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MINI-REVIEW

Clavulanic acid biosynthesis and genetic manipulation for its overproduction

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Abstract Clavulanic acid, a β-lactamase inhibitor, is used together with β-lactam antibiotics to create drug mixtures possessing potent antimicrobial activity. In view of the clinical and industrial importance of clavulanic acid, identification of the clavulanic acid biosynthetic pathway and the associated gene cluster(s) in the main producer species, *Streptomyces clavuligerus*, has been an intriguing research question. Clavulanic acid biosynthesis was revealed to involve an interesting mechanism common to all of the clavam metabolites produced by the organism, but different from that of other β-lactam compounds. Gene clusters involved in clavulanic acid biosynthesis in *S. clavuligerus* occupy large regions of nucleotide sequence in three loci of its genome. In this review, clavulanic acid biosynthesis and the associated gene clusters are discussed, and clavulanic acid improvement through genetic manipulation is explained.

Keywords Clavulanic acid · Clavam · β-lactam antibiotics · *Streptomyces clavuligerus*

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Introduction

Numerous antibiotics have been discovered as natural products from the genus *Streptomyces* and were developed into valuable therapeutic agents. However, although antibiotics are valued for their ability to prevent bacterial growth by targeting essential cellular processes, antibiotic overuse has led to emergence of pathogens resistant to many if not all antibiotics. β-lactam antibiotics such as penicillins, cephalosporins, and monobactams possess activities that disrupt the integrity of the bacterial cell wall, which makes them useful against infections caused by a broad variety of pathogens. However, as the ability to produce β-lactamases has spread within pathogenic bacteria, new antibiotics have been required to evade and to overcome these destructive enzymes. The approach of inhibiting β-lactamase activity by incorporating β-lactamase inhibitors into antibiotic mixtures has been a particularly effective strategy made possible by the discovery or development of novel antibiotics like clavulanic acid, sulbactam, and tazobactam, which restore effectiveness to β-lactam antibiotics.

Clavulanic acid was first investigated in *Streptomyces clavuligerus*, which is a filamentous, Gram-positive soil bacterium from the genus *Streptomyces* in the family Actinomycetes, and a veritable biological antibiotic "factory". *S. clavuligerus* was first isolated during a screen for strains producing β-lactam antibiotics, as a result of its ability to produce cephamycin C and penicillin N (Nagarajan et al. 1971; Higgens and Kastner 1971). The existence of clavulanic acid with β-lactamase inhibitory activity was not recognized until its discovery by Brown et al. in 1976, and the discovery of the other clavams produced by this species, including clavam 2-carboxylate, 2-hydroxymethylclavam, 2-formyloxymethylclavam, and alanylclavam followed after

that (Brown et al. 1976; Brown et al. 1979; Pruess and Kellett 1983).

Clavulanic acid is one of the so-called non-classical β-lactam compounds, distinct from classical β-lactam antibiotics such as penicillins, cephalosporins, and cephamycins. This compound has remarkable inhibitory activity on group 2 β-lactamases of molecular classes A or D, which include penicillinases, cephalosporinases, and broad spectrum β-lactamases, functioning as an active site-directed suicide inhibitor (Bush et al. 1995; Shahid et al. 2009). This inhibitory property is directly applicable to clinical therapy, and consequently, clavulanic acid has become an important commercial product available under trade names such as Augmentin™ and Timentin™, products containing clavulanic acid combined with β-lactam antibiotics such as amoxicillin or ticarcillin, respectively. Although newer β-lactamase inhibitors have been developed such as penems, olivanic acids, ME1071, NXL1071, thiazolidinecarboxylic acid, and *m*-aminophenylboronic acid, most of them are not yet available in the clinic and are still at the experimental stage (Shahid et al. 2009; Lee et al. 2010). Thus, clavulanic acid still plays in important role as an agent to inhibit β-lactamases, and development of clavulanic acid and β-lactam antibiotic combination drugs has presented additional challenges to enhance pharmacokinetics of the formulations (Geddes et al. 2007).

Studies on clavulanic acid production by *S. clavuligerus* have been actively pursued due to the commercial and clinical importance of clavulanic acid. Initially, studies were directed towards locating and identifying genes involved in its production and then using this information to deduce the specific pathway involved. Subsequently, the genetic studies have been directed towards genetic manipulation of the producer organism resulting in development of improved producer strains.

Clavulanic acid and its producers

Clavulanic acid was first identified from *S. clavuligerus* and later from other *Streptomyces* species, *Streptomyces jumonjinensis* and *Streptomyces katsurahamanus* (Brown et al. 1976; Jensen and Paradkar 1999). In addition, several species of *Streptomyces* have been identified as producing other clavam metabolites structurally similar to clavulanic acid, such as clavaminic acid, 2-hydroxyethylclavam, valclavam, and clavamycins. Interestingly, all *Streptomyces* species described to date that produce clavulanic acid also produce conventional β-lactam antibiotics, but those that produce only the other clavam metabolites do not. Furthermore, the number of species described as producing non-clavulanic acid clavams exceeds those producing clavulanic acid, implying that the ability to biosynthesize clavulanic acid is more restricted in distribution (Jensen and Paradkar 1999). *Streptomyces lipmanii* appears to occupy a position intermediate among the clavam producers since it produces only clavaminic acid, a biosynthetic intermediate common to both clavulanic acid and the other clavams. *S. lipmanii* also produces conventional β-lactam antibiotics but cannot produce either clavulanic acid or any of the other clavam end products (Jensen and Paradkar 1999).

