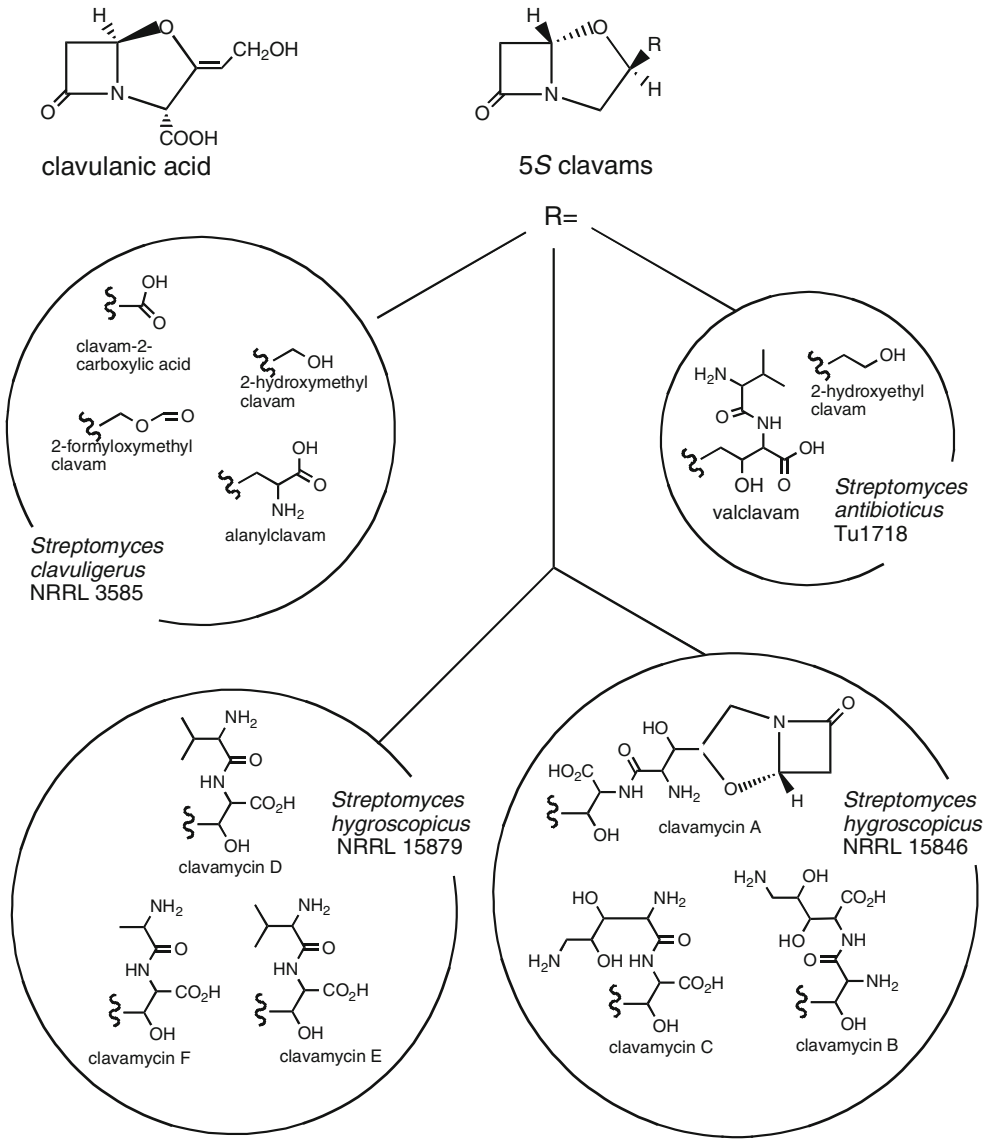

Fig. 1 Naturally occurring clavam metabolites

range of species, including *S. flavogriseus* ATCC 33331 (accession number CP002475) and *Saccharomonospora viridis* DSM 43017 (accession number CP001683).

Clavam compounds have a bicyclic nucleus with a β-lactam ring fused to an oxazolidine ring. In the case of clavulanic acid, this nucleus is modified by the addition of a carboxyl group attached at the C3 position and a side chain at C2 [24] (Fig. 1). The 3*R*, 5*R*, stereochemistry of clavulanic acid is believed to be essential for its inhibition of β-lactamases [6]. In agreement with this, all of the other clavam metabolites found in nature have 5*S* stereochemistry and do not exhibit β-lactamase inhibitory activity. These clavams are thus called the 5*S* clavams and comprise the basic bicyclic clavam nucleus, but with no C3 carboxyl group, and with a range of side chains at C2 (Fig. 1). Although the 5*S* clavams do not inhibit β-lactamases, they are biologically active. 5*S* clavam metabolites can have antifungal properties [11] due to effects on RNA synthesis [60] and some can also be bacteriostatic through the inhibition of methionine biosynthesis [59]. Despite this range of biological activities, none of the 5*S* clavams is currently of medical importance.

All of the clavulanic acid used clinically is produced by fermentations of the producer species *S. clavuligerus,* and this organism also produces the 5*S* clavams, clavam-2-carboxylate, 2-hydroxymethylclavam, 2-formyloxymethylclavam, and alanylclavam (Fig. 1). Because of the industrial importance of the clavulanic acid fermentation, essentially all of the studies on the biosynthesis of clavam metabolites have been conducted in this species. However, *S. clavuligerus* is unique among the clavam producers so far described in its ability to produce both clavulanic acid

and 5*S* clavams. All other producers synthesize either clavulanic acid or 5*S* clavams alone, and so clavam biosynthesis in *S. clavuligerus* may be inordinately complex when compared to other producer species.

The structural similarities between clavulanic acid and the 5*S* clavams reflect shared elements of a common biosynthetic pathway. The shared steps in their biosynthetic pathways are referred to as the "early steps," leading to the last common intermediate, clavaminic acid, while the steps specific to either clavulanic acid or 5*S* clavam production are called the "late steps."

The early steps of clavam biosynthesis

The genes responsible for clavulanic acid biosynthesis by *S. clavuligerus* are clustered together on the chromosome adjacent to the cephamycin C biosynthetic gene cluster [75] (Fig. 2). This clavulanic acid gene cluster includes genes encoding enzymes involved in the early steps required for both clavulanic acid and 5*S* clavam biosynthesis, as well as genes encoding enzymes specific for clavulanic acid biosynthesis alone.

