



The CagRS Two-Component System Regulates Clavulanic Acid Metabolism via Multiple Pathways in *Streptomyces clavuligerus* F613-1

Jiafang Fu¹, Ronghuo Qin¹, Gongli Zong¹, Cheng Liu², Ni Kang¹, Chuanqing Zhong^{2*} and Guangxiang Cao^{1*}

¹ Shandong Medicinal Biotechnology Center, Shandong Academy of Medical Sciences, Jinan, China, ² School of Municipal and Environmental Engineering, Shandong