Clavulanic acid is classified as a clavam metabolite and characterized by a bicyclic nucleus comprising a four-membered β-lactam ring fused to a five-membered oxazolidine ring. In terms of its molecular structure, the nucleus of clavulanic acid is similar to that of the classical antibiotic, penicillin, except that sulfur in the thiazolidine ring of penicillin is replaced with oxygen in the oxazolidine ring of clavulanic acid, an acylamino substituent is absent at C-6, and an exo-β-hydroxyethylidene group is attached at C-2, resulting in a compound that irreversibly inactivates β-lactamase rather than being cleaved by the enzyme (Baggaley et al. 1997).

The activity of clavulanic acid is also different from that of the other clavam metabolites, despite their similar structures. In contrast to the other clavams, which have a 3*S*,5*S* stereochemistry and which show only antifungal and antibacterial activity, clavulanic acid has 3*R*,5*R* stereochemistry, which gives it β-lactamase inhibitory activity but poor antibacterial activity (Baggaley et al. 1997). Reflecting this difference in stereochemistry, the non-clavulanic acid clavams are hereafter referred to collectively as the 5*S* clavams.

Clavulanic acid biosynthesis

Both clavulanic acid and the 5*S* clavams arise from a shared biosynthetic pathway which gives rise to the clavam nucleus (Fig. 1). Early studies of clavulanic acid biosynthesis focused on the identification of biosynthetic precursors as a way to predict the enzymes that might be involved. It soon became clear that the mechanism for clavulanic acid biosynthesis is different from that of conventional β-lactam antibiotics such as penicillins and cephalosporins produced by a non-ribosomal peptide synthetase in that a tripeptide precursor is not involved despite the presence of a β-lactam ring in clavulanic acid. Instead, arginine and ornithine were proposed to be precursors providing the nitrogen atom to clavulanic acid, based on feeding experiments employing labeled amino acids (Romero et al. 1986) and ultimately, Valentine et al. (1993) showed that arginine is a more direct precursor than ornithine. In addition, feeding labeled 3-carbon intermediates of glycolysis first suggested that the β-lactam ring of clavulanic acid might be derived from glycerate and pyruvate (Townsend and Ho 1985; Pitlik and Townsend 1997; Thirkettle et al. 1997), and subsequently,

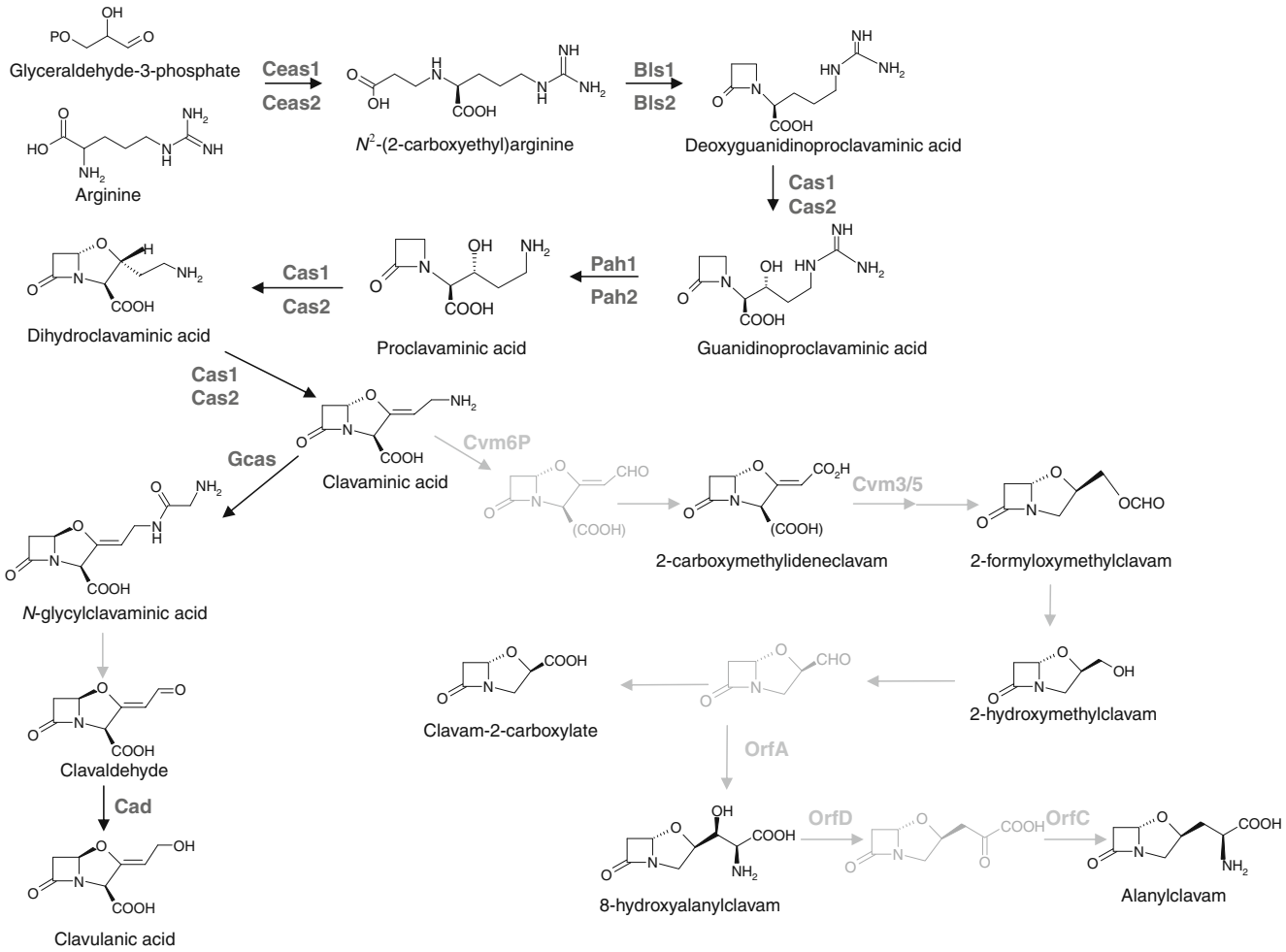

Fig. 1 The biosynthetic pathway for clavam metabolites including clavulanic acid. Hypothetical structures, lines, and proteins are represented in *gray*

Khaleeli et al. (1999) showed that glyceraldehyde-3-phosphate is incorporated as a direct substrate into the pathway.