The first reaction of the shared part of the clavam biosynthetic pathway is the condensation of L-arginine and glyceraldehyde-3-phosphate to produce N^2-(2-carboxyethyl)arginine [31, 74] (Fig. 3). This reaction is catalyzed by carboxyethylarginine synthase (CEAS), which requires thiamine pyrophosphate and Mg^{2+} for activity. Mutation of *ceaS2*, the first gene in the clavulanic acid gene cluster in *S. clavuligerus*, essentially abolishes production of all clavams [28, 57]. The crystal structure of the CEAS2 enzyme reveals that it exists as a dimer of dimers, two dimers that associate loosely with each other to form a tetramer [12].

N^2-(2-carboxyethyl)arginine next undergoes cyclization to form a β-lactam ring-containing intermediate called deoxyguanidinoproclavaminate. This reaction is catalyzed by β-lactam synthetase (BLS), an ATP and Mg^{2+}-requiring enzyme encoded by *bls* [5, 23, 45]. Disruption of *bls2,* the second gene in the clavulanic acid gene cluster in *S. clavuligerus,* also results in greatly decreased clavam metabolite yields [5, 28]. Structural studies have shown that BLS2 exists in solution as a dimer and is similar to a class of asparagine synthetases [48].

Clavaminic acid synthase (CAS) catalyzes the hydroxylation of deoxyguanidinoproclavaminate to form guanidinoproclavaminate [7]. CAS is an intermolecular dioxygenase that requires α-ketoglutarate, O_2, and Fe^{2+} to carry out catalysis [44, 62]. Mutation of *cas2*, the fourth gene in the clavulanic acid gene cluster, causes mutants to produce reduced amounts of clavams [28]. The crystal structure of Cas2 reveals a monomeric structure with a β-barrel core in which the active site resides [78].

The guanidino group of guanidinoproclavaminate is then removed by hydrolysis through the action of proclavaminate amidinohydrolase (PAH) [76] in a reaction giving rise to proclavaminic acid. Mutation of *pah2*, the third gene in the clavulanic acid gene cluster [1], again causes partial loss of clavam production. Structural studies of

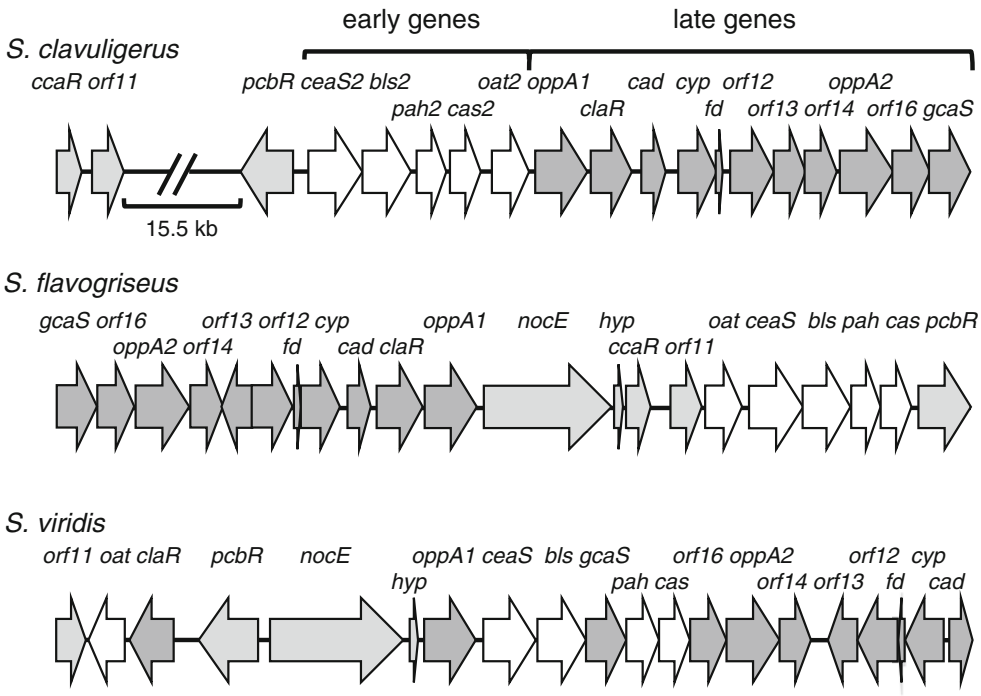

Fig. 2 Clavulanic acid gene clusters from known and presumed clavulanic acid producer species. Early genes are shown as *white arrows*, late genes as *dark gray arrows*, and related genes with regulatory or unknown functions are *light gray*