Identification of carboxyethylarginine synthase (Ceas) as the enzyme responsible for combining these two precursors in the first dedicated step of clavulanic acid biosynthesis was determined from the study of the *ceaS* gene located adjacent to the genes involved in penicillin and cephalosporin biosynthesis and at one extreme end of the cluster of genes for clavulanic acid biosynthesis (Khaleeli et al. 1999; Pérez-Redondo et al. 1999). The amino acid sequences of Ceas deduced from the *ceaS* gene shows similarity to acetohydroxyacid synthases with motifs that recognize pyruvate and thiamine pyrophosphate (Pérez-Redondo et al. 1999). The arginine and glyceraldehyde-3-phosphate precursors are initially condensed by Ceas, which catalyzes a thiamine diphosphate (ThDP)-dependent reaction to give rise to N^2-(2-carboxyethyl)arginine (Khaleeli et al. 1999). Ceas is a significant protein catalyzing an unusual reaction where an N–C bond is formed involving an electrophilic acryloyl-thiazolium intermediate of ThDP, unlike the nucleophilic intermediates generated by typical ThDP-dependent processes (Merski and Townsend 2007). X-ray crystallographic analysis of Ceas showed similarity to the crystal structure of pyruvate decarboxylase and demonstrated that the enzyme had a tetrameric form with cofactors Mg^{2+} and ThDP present in the active site (Caines et al. 2004). Based on the crystal structure, Caines et al. (2009) further suggested that the overlapping binding sites for the substrates indicated that the ThDP-mediated condensation of glyceraldehyde-3-phosphate and arginine must occur sequentially. That is, glyceraldehyde-3-phosphate binds first in the active site of Ceas with release of phosphate, and then arginine binds in a position that allows reaction with the newly formed acryloyl-ThDP intermediate (Caines et al. 2009).

β-lactam synthetase (Bls) catalyzes the second step of the pathway converting N^2-(2-carboxyethyl)arginine to

deoxyguanidinoproclavaminate, thereby generating the monocyclic β-lactam ring (Bachmann et al. 1998). This step is completely distinct from the reaction that generates the β-lactam ring of penicillins and cephamycins. That reaction involves cyclization of a non-ribosomally generated δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine tripeptide, by a single protein viz isopenicillin N synthase. In contrast, the catalytic reaction of Bls proceeds by adenylation of the N^2-(2-carboxyethyl)arginine β-carboxylate followed by cyclization via an oxoanion intermediate in the presence of ATP and Mg^{2+} (Bachmann et al. 1998; Bachmann and Townsend 2000; Miller et al. 2002). Bls is similar to class B asparagine synthetases in peptide sequence and in terms of amino acid adenylation via a ATP/Mg^{2+}-dependent reaction, but Bls catalyzes an intramolecular ring closure reaction to form the β-lactam, whereas asparagine synthetases carries out intermolecular amide bond formation. Bls structural analysis shows that the C-terminal active site expanded in Bls can accommodate N^2-(2-carboxyethyl)arginine, a larger substrate than aspartic acid, suggesting that through evolutionary change, Bls activity has become biased toward formation of the β-lactam ring, while the interdomain reaction typical of asparagine synthetases is disfavored (Miller et al. 2001, 2002).

Two enzymes, clavaminate synthase (Cas) and proclavaminate amidinohydrolase (Pah), follow Bls catalyzing reactions to generate the bicyclic intermediate, clavaminate. Deoxyguanidinoproclavaminate is first hydroxylated by Cas to give guanidinoproclavaminate (Baldwin et al. 1993), and then subsequently Pah catalyzes the conversion of guanidinoproclavaminate to proclavaminate by removing the arginine-derived guanidino group (Elson et al. 1993; Aidoo et al. 1994). Cas then participates again in the next two reactions to catalyze the formation of the first bicyclic intermediate through oxidative ring closure of proclavaminate to give dihydroclavaminate, followed by desaturation to form clavaminate (Elson et al. 1987; Baldwin et al. 1991; Salowe et al. 1991). The Cas has unusual functions as a non-heme iron, 2-oxoglutarate-dependent oxygenase which carries out distinct oxidative catalytic reactions, hydroxylation, cyclization, and desaturation at the single catalytic site during the process of clavulanic acid biosynthesis (Busby and Townsend 1996). Crystallographic study of the Cas presented that it has a jelly roll β-barrel core found in 2-oxoglutarate and related oxygenases, implying divergent evolution of the family (Zhang et al. 2000). The Cas protein has a 2-His-1-carboxylate binding motif in the active site which serves to anchor the iron and provides a platform for the reactive intermediates. The flexibility of the Cas results from that the carboxylate for an iron ligand is provided by a glutamate, not an aspartate preferred by other 2-oxoglutarate oxygenases (Zhang et al. 2000). Furthermore, the mutagenesis studies identified the iron binding ligands and suggested that amino acids extended between two histidines could provide the versatility for the Cas (Khaleeli et al. 2000).

Pah, as deduced from sequence analysis of the corresponding gene, shows significant homolgy to agmatine ureohydrolase and other arginases, and its enzymatic functional behavior is also similar to arginases in terms of Mn^{2+} requirement, pH activity profile, and kinetic properties (Elson et al. 1993; Aidoo et al. 1994; Wu et al. 1995). Thereby, the Pah protein was proposed to belong to the arginase family but to have evolved to accept β-lactams, not arginine as its substrate (Ouzounis and Kyrpides 1994; Wu et al. 1995; Elkins et al. 2002). The crystal structure also showed that the active site of Pah possessed a di-Mn^{2+} site, where hydrolytic attack of the carbon atom of the guanidino group is facilitated by the di-Mn^{2+}-bridging water molecule (Elkins et al. 2002). With the formation of clavaminate, this marks the end of the shared portion of the pathway common to both clavulanic acid and the 5*S* clavams, and from here, the pathways diverge.

N-glycyl-clavaminic acid synthetase (Gcas) has been identified as a protein encoded by *orf17*, a gene located near the cluster of genes encoding enzymes for the early steps of clavulanic acid biosynthesis (Mellado et al. 2002; Jensen et al. 2004a). Gcas was initially presumed to be a carboxylase based on similarity and was shown to be important for clavulanic acid production since clavulanic acid was undetectable in a Gcas defective mutant (Jensen et al. 2004a). Arulanantham et al. (2006) showed that Gcas (also referred to as Orf17) is a member of the ATP-grasp fold superfamily and that it can catalyze conversion of clavaminic acid to *N*-glycyl-clavaminic acid in an ATP-dependent manner; hence, its designation as *N*-glycyl-clavaminic acid synthetase (Gcas). *N*-glycyl-clavaminic acid represents the first identified biosynthetic intermediate of the pathway that is specific only for clavulanic acid and not the 5*S* clavams.

A double epimerization and an oxidative deamination reaction are predicted to follow Gcas in the pathway and ultimately to yield clavaldehyde; however, the mechanism by which *N*-glycyl-clavaminic acid is converted to clavaldehyde is still not clear. Clavaldehyde (3*R*,5*R*-clavulanate-9-aldehyde), the last known intermediate of the pathway and the immediate precursor of clavulanic acid, is an unstable α,β-unsaturated aldehyde with β-lactamase inhibitory activity (Nicholson et al. 1994; Fulston et al. 2001). The observation that clavaldehyde shows β-lactamase inhibitory activity supports the contention that inhibitory activity is a feature associated with the 3*R*,5*R* stereochemistry of the β-lactam ring. The highly unstable clavaldehyde intermediate finally gets reduced to give clavulanic acid in an NADPH-dependent reaction catalyzed without degradation of the bicyclic β-lactam ring by clavulanic acid dehydrogenase (Cad) which belongs to the

short chain dehydrogenase/reductase family (Fulston et al. 2001; MacKenzie et al. 2007).

Gene clusters for clavulanic acid biosynthesis

Clavulanic acid gene cluster

The earliest studies to search for genes involved in clavulanic acid biosynthesis were performed by complementing a clavulanic acid non-producing strain of *S. clavuligerus* with chromosomal DNA fragments from wild type *S. clavuligerus* (Bailey et al. 1984). Subsequently, the *cas* and *pah* genes were located using reverse genetics techniques, and then DNA sequencing of flanking regions eventually revealed the components of the complete clavulanic acid gene cluster. However, it has since been shown that the genetic information needed for production of clavulanic acid and the 5*S* clavams is distributed among three biosynthetic gene clusters located in unlinked regions of the *S. clavuligerus* chromosome (Fig. 2). These gene clusters, which give rise to all of the various clavam metabolites, have been designated the clavulanic acid gene cluster, the clavam gene cluster, and the paralog gene cluster (Tahlan et al. 2004a). The loci of the gene clusters were subsequently identified from the draft sequence of *S. clavuligerus* ATCC 27064. The supercluster of clavulanic acid–cephamycin C and the clavam gene cluster both lie on the chromosome, whereas the paralog gene cluster is located in pSCL4 (Medema et al. 2010).

The existence of two copies of the *cas* gene was first demonstrated by a reverse genetics approach in which the amino acid sequences of the two purified Cas isozymes were used to design probes to locate the corresponding *cas* genes (Marsh et al. 1992). The two *cas* genes came from unlinked fragments of chromosomal DNA, providing the first clues to the existence of the *cas1*-containing clavam gene cluster in addition to the *cas2*-containing clavulanic acid gene cluster. On the other hand, the clavulanic acid gene cluster was shown to be located adjacent to the gene cluster for cephamycin biosynthesis, creating a "supercluster" of genes (Ward and Hodgson 1993). Characterization of Pah activity by Elson et al. (1993) contributed to the identification of the *pah* gene, and this sequence analysis provided further evidence that the gene clusters for clavulanic acid and the classical penicillin/cephamycin C compounds are linked, as *pah* was found located a short distance downstream of the *pcbC* gene needed for penicillin biosynthesis. Additionally, disruption of *pah* resulted in blocked production of clavulanic acid, confirming that the *pah* gene is necessary to biosynthesize clavulanic acid (Aidoo et al. 1994).