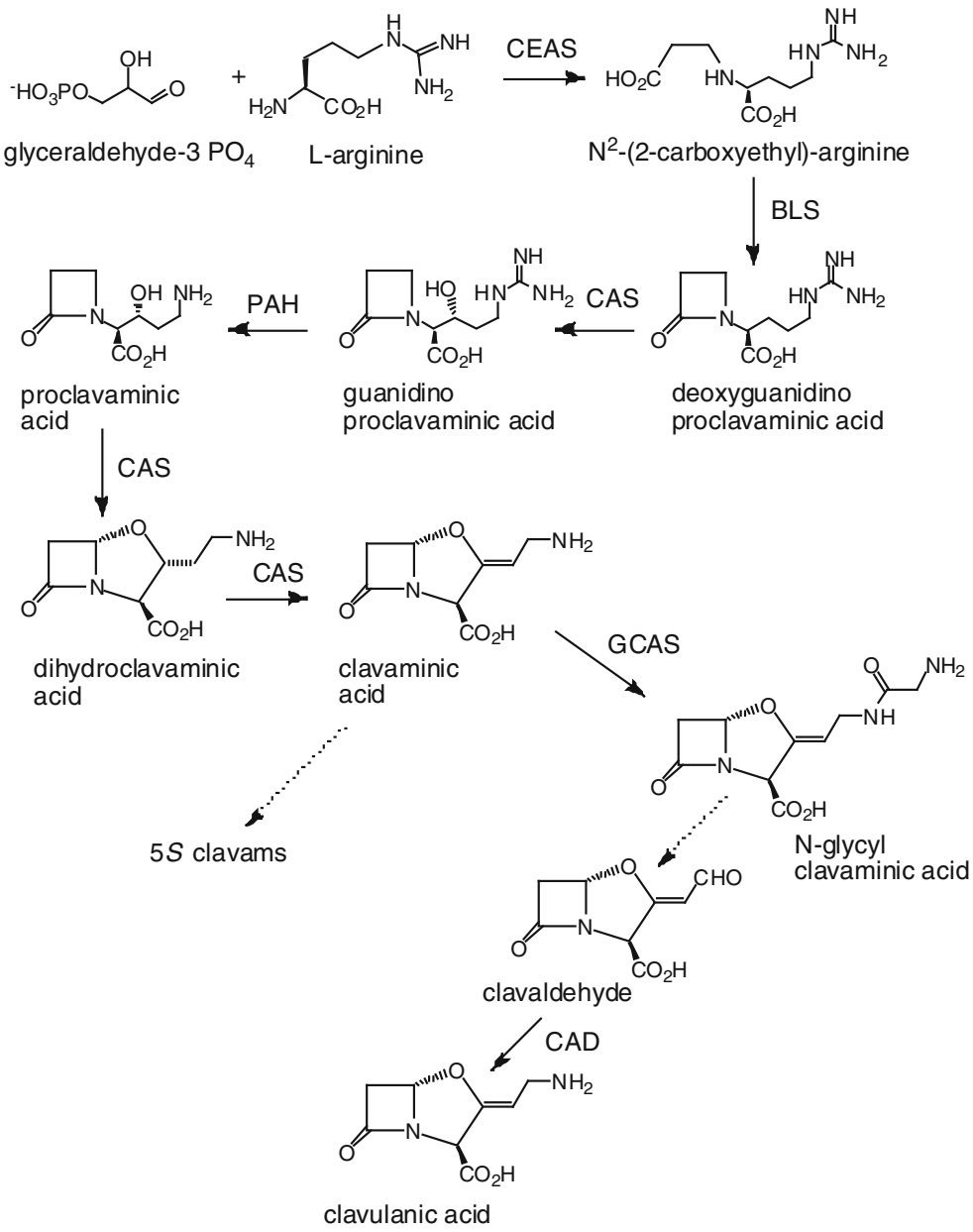

Fig. 3 Biosynthetic pathway to clavulanic acid in *S. clavuligerus*. *CEAS* carboxyethylarginine synthase, *BLS* beta-lactam synthetase, *CAS* clavaminic acid synthase, *PAH* proclavaminic acid amidinohydrolase, *GCAS* glycylclavaminic acid synthase, *CAD* clavulanic acid dehydrogenase

PAH2 show that it is a hexameric protein that requires two Mn^{2+} atoms in its active site for hydrolytic activity [17].

The final two early steps leading to clavaminic acid formation are both catalyzed by CAS. First, proclavaminic acid undergoes cyclization to produce dihydroclavaminic acid, which is then desaturated to produce clavaminic acid [61].

Although its exact role in clavam biosynthesis is unknown, the next gene in the clavulanic acid gene cluster, *oat2*, is categorized as an early gene because it is expressed in the same temporal and regulatory pattern as the other early genes [28]. *oat2* encodes an ornithine acetyltransferase (OAT2) that can catalyze the transfer of an acetyl group from *N*-acetylornithine to glutamate [30]. Like other ornithine acetyltransferases, OAT2 undergoes autoproteolysis to produce α and β fragments that associate to form an $\alpha_2\beta_2$ heterotetramer. Insertional mutagenesis of *oat2* resulted in decreased levels of clavam biosynthesis [13, 30] unless mutants were supplemented with high levels of arginine, leading to the hypothesis that OAT2 may affect clavam production by increasing cellular pools of arginine.

Clavaminic acid is the most advanced intermediate common to both the clavulanic acid and the 5*S* clavam biosynthetic pathways [15]. Although the enzymes giving rise to clavaminic acid have been well characterized, many of the subsequent steps leading to clavulanic acid remain unclear. However, the entire clavulanic acid gene cluster from *S. clavuligerus* has been sequenced, and examination

of the types of gene products involved provide insights as to the nature of the steps leading from clavaminic acid to clavulanic acid.

Late stages in clavulanic acid biosynthesis

The next gene in the clavulanic acid gene cluster is *oppA1* [23, 28] (Fig. 2). Interestingly, *oppA1* has significant similarity (47 % identity at the amino acid level) to *oppA2* found further downstream in the clavulanic acid gene cluster [47]. Both of these genes encode predicted oligopeptide permeases-type binding proteins and both contain a domain found in bacterial extracellular solute-binding proteins. Mutants defective in either gene cannot produce any clavulanic acid [41]. Analysis of *oppA1* and *oppA2* mutants provided evidence that both are involved in peptide transport, but how this translates into their having essential roles in clavulanic acid production is still unclear. Structural studies show that OppA2 has a two-lobed structure encompassing a substrate binding cleft, and cellular localization studies suggest that the protein is predominantly soluble and intracellular [42].

In the first enzymatic step devoted specifically to producing clavulanic acid, clavaminic acid is converted to *N*-glycylclavaminic acid by glycylclavaminic acid synthase (GCAS) [4] (Fig. 3). Mutants in *gcaS,* the last gene of the clavulanic acid cluster, are unable to produce clavulanic acid [26]. GCAS is a member of the ATP-grasp fold superfamily and catalyzes a carboxylate-amine ligation reaction using ATP and glycine in the presence of Mg^{2+} and K^{+}.

The final reaction in the biosynthesis of clavulanic acid is carried out by clavaldehyde dehydrogenase (CAD), encoded by *cad* [58]. CAD reduces clavaldehyde to form clavulanic acid [50], and mutation of *cad* blocks clavulanic acid production, whereas overexpression of *cad* increases clavulanic acid production [28, 58]. Structural studies reveal that CAD acts as a tetramer and uses NADPH as a source of reducing power [43].

The intervening reactions that convert *N*-glycylclavaminic acid to clavaldehyde, thus completing the biosynthetic pathway to clavulanic acid, have not yet been elucidated, including the intriguing reaction(s) that inverts the 3*S*, 5*S* stereochemistry of *N*-glycylclavaminic into the 3*R*, 5*R* stereochemistry of clavaldehyde. Six additional genes of unknown function in the clavulanic acid cluster have been subjected to mutagenic analysis and all were shown to be essential or very important for clavulanic acid biosynthesis, so the solution to this biosynthetic puzzle presumably lies in the activities of their gene products.

cyp, the ninth gene in the clavulanic acid gene cluster (Fig. 2), encodes a protein with high similarity to cytochrome P-450 s, and disruption of *cyp* blocks the production of clavulanic acid [28, 36, 47]. Cytochrome P-450 s typically carry out oxidative reactions in cooperation with a ferredoxin protein, and the presence of the ferredoxin-encoding gene, *fd*, lying adjacent to *cyp* supports this premise. Mutation of *fd* results in markedly decreased clavulanic acid biosynthesis [26]. CYP and FD are attractive candidates to perform the double enantiomerization in the conversion of the 3*S*, 5*S* stereochemistry of *N*-glycylclavaminic acid to the 3*R*, 5*R* stereochemistry of clavaldehyde, but in vitro demonstration of this activity remains elusive.

orf12 lies beyond *fd* in the gene cluster. The predicted amino acid sequence encoded by *orf12* shows some similarity to class A β-lactamases. While this argues against a role in clavulanic acid biosynthesis, mutation of *orf12* abolishes clavulanic production [26, 36]. The formation of the β-lactam ring in all clavam metabolites results from catalysis by Bls of a reaction that is in effect a reversal of β-lactamase activity. However, any suggestion that ORF12 might be involved in the opening or reclosure of the β-lactam ring during stereochemical inversion is weakened by the finding that ORF12 lacks three of the four highly conserved motifs associated with β-lactamase activity, retaining only the SDN motif.

The remainder of the genes in the main clavulanic acid gene cluster (*orf13* to *orf16*) [26, 47] have also been subjected to insertional inactivation. *orf13* encodes a protein similar to amino acid metabolite efflux pumps from a number of bacterial species. Disruption of *orf13* gives a mutant that produces only low levels of clavulanic acid and 5*S* clavams (5 % relative to wild-type), suggesting a role in the transport of all clavam metabolites out of the cell.

ORF14 shares significant similarity with acetyltransferases, and crystallographic analyses indicates that it belongs to the Gcn5-related acetyl transferase family of proteins [25]. Although acetyltransferases can be involved in the inactivation of antibiotics via chemical modification, mutation of *orf14* results in a phenotype varying from partial [47], to almost complete loss of clavulanic acid production [26], suggesting a role in biosynthesis rather than as a resistance gene. The variable nature of these mutants further suggests that the phenotype may be growth medium-dependent.

orf16 shows similarity only to genes encoding hypothetical proteins from other putative clavulanic acid producing microorganisms such as *S. viridis* and *S. flavogriseus*. Although the ORF16 protein carries a putative sortase recognition sequence near its C-terminus, believed to be required for attaching proteins to the cell wall in Gram-positive organisms, little else can be deduced from its nucleotide sequence. Mutants in *orf16* are not only completely disabled in clavulanic acid production, but they

accumulate two novel clavams: *N*-acetylglycylclavaminic acid and trace amounts of *N*-glycylclavaminic acid, metabolites also produced by *oppA2* mutants. While *N*-glycylclavaminic acid is believed to be an intermediate in clavulanic acid biosynthesis, it is also possible that *N*-acetylglycylclavaminic acid represents a subsequent intermediate that has accumulated due to disruption of the ORF16 gene product.

In addition to *N*-glycylclavaminic acid and *N*-acetylglycylclavaminic acid, *N*-acetylclavaminic acid has also been detected in culture broths of an uncharacterized *S. clavuligerus* mutant unable to produce clavulanic acid [18]. Since clavaminic acid is converted to *N*-glycylclavaminic acid by GCAS, the next step in the pathway may be the *N*-acetylation of *N*-glycylclavaminic acid by ORF14 to produce *N*-acetylglycylclavaminic acid. Because *orf16* mutants accumulate appreciable amounts of *N*-acetylglycylclavaminic acid, ORF16 may normally function to convert this intermediate to another metabolite, possibly *N*-acetylclavaminic acid.

Analysis of the region downstream of the clavulanic acid gene cluster reveals a pair of genes that encode proteins with high similarity to penicillin-binding proteins (PBPs), but mutational analyses of *orf18* and *orf19* indicate that they have no role in clavulanic acid production or resistance. Further downstream lies *orf20*, encoding an apparent cytochrome P-450 not involved in clavulanic acid biosynthesis, and beyond it, three genes of potential regulatory importance, encoding a type E sigma factor, and the elements of a two-component regulatory system. Disruption of these genes had relatively minor effects, suggesting that any involvement in clavulanic acid production must be conditional [69].

While *S. clavuligerus* is the only species in which clavulanic acid biosynthesis has been studied to any significant extent, the availability of genomic sequence information for two additional putative clavulanic acid producers, *S. viridis* and *S. flavogriseus*, allows a comparison of the elements of the clavulanic acid gene clusters in these three species (Fig. 2). All three species contain all of the genes described above as comprising the *S. clavuligerus* gene cluster. In addition, the clavulanic acid gene clusters from both *S. viridis* and *S. flavogriseus* contain a *pcbR* orthologue. In *S. clavuligerus*, *pcbR*, encoding a penicillin binding protein, lies at the interface between the cephamycin and clavulanic acid gene clusters. Mutation of *pcbR* was only possible in a cephamycin C non-producer background, and in those mutants, resistance to β-lactam antibiotics was reduced with no obvious effects on clavulanic acid production. On this basis, the gene was considered part of the cephamycin C gene cluster [53]. However, neither *S. viridis* nor *S. flavogriseus* contains any other elements of a gene cluster for cephamycin C biosynthesis, and so the possible involvement of *pcbR* in production of clavulanic acid should be examined further. The clavulanic acid gene cluster from *S. flavogriseus* also includes an orthologue of *ccaR*, a gene encoding a *Streptomyces* antibiotic regulatory protein (SARP)-type transcriptional regulator, and located in the cephamycin gene cluster of *S. clavuligerus*, from where it regulates both cephamycin C and clavulanic acid production [2, 56]. No clear counterpart to *ccaR* is found in *S. viridis*. However, the clavulanic acid gene clusters from *S. viridis*, and *S. flavogriseus* both contain orthologues of *orf11* (also known as *orf10,* [63]) from the cephamycin gene cluster. This gene is located just downstream from, and transcriptionally linked to *ccaR* in the *S. clavuligerus* cephamycin gene cluster, but is otherwise poorly understood [2]. ORF11 shows similarity to metallo-beta-lactamase superfamily domain proteins.

Interestingly, while no cephamycin C gene cluster was evident in the vicinity of the clavulanic acid gene cluster of *S. flavogriseus* or anywhere else in the genome, the region flanking the clavulanic acid gene cluster did contain a beta-lactamase inhibitor protein (BLIP)-encoding gene showing 100 % identity to the *bliA* gene from *Streptomyces exfoliatus* and slightly lower similarity to the *bli* and *blp* genes of *S. clavuligerus*. This may indicate a previously unrecognized association between clavulanic acid production and the presence of BLIP-encoding genes, at least in *Streptomyces*-producer species. No *bli* homologues were present in the genome of *S. viridis*.