DNA sequence analysis of larger stretches of the clavulanic acid gene cluster was first reported by Jensen et al. (1993) and Hodgson et al. (1995), which gave information for the region extending from *ceaS2* (*orf2*) to *cyp* (*orf10*). Since that time, genetic studies have been

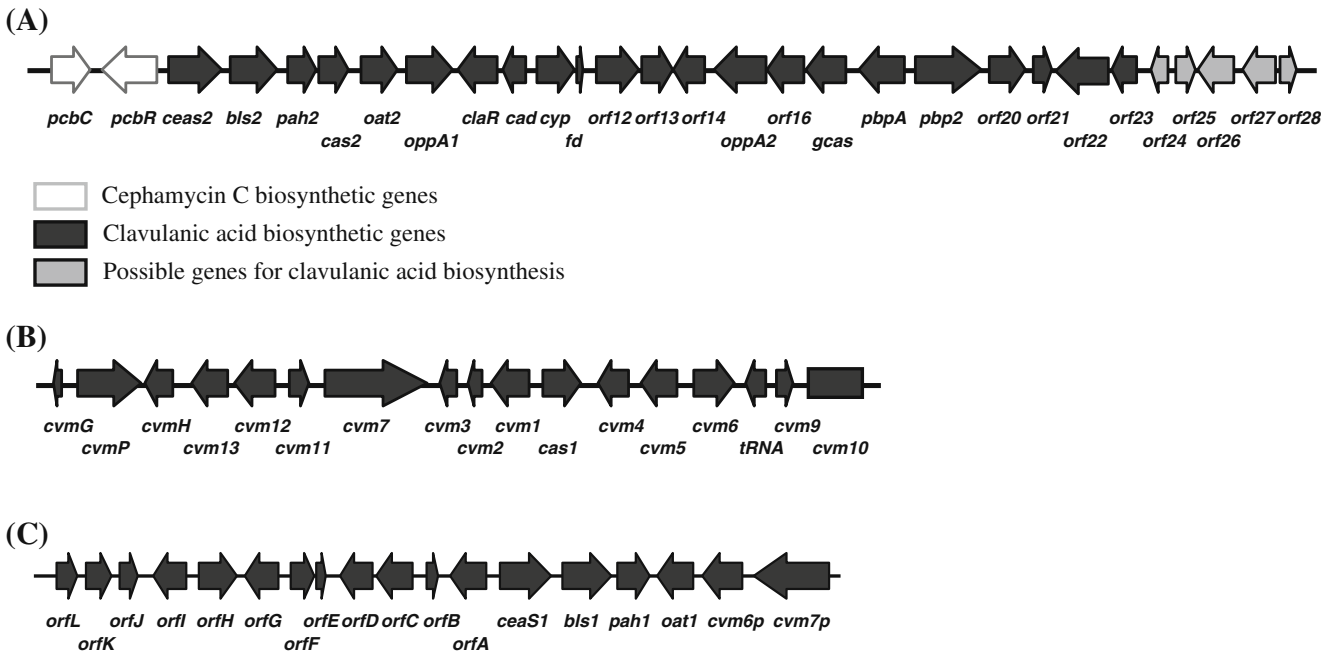

Fig. 2 Three gene clusters for biosynthesis of clavulanic acid and other clavam metabolites. **a** Clavulanic acid biosynthetic gene cluster. **b** Clavam biosynthetic gene cluster. **c** Paralog gene cluster

undertaken to use the DNA sequence information to understand clavulanic acid biosynthesis and ultimately to exploit these genes to enhance and improve production. Several research groups have contributed additional sequenced information leading to the identification of the complete set of clavulanic acid biosynthetic genes; *cyp*, *fd*, and *orf12* by Li et al. (2000), *cyp* through *pbpA* (*orf18*) and into *pbp2* (*orf19*) by Mellado et al. (2002), *fd* (*orf11*) to *orf20* by Jensen et al. (2004a), *orf21* to *orf23* by Song et al. (2009), and *orf20* to *orf28* by Jnawali et al. (2008).

In early studies, several significant genes such as *ceaS2*, *bls2*, *pah2*, *cas2*, and *cad* were deduced to be involved in clavulanic acid biosynthesis because they apparently encoded proteins with activities consistent with the predicted pathway. The involvement of each was then confirmed in vivo by generating gene-disrupted mutants. Surprisingly, mutants defective in *ceaS2*, *bls2*, *pah2*, or *cas2* only lost part of their ability to produce clavulanic acid and 5*S* clavam metabolites depending on the type of medium in which they were cultivated (Paradkar and Jensen 1995; Pérez-Redondo et al. 1999; Jensen et al. 2000). In contrast, mutants defective in *oppA* (*orf7*), *claR*, *cad*, or *cyp* produced no clavulanic acid under any growth conditions while production of 5*S* clavam metabolites was relatively unaffected. These results suggested that paralogous genes encoding functionally equivalent proteins exist for the genes that are involved in the early steps of the pathway essential to production of both clavulanic acid and 5*S* clavam metabolites (Jensen et al. 2000). Subsequently, the existence of the paralogous gene set was proved when the so-called paralog gene cluster was found in another location (Tahlan et al. 2004b).

A gene, *oat2* (*orf6*) encodes a protein with a monofunctional ornithine acetyltransferase activity catalyzing the transfer of an acetyl group from *N*-acetylornithine to L-glutamate with the generation of ornithine (Hodgson et al. 1995; Kershaw et al. 2002). Thus, the Oat protein is involved in the arginine biosynthesis and ultimately becomes responsible for clavulanic acid production (Kershaw et al. 2002; Elkins et al. 2005). Furthermore, the presence of Arg boxes in the *oat2* gene sequence suggests that transcription may be governed by ArgR, and since the *oat2* defective mutant shows different phenotypes depending on arginine concentrations, this suggests that Oat2 may regulate clavulanic acid production by affecting availability of arginine (de la Fuente et al. 2004).