Another intriguing feature of the clavulanic acid gene clusters from both *S. viridis* and *S. flavogriseus* is the presence of a large gene (4,248 bp and 4,068 bp, respectively) encoding a putative protein similar to NocE from *Nocardia uniformis*. This protein has a hydrolase domain near its C-terminus but is otherwise uncharacterized. Although no such gene is found in the clavulanic acid gene cluster from *S. clavuligerus,* a highly similar orthologue is present in *S. clavuligerus,* but located well away from any of the gene clusters associated with biosynthesis of clavam metabolites. Clavulanic acid biosynthesis has been the subject of considerable research activity in recent years, but there are no reports in the literature of successful transplantation of clavulanic acid producing ability from *S. clavuligerus* into heterologous hosts. We have made several unsuccessful attempts over the years, but whether this reflects an unanticipated requirement for a *nocE* homologue in addition to the main clavulanic acid gene cluster, or some other difficulty, remains to be determined.

Late stages in 5*S* clavam biosynthesis

As in clavulanic acid biosynthesis, the late stages in 5*S* clavam biosynthesis are largely unknown. Egan et al.

[15] proposed a mechanism by which clavaminic acid would be converted into an aldehyde intermediate via decarboxylation and deamination, and from there into 2-formyloxymethylclavam, 2-hydroxymethylclavam, and clavam-2-carboxylate by oxidation and hydrolysis reactions.

Genes specifically involved in the production of 5*S* clavams are found in *S. clavuligerus* in two gene clusters located apart from the clavulanic acid gene cluster. The first indication that genes involved in the biosynthesis of clavulanic acid and the 5*S* clavams are separate came when two *cas* genes (*cas1* and *cas2*) were isolated by Marsh et al. [44]. Since the genes for clavulanic acid production are clustered together around *cas2*, genes specifically involved in 5*S* clavam biosynthesis were sought by sequencing the region around *cas1*. Mosher et al. [49] found a number of new genes (*cvm1*, *cvm2*, *cvm3*, *cvm4*, *cvm5*, and *cvm6*) in what is now called the clavam gene cluster (Fig. 4). Tahlan et al. [70] sequenced further outward to extend the clavam gene cluster and carried out mutagenesis of the genes. Analysis of these genes suggested a possible pathway of 5*S* clavam biosynthesis (Fig. 5).

cvm1 is located immediately upstream of *cas1* (Fig. 4) and shows strong similarity to genes encoding aldo–keto reductases. Mutation of *cvm1* has no effect on clavulanic acid production but eliminates clavam-2-carboxylate, 2-hydroxymethylclavam, and alanylclavam production [49]. *cvm2* encodes a predicted protein with limited similarity to isomerases, and mutants in *cvm2* were severely compromised in 5*S* clavam production [49, 70]. *cvm3* encodes a putative flavin reductase predicted to provide reduced flavin for the flavin-dependent mono-oxygenase encoded by *cvm5* [49, 70]. While mutagenesis of *cvm3* did not affect clavam production, mutants defective in *cvm5* were unable to produce the normal 5*S* clavams and instead accumulated a possible intermediate in the pathway, 2-carboxymethylideneclavam [70] (Fig. 5). The protein encoded by *cvm5* may carry out a Baeyer–Villiger oxidation in the eventual conversion of 2-carboxymethylideneclavam to 2-formyloxymethylclavam. Genes further upstream of *cas1* beyond *cvm3* were found not to be required for clavam production [70].

Of the genes downstream from *cas1*, only *cvm5* is essential for 5*S* clavam production. *cvm6* and *cvm7* lie immediately downstream of *cvm5* and show similarity to aminotransferases and transcriptional regulators, respectively, and were initially viewed as likely candidates to be involved in 5*S* clavam biosynthesis, but mutational analysis ruled this out [70]. Furthermore, although *cvm1*, *cvm2*, and *cvm3/5* are all involved in 5*S* clavam biosynthesis, additional gene products seemed necessary to account for production of all of the 5*S* clavam products.

The identification of these additional genes followed upon the realization that all of the genes for the early stages of clavam production (not just *cas1* and *cas2*) exist as paired copies or paralogues [28]. Subsequently, a second

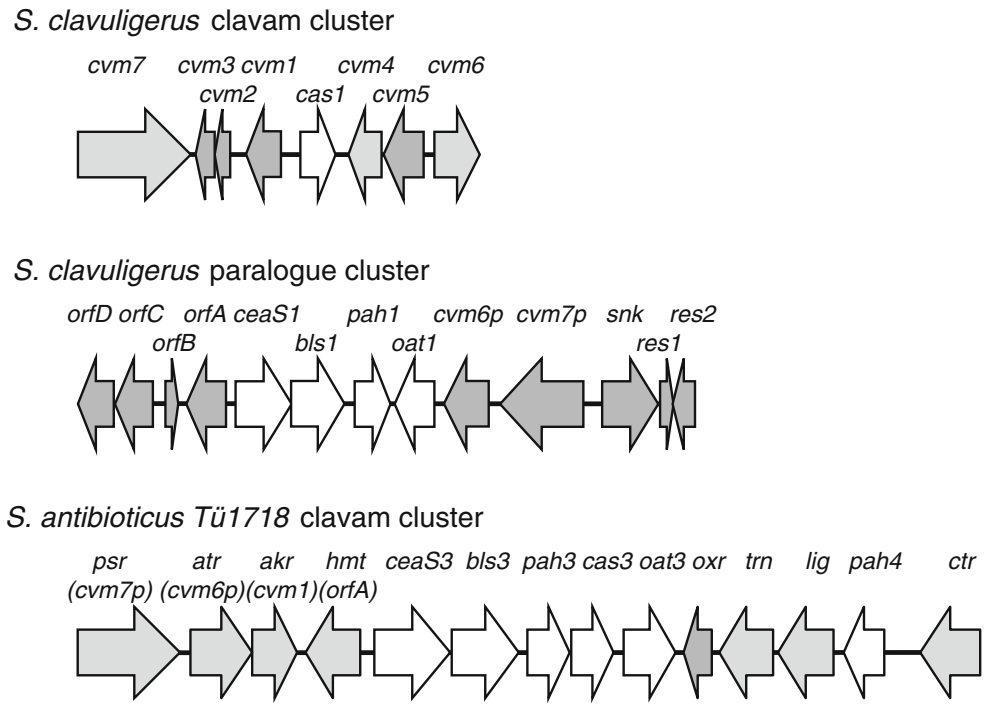

Fig. 4 Gene clusters associated with the production of 5*S* clavams in *S. clavuligerus* and *S. antibioticus*. Early genes are shown as *white arrows*, late genes as *dark gray arrows*, and genes with unknown functions are *light gray*

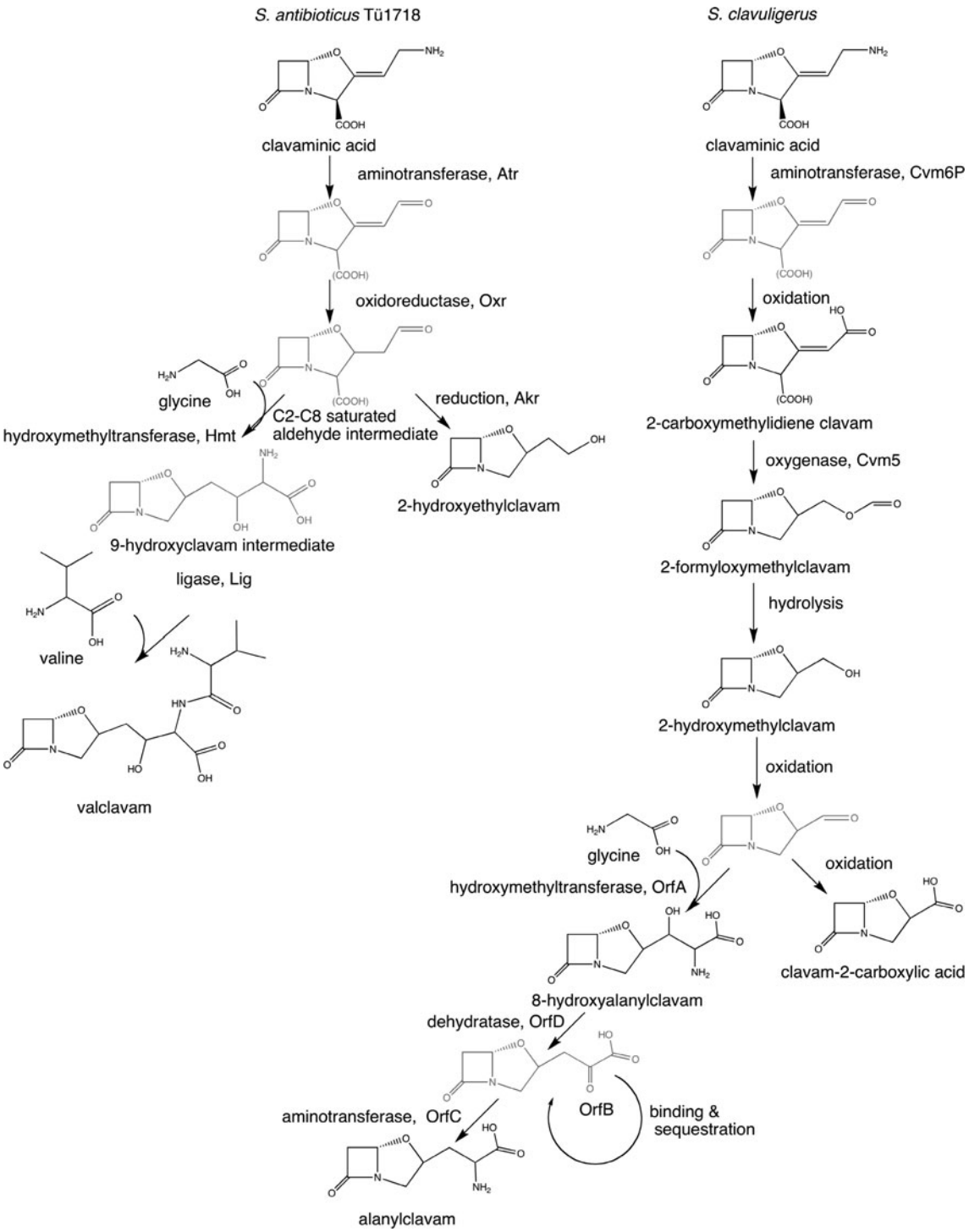

Fig. 5 Proposed late steps in the biosynthetic pathway to 5*S* clavam metabolites in *S. clavuligerus* and *S. antibioticus.* Known metabolites are shown in *black*; proposed metabolites are shown in *gray*. Taken with permission from [20]

copy of *pah2,* called *pah1*, was located. Sequencing the region surrounding *pah1,* now referred to as the paralogue cluster, revealed the presence of duplicate copies of the rest of the early genes, as well as additional late 5*S* clavam biosynthetic genes (Fig. 4) [27]. In view of the apparently extensive occurrence of lateral gene transfer in the genus *Streptomyces,* the cluster should perhaps more correctly have been named the orthologue gene cluster, but given its acceptance in the published literature, the term paralogue cluster will continue to be used here.

The central region of the paralogue cluster includes *ceaS1*, showing 66 % amino acid identity to *ceaS2*, *bls1*, with 49 % identity to *bls2*, *pah1,* with 71 % identity to *pah2*, and *oat1*, with 47 % identity to *oat2*. Mutation of any one copy of these pairs of genes decreased clavam production, and double mutants were completely devoid of all clavam production, with the exception of *oat1/oat2* mutants, which still produced clavams but in reduced amounts. This supports a non-essential role for the *oat* genes in increasing precursor flux into the clavam biosynthetic pathways.

Beyond *oat1,* the paralogue gene cluster contains two additional paralogues, *cvm6p* and *cvm7p,* which resemble *cvm6* and *cvm7* from the clavam cluster (Fig. 4). However, unlike *cvm6* and *cvm7*, mutation of either *cvm6p* (encoding a putative aminotransferase) or *cvm7p* (encoding a large bi-domain transcriptional regulator) abolishes 5*S* clavam production [70] with no effect on clavulanic acid production. Cvm6p is proposed to deaminate clavaminic acid to produce the aldehyde intermediate predicted by Egan et al. [15] (Fig. 5).

Cvm7p is a pathway-specific regulator for the late steps of 5*S* clavam biosynthesis, and furthermore, *cvm7p* lies just upstream of a set of three genes, *snk, res1* and *res2*, that encode elements of an atypical two-component regulatory system. Mutations in either *snk* or *res2* cause loss of 5*S* clavam production with no effect on clavulanic acid production whereas mutation of *res1* causes increased production of 5*S* clavams [35]. Genes beyond *res2* in the paralogue cluster appear unrelated to clavam production.