Lorenzana et al. (2004) investigated *oppA1* (*orf7*) and *oppA2* (*orf15*), whose gene products showed similarity to periplasmic oligopeptide-binding proteins of other *Streptomyces* and notably resembled BldKB, an ABC transporter involved in differentiation of *Streptomyces coelicolor* (Nodwell et al. 1996; Lorenzana et al. 2004; Mackenzie et al. 2010). Both of the genes are located in the clavulanic acid gene cluster, and mutants defective in either *oppA1* or *oppA2* are defective for clavulanic acid production thereby showing that they are not only required to produce clavulanic acid but also that they are not functionally equivalent to each other in terms of their roles in clavulanic acid biosynthesis like the other paralogous genes. The two OppA proteins have substrate specificities for different peptides and function to transport the peptides, suggesting that their ability to bind and transport signaling peptides may influence clavulanic acid biosynthesis (Lorenzana et al. 2004). Recently, a crystal structure has been obtained for OppA2 displaying a peptide/arginine moiety bound in the cleft between the two lobes of the protein. Biochemical studies also proposed that OppA may be involved in import of arginine and/or peptides to supply substrates for production of clavulanic acid and 5*S* clavams, and that it might also be responsible for sensing signaling peptides (Mackenzie et al. 2010).

DNA sequence analysis of *claR*, located in the clavulanic acid gene cluster, indicated that it likely encodes a transcriptional regulatory protein. ClaR is a protein of 431 amino acids that includes a domain showing significant similarity to the helix-turn-helix motif region of LysR transcriptional regulators (Paradkar and Jensen 1998; Pérez-Redondo et al. 1998). A *claR* disrupted mutant lost its ability to produce clavulanic acid, suggesting that *claR* is essential for clavulanic acid biosynthesis. Furthermore, the accumulation of clavaminic acid in the *claR* mutant, and comparison of the *claR* mutant to the wild type by transcriptional analysis suggested that ClaR regulates genes involved in the late steps of the clavulanic acid pathway such as *oppA1*, *cad*, and *cyp* (Paradkar and Jensen 1998). CcaR is another regulator for clavulanic acid production and a member of the *Streptomyces*-activator regulatory protein family. CcaR is located in the cephamycin C biosynthetic gene cluster, but it acts as a positive regulator that directly effects transcription of the *ceaS2*, *bls2*, *pah2*, and *cas2* genes involved in the early stage of clavulanic acid biosynthesis and also regulates the activity of late stage genes indirectly by controlling expression of *claR* (Pérez-Llarena et al. 1997; Paradkar and Jensen 1998; Pérez-Redondo et al. 1998; Santamarta et al. 2002) (Regulation of clavulanic acid biosynthesis was reviewed by Liras et al. 2008).

Li et al. (2000) reported that *cyp*, *fd*, and *orf12* resemble genes encoding cytochrome P-450, ferredoxin, and β-lactamase-like proteins, respectively. Mutational inactivation of *cyp* and *orf12* resulted in complete loss of clavulanic acid production, while production was only partially reduced in a *fd* disrupted mutant, suggesting that *cyp* and *orf12* are essential, and *fd* is beneficial but not absolutely required for clavulanic acid biosynthesis (Li et al. 2000; Mellado et al. 2002; Jensen et al. 2004a). Since cytochrome P-450 and ferredoxin-requiring enzymes carry out a wide range of

oxidative reactions in organisms, *cyp* and *fd* are expected to be responsible for some type of oxidation between *N*-glycylclavaminic acid and clavaldehyde in the late steps of clavulanic acid biosynthesis. However, their biochemical functions are still unclear, and so the nature of their involvement remains to be determined along with that of *orf12*. *orf13* and *orf14* resemble genes encoding an acetylated amino acid export pump from *Escherichia coli* and an acetyltransferase, respectively (Mellado et al. 2002; Jensen et al. 2004a). Mutation of each gene indicated that they are both involved in clavulanic acid and 5*S* clavam production, as evidenced by the severe reduction (in the *orf13* mutant) or complete loss (in the *orf14* mutant) of the target compounds (Jensen et al. 2004a). Mellado et al. (2002) suggested that acetylated metabolites such as the *N*-acyl derivatives of clavaminic acid might be generated by Orf14, and that Orf13 might transport the acetylated clavam metabolites resulting in their excretion into the medium as described by Elson et al. (1988).

Jensen et al. (2004a) examined *orf15* (now designated *oppA2)*, *orf16* (encoding a hypothetical protein), and *orf17* (now designated *gcaS)* genes. Each of these disrupted mutants failed to produce clavulanic acid, but it was observed that *N*-acetyl-glycyl-clavaminic acid and *N*-glycyl-clavaminic acid were accumulated in both *oppA2* and *orf16* mutants. Gcas has been shown to convert clavaminic acid to *N*-glycyl-clavaminic acid, and so it was suggested that the compounds accumulated by the mutants are biosynthetic intermediates, with *N*-glycyl-clavaminic acid followed by *N*-acetyl-glycyl-clavaminic acid in the clavulanic acid pathway (Arulanantham et al. 2006). Both *orf18* and *orf19* genes encode proteins similar to penicillin binding proteins. Inactivation of the *orf19* gave no apparent effects on growth or clavulanic acid production, but *orf18* is essential for cell growth. However, in view of the fact that they are likely to encode penicillin binding proteins involved in cell wall biosynthesis, and therefore give rise to targets for β-lactam antibiotics, it was suggested they are part of the clavulanic acid gene cluster (Jensen et al. 2004a).

Recently, three additional genes viz *orf21*, *orf22*, and *orf23,* were identified to encode a putative sigma factor, a sensor kinase, and a response regulator, respectively (Jnawali et al. 2008; Song et al. 2009). *orf22* and *orf23* disrupted mutants displayed modest reductions of clavulanic acid production to different levels and overexpression of Orf23 and Orf22/Orf23 resulted in increase of clavulanic acid production compared with control cultures, suggesting that they are a two-component system that could control antibiotic production in *S. clavuligerus*. Recently, the clavulanic acid gene cluster was extended to *orf28*, but the potential involvement of the newly sequenced orfs in clavulanic acid production remains to be assessed (Jnawali et al. 2008).

Clavam gene cluster

Following the recognition that Cas1 and Cas2 exist as a pair of isozymes (Salowe et al. 1990; Marsh et al. 1992), *cas1* was cloned and sequenced and determined to reside in a location outside of the clavulanic acid gene cluster. *cas1* and *cas2* are paralogous genes that are functionally equivalent, but their transcription is regulated by different nutritional conditions (Paradkar and Jensen 1995). The loci for the two genes were shown to be separate, and DNA sequence determination of the region encompassing *cas1* led to the discovery of the clavam gene cluster surrounding *cas1* (Mosher et al. 1999). Gene disrupted mutants were constructed for each of the genes of the clavam cluster and investigated as to whether they have the ability to produce clavam metabolites including clavulanic acid by Mosher et al. (1999) and Tahlan et al. (2007). Among them, *cvm1*, *cvm2*, and *cvm5* disruptants showed altered ability to produce 5*S* clavam metabolites but normal production of clavulanic acid. Disruption of *cvm1*, which has similarity to aldo-keto reductases, caused loss of production of the 5*S* clavams (Mosher et al. 1999). Inactivation of Cvm2, similar to proteins possessing ketosteroid isomerase-like domains and involved in the degradation of steroids in bacteria, caused greatly reduced alanylclavam and 2-hydroxymethylclavam and completely eliminated clavam-2-carboxylate production (Tahlan et al. 2007). While the biochemical reactions catalyzed by Cvm1 and Cvm2 are unknown, the enzymes were found to be essential for biosynthesis of one or more of the 5*S* clavams, suggesting their involvement in the later unknown steps leading to the clavam products. Cvm5 resembles flavin-dependent monooxygenases that utilize reduced flavin provided by a flavin reductase, and Cvm3 resembles flavin reductases, suggesting that they may function in a coupled reaction. Disruption of *cvm3* caused no changes in 5*S* clavam metabolite profile; however, *cvm5* disruption abolished production of all of the known 5*S* clavams, while the novel compound, 2-carboxymethylideneclavam, was accumulated. Since production of 2-carboxymethylideneclavam is observed in the wild type, although in very small amounts, the compound was presumed to be an intermediate following clavaminic acid (Tahlan et al. 2007).

Paralog gene cluster

The existence of second copies of the genes required for the early steps of clavulanic acid biosynthesis (other than *cas1* and *cas2*) was proposed when inactivation of several seemingly essential genes in the clavulanic acid gene cluster did not result in complete loss of clavulanic acid production (Jensen et al. 2000). Subsequent studies found the second gene set, designated the paralog gene cluster, which included *ceaS1*, *bls1*, *pah1*, and *oat1* (Jensen et al.

2004b; Tahlan et al. 2004b). The *pah1* gene was discovered by cross-hybridization using a *pah2* specific probe and found to encode a protein functionally equivalent to Pah2 and 72% identical at the nucleotide level to *pah2*. *pah1* disruption showed no significant effects on production of any of the clavam metabolites including clavulanic acid; however, double mutants with defects in both *pah1* and *pah2* did not produce any clavam metabolites (Jensen et al. 2004b).

Three genes located adjacent to *pah1* also displayed 73%, 60%, and 63% identities respectively, to the *ceaS2*, *bls2*, and *oat2* genes, indicating that the products of paralogous genes are those enzymes responsible for the early shared steps of clavulanic acid and 5*S* clavam biosynthesis. Whereas mutants defective in only one copy of the paired genes showed production of clavulanic acid and 5*S* clavam metabolites to variable degrees, double mutants defective in both copies of the genes were completely blocked in clavulanic acid and 5*S* clavam metabolite biosynthesis, except for the *oat1/oat2* double mutants. These mutants produced a reduced yield of clavulanic acid and some 5*S* clavam metabolites, suggesting that Oat1/Oat2 serve only to increase arginine levels. The fact that these double mutants were not auxotrophic for arginine further suggests that ArgJ, another ornithine acetyltransferase enzyme found in *S. clavuligerus*, is of primary importance in the arginine biosynthetic pathway (de la Fuente et al. 2004; Tahlan et al. 2004b).

Genes similar to *cvm6* and *cvm7* from the clavam cluster also appeared in the paralog gene cluster where they were designated *cvm6P* and *cvm7P*. These genes encode putative aminotransferases and transcriptional regulators, respectively, but mutants defective in *cvm6* and *cvm7* had no effect on clavam metabolite production. However, mutants defective in *cvm6P* and *cvm7P* completely lost the ability to produce 5*S* clavams, suggesting that Cvm7P is a transcriptional regulator specific for 5*S* clavam metabolite biosynthesis (Tahlan et al. 2007). Therefore, the paralog gene cluster includes genes involved in the early steps common to both clavulanic and other clavam metabolite biosynthesis, but also includes genes specific for the late stages of 5*S* clavam biosynthesis (Zelyas et al. 2008). *orfA*, *orfB*, *orfC*, and *orfD* from the paralog gene cluster encode a predicted hydroxymethyltransferase, a YjgF/YER057c/UK114 family regulatory protein, an aminotransferase, and a dehydratase, respectively, all of which are similar to proteins involved in amino acid regulation or biosynthesis. Mutants with defects in these genes were unable to produce alanylclavam but had no effect on the other 5*S* clavams and clavulanic acid. Moreover, the *orfC* disruptant accumulated 8-*OH*-alanylclavam, which is proposed to be an intermediate in the alanylclavam biosynthetic pathway. Thus, the four genes in the paralog cluster appear to be involved in the reactions which specifically convert 2-hydroxymethylclavam to alanylclavam via 8-hydroxyalanylclavam (Zelyas et al. 2008).