Beyond *ceaS1* on the other flank of the paralogue cluster, lie four genes, *orfA*, *orfB*, *orfC*, and *orfD*, that have roles specifically in alanylclavam biosynthesis [77]. Mutation of any of genes *orfA–orfD* abolishes alanylclavam production specifically without affecting production of any of the other clavams, including clavulanic acid. Furthermore, mutants in *orfC* and *orfD* accumulate a clavam intermediate, 8-hydroxyalanylclavam, not found in wild-type cells. The nature of this intermediate, together with a consideration of the types of reactions that proteins resembling ORFs A, B, C, and D catalyze in serine-threonine biosynthesis has allowed a putative pathway to alanylclavam to be proposed (Fig. 5). Genes beyond *orfD* appear unrelated to clavam biosynthesis.

Alanylclavam biosynthesis is proposed to branch off from the central clavam pathway via a hydroxymethyltransferase-type reaction catalyzed by ORFA in which glycine is attached to an aldehyde group-bearing intermediate, resulting in the formation of 8-OH alanylclavam. ORFD, which resembles threonine dehydratases, then catalyzes the dehydration/deamination of 8-OH alanylclavam to produce a clavam intermediate with a pyruvyl side chain at C2. Alanylclavam is finally produced by transamination through the action of ORFC, which shows similarity to family I aminotransferases. The proposed role of ORFB in this pathway is regulatory. ORFB resembles members of the YjgF/YER057c/UK114 protein family, which includes proteins typically involved in purine and amino acid biosynthesis in prokaryotes and associated with non-enzymatic roles, perhaps involving sequestration of labile or toxic intermediates [55, 66]. ORFB may bind the pyruvylclavam intermediate, expected to be a highly reactive and possibly toxic compound, to enable it to serve as the substrate for ORFC.

Genetic analyses indicated that the clavulanic acid gene cluster, the clavam gene cluster, and the paralogue gene cluster are unlinked in *S. clavuligerus* [72]. Recent full-genome sequencing has validated those analyses and shown that the paralogue cluster is located on a linear megaplasmid while the clavulanic acid and clavam clusters are separated on the chromosome by more than 1 Mb, an unusual arrangement in view of the usual clustering of secondary metabolite biosynthetic genes [46, 68].

The complexity of clavam biosynthesis in *S. clavuligerus* has made it difficult to evaluate the importance of the various genes found in the clavam and paralogue clusters in the synthesis of 5*S* clavam metabolites and suggested that an examination of clavam biosynthetic genes in a species producing only 5*S* clavam metabolites might provide a useful comparison. Analysis of the clavam gene cluster from *Streptomyces antibioticus* Tü1718, a producer of 8-OH-ethylclavam and valclavam showed conservation of the early clavam biosynthetic genes *ceaS, bls, pah*, *cas*, and *oat*, as well as the 5*S* clavam-specific genes *cvm1*, *orfA, cvm6p*, and *cvm7p* (Fig. 4), thereby confirming their importance for 5*S* clavam biosynthesis in general [20]. In contrast, *cvm5,* essential for 5*S* clavam biosynthesis in *S. clavuligerus*, is not represented in *S. antibioticus* Tü1718 nor is its accompanying gene, *cvm3*. Conversely, *oxr*, essential for valclavam biosynthesis in *S. antibioticus* Tü1718, is not represented in *S. clavuligerus*. Together, this information suggests how 5*S* clavam biosynthesis might differ between these two species (Fig. 5).

Regulation of clavam biosynthesis in *S. clavuligerus*

The molecular mechanisms behind the regulation of antibiotic biosynthesis are of considerable interest due to the economic and medical benefits that accrue from increased antibiotic production. In *S. clavuligerus*, a variety of regulatory factors have been identified that play important roles in the biosynthesis of clavulanic acid, of the 5*S* clavams, or of all clavams.

In *S. clavuligerus*, a SARP called CcaR (cephamycin and clavulanic acid regulator), encoded in the cephamycin C gene cluster, is essential for the production of

cephamycin C and clavulanic acid, but not the 5*S* clavams [2, 56, 63, 71]. CcaR is required for the expression of a polycistronic transcript including *ceaS2*, *bls2*, *pah2*, and *cas2*, the early genes for clavulanic acid biosynthesis [54] (Fig. 2) as well as for activating its own transcription by binding to its promoter region [65].

CcaR also regulates expression of *claR*, a gene from within the clavulanic acid gene cluster, which encodes a LysR-type transcriptional regulator [52, 58, 71]. Mutants defective in *claR* are unable to produce clavulanic acid (but can still produce cephamycin C, clavaminic acid, and the 5*S* clavams) while strains carrying multiple copies of *claR* overproduce clavulanic acid [52, 58]. ClaR is necessary for the transcription of *oppA1*, *cad*, and *cyp*, which are all late genes required for clavulanic acid production [52]. CcaR mutants cannot express *claR* transcripts; thus CcaR not only affects the early stages of clavulanic acid biosynthesis by activating expression of the early genes but is also necessary for the late stages of clavulanic acid production through *claR* [58, 71]. A recent comprehensive study of targets for CcaR confirmed it role in regulating expression of the *ceaS2* operon, *claR* and its own gene, as well as genes within the cephamycin C gene cluster [63]. Inspection of promoter regions upstream of genes transcriptionally regulated by CcaR, such as *cefD-cmcI* and *lat* (genes involved in cephamycin C biosynthesis) and *ceaS2*, reveals that they contain sequence repeats characteristic of SARP transcriptional regulators [65, 71].