Genetic manipulation for overproduction of clavulanic acids

Random mutagenesis and selection techniques have traditionally been used in strain improvement programs to generate production strains better suited to industrial fermentations. However, genetic engineering to construct improved strains in a targeted manner has recently become possible based on a growing body of knowledge regarding antibiotic biosynthesis and the relevant gene clusters, together with the development of techniques for genetic manipulation of the producer organisms (Paradkar et al. 2001). Our understanding of the pathway for clavulanic acid biosynthesis has largely been deduced from genetic studies performed in *S. clavuligerus*, and subsequently, this knowledge has been applied to the metabolic engineering of the species for improvement of clavulanic acid production. Most examples of clavulanic acid overproduction have resulted from manipulation of genes encoding biosynthetic enzymes or transcriptional regulators.

Amplification of biosynthetic genes encoding specific enzymes can lead to an earlier and more rational way to improve strains with higher antibiotic production. The specific amplification of *ceaS2*, encoding the first enzyme in the biosynthetic pathway resulted in increased production of clavulanic acid (Pérez-Redondo et al. 1999). In addition to effects caused by manipulation of biosynthetic genes, amplification of the gene encoding the transcriptional regulator CcaR in *S. clavuligerus* resulted in production of clavulanic acid as well as cephamycin C at levels that were increased by about threefold, whereas inactivation of CcaR gave rise to accumulation of clavaminic acid (Pérez-Llarena et al. 1997). In the case of the clavulanic acid-specific regulator, ClaR, amplification of *claR* on a multicopy plasmid showed significant enhancement in both clavulanic acid and alanylclavam production, but cephamycin C production was reduced (Pérez-Redondo et al. 1998). Recently, Hung et al. (2007) showed that clavulanic acid production was increased by combined overexpression of a positive regulator and a rate-limiting enzyme viz CcaR and Cas2. CcaR and Cas2, when integrated into the *S. clavuligerus* chromosome, induced an outstanding improvement of 23.8-fold in clavulanic acid production, which represented a greater yield than any seen previously through amplification of a single gene or use of multicopy plasmids. In addition to pathway-specific regulators, the pleiotropic regulator, AdpA has been shown to be responsible for both morphological differentiation and antibiotic production in

Streptomyces. Increased dosage of *adpA* in *S. clavuligerus* effected an enhancement of clavulanic acid production (López-García et al. 2010). Similarly, increasing the copy number of certain clavulanic acid-specific biosynthetic genes such as *cyp*, *fd*, *orf12*, and *orf14*, by introduction on multiple copy expression plasmids, resulted in positive effects on production of clavulanic acid, although the functions of the *orf*s in clavulanic acid production are still not clear (Mellado et al. 2002).

Gene replacement technologies used to prevent undesired metabolites from being produced were also applied to give overproduction of clavulanic acid while preventing production of cephamycin C. A specific deletion of the *lat* gene encoding lysine epsilon-aminotransferase, which is an essential enzyme involved in cephamycin C biosynthesis, increased clavulanic acid production 2- to 2.5-fold (Paradkar et al. 2001).

Metabolic engineering approaches to control precursor flux have also resulted in enhancement of clavulanic acid production. Li and Townsend (2006) focused on the glycolytic pathway to overcome glyceraldehyde-3-phosphate which was suggested to be a limiting factor in clavulanic acid biosynthesis. Two genes, *gap1* and *gap2*, encoding glyceraldehyde-3-phosphate dehydrogenases were inactivated by genetic engineering and consequently, a *gap1* disruptant showed twofold improvement of clavulanic acid production, suggesting that the rational engineering was achieved by glyceraldehyde-3-phosphate flux channeled to the clavulanic acid biosynthesis rather than to the glycolytic pathway. Moreover, when arginine was fed to the mutant where the intracellular arginine pool might be decreased, clavulanic acid production was elevated a further threefold over the wild type strain (Li and Townsend 2006). Overexpression of CcaR and ClaR in this *gap1* deletion mutant led to an increase in clavulanic acid and cephamycin C production. Furthermore, a greatly increased yield of the antibiotics resulted from fermentation through the feeding of ornithine and glycerol (Jnawali et al. 2010).

Baños et al. (2009) recently identified a glycerol-utilizing gene cluster (*gylR-glpF1K1D1*) in *S. clavuligerus*, and transformants carrying additional copies of the *glp* genes were improved with respect to clavulanic acid production as well as glycerol utilization. Amplification of *glpF1K1D1* and supplement of high glycerol concentrations led to 7.5-fold increased clavulanic acid production in various backgrounds including the wild type strain, a *gap1* disrupted mutant and a *relA* disrupted mutant, providing several examples of metabolic engineering approaches to construct overproducer strains.

S. clavuligerus is rightly regarded as a valuable microorganism, both industrially and clinically. The organism produces not only β-lactam antibiotics, including clavulanic acid and conventional penicillin and cephamycin compounds, but also holomycin, a member of the pyrrothine class antibiotics, and an antibiotic related to tunicamycin, a glucosamine-containing antibiotic (Kenig and Reading 1979). Recently, the draft genome sequence of *S. clavuligerus* ATCC 27064 was published, and the presence of many gene clusters for secondary metabolites, more than have been found in any of the other completed *Streptomyces* genomes, was revealed (Medema et al. 2010). The genome sequence should provide further insights to the mechanisms used by this fascinating microorganism to produce its broad array of metabolites and is likely to provide information on additional metabolic capabilities not yet recognized. Application of this genetic information to manipulation of the organism should facilitate the development of *S. clavuligerus* into an antibiotic "factory" for production of valuable metabolites.

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