While CcaR and ClaR represent a pathway-specific regulatory cascade controlling clavulanic acid biosynthesis specifically, a parallel system specifically controlling 5*S* clavam biosynthesis also exists. A new class of antibiotic regulatory proteins that has an N-terminal SARP-like domain and a C-terminal ATPase domain has been described recently, with PimR, from *S. natalensis* [3], and SanG, from *S. ansochromogenes* [39] as typical representatives. Within the paralogue cluster, *cvm7p*, a gene similar to *pimR* and *sanG,* regulates 5*S* clavam production specifically without affecting morphological differentiation or clavulanic acid production [70]. Interestingly, *cvm7*, in the clavam gene cluster, is a paralogue of *cvm7p* and so *cvm7* also belongs in this new class of antibiotic regulatory genes, but its mutation does not affect clavam biosynthesis. Cvm7p regulates transcription of several of the late genes essential for biosynthesis of 5*S* clavams [35] and so it represents a 5*S* clavam functional counterpart to ClaR.

snk, a gene encoding a putative sensor kinase, lies immediately downstream of *cvm7p* in the paralogue cluster, and beyond it lies two response regulators, *res1* and *res2* (Fig. 4). Sensor kinases typically detect a specific stimulus, autophosphorylate, and then transfer the phosphate to another protein called a response regulator. Response regulators are DNA-binding proteins that, upon phosphorylation, activate or repress the transcription of genes. Res1 differs from Res2 and other typical response regulators in that it is truncated and contains only a phosphorylation site but no DNA-binding domain. Mutants defective in *snk* and or *res2* were unable to produce any 5*S* clavams whereas mutants defective in *res1* overproduced 5*S* clavams [35]. Rather than directly affecting expression of 5*S* clavam biosynthetic genes, however, this atypical two-component system exerts its effect indirectly by regulating expression of *cvm7p*. Therefore, it represents the 5*S* clavam counterpart of *ccaR*, acting to regulate expression of the direct pathway-specific transcriptional regulator, Cvm7p.

In addition to control by pathway-specific transcriptional regulators, clavulanic acid and 5*S* clavam biosynthesis are also subject to multiple systems of higher level or global regulation. One such higher level of regulation, the BldA system, mediated by a tRNA molecule recognizing UUA codons, has unusual features in *S. clavuligerus*. Because of the high G + C content of *Streptomyces* spp. genomes, genes containing TTA codons are rare and largely restricted to non-essential genes associated with differentiation or secondary metabolism, such that *bldA* mutants are bald (cannot undergo differentiation) and unable to produce antibiotics. In keeping with this paradigm, *bldA* null mutants of *S. clavuligerus* do not form aerial hyphae, and *ccaR* carries a TTA codon [56]. However, these *bldA* mutants are still capable of producing both cephamycin C and clavulanic acid, suggesting that some TTA codon-containing genes are dependent upon *bldA* in *S. clavuligerus* while others are not [73]. Until recently, *ccaR* was the only *S. clavuligerus* gene known to contain a TTA codon, but now *res1*, *res2*, and *orfA* (encoding the first enzyme of the alanylclavam pathway) from the paralogue cluster have also been found to contain TTA codons. Interestingly, *bldA* mutants of *S. clavuligerus* produce and even overproduce some 5*S* clavams when compared to the wild-type, but do not produce any alanylclavam. This suggests that *res2,* like *ccaR,* is expressed in the *bldA* mutant, whereas *res1* and *orfA* are not. It was hypothesized that TTA-containing genes might be expressed in *bldA* mutants through mistranslation by a non-cognate leucyl tRNA, depending on the context of the TTA codon [73], such that TTA codons followed by G or A would be more likely to be mistranslated in-frame whereas TTA codons followed by C or T would result in a frame shift. In keeping with this theory, *ccaR* and *res2* have TTA codons followed by G residues, whereas the TTA codons in *res1* and *orfA* are followed by C residues. Also consistent with this theory, the *adpA* gene of *S. clavuligerus,* responsible for the bald phenotype of *bldA* mutants, contains a TTA codon followed by a C residue [40].

Even though *ccaR* expression was found to be independent of *bldA*, another "bald" gene, *bldG*, is required for the transcription of *ccaR* [9]. BldG, first characterized in *S. coelicolor*, shows similarity to anti-anti-sigma factors from *Bacillus subtiltis* and *Staphylococcus aureus* [8]. In *S. clavuligerus*, a *bldG* homologue was found to be required for aerial hyphae formation and for the production of cephamycin C, clavulanic acid, and 5*S* clavams. BldG may direct the production of cephamycin C and clavulanic acid by regulating expression of *ccaR*, but since CcaR does not regulate 5*S* clavam biosynthesis, BldG must also control expression of at least one other gene involved in 5*S* clavam production [71].

For a number of *Streptomyces* spp., γ-butyrolactone-type hormones also play an important role in regulating secondary metabolism [21, 32]. The binding sites of γ-butyrolactone receptors have been identified and characterized as autoregulatory elements (ARE) [34, 51] and an ARE has been identified upstream of *ccaR* in *S. clavuligerus* [19]. Additionally, *S. clavuligerus* has been found to produce a γ-butyrolactone autoregulator [22], and it also carries a gene, *scaR* (also known as *brp*), which encodes a γ-butyrolactone receptor protein [33]. ScaR binds not only to the ARE upstream of *ccaR* but also to an ARE upstream of its own gene [64]. Since a *scaR* mutant overproduces both cephamycin C and clavulanic acid, it is probable that ScaR, like ArpA, acts as a repressor and inhibits the expression of *ccaR* by binding the upstream ARE.

Concluding remarks

Like many secondary metabolites, clavam compounds arise as a family of structurally related metabolites from a shared biosynthetic pathway. Unlike most families of secondary metabolites, however, the genes encoding the enzymes for clavam biosynthesis in *S. clavuligerus* are distributed across three separate locations in the genome. This likely reflects the fact that clavulanic acid and the 5*S* clavams are actually produced from two separate pathways, and it is only by chance that these two pathways co-exist in *S. clavuligerus*. The genetic information specifying these processes was likely acquired by *S. clavuligerus* through separate lateral gene transfer events, resulting in their residing in separate locations in the genome. Over evolutionary time, the two pathways have developed cross-regulatory features, such that mutation of *ceaS2* has an inordinately severe effect on production of all clavam metabolites, but otherwise, they still function as two largely independent processes. This complexity of clavam biosynthesis in *S. clavuligerus* may have hampered the elucidation of these pathways. Species producing only one or the other type of clavam compounds may provide less complicated systems with which to work. On the other hand, it is likely very fortunate that clavulanic acid biosynthesis was first discovered, and the commercial fermentation developed, in *S. clavuligerus.* The twinning of the early steps of the pathway may give this species an intrinsically higher capacity for the production of clavaminic acid and make it a particularly suitable platform from which to launch a strain improvement program. Intriguing questions remain in both the biosynthesis of clavulanic acid and the 5*S* clavams, notably the mechanism behind the double stereochemical inversion of the clavam nucleus that separates clavulanic acid from the rest of the 5*S* clavam metabolites. Perhaps conducting future studies in less complex producer species will provide answers to this and other elusive questions.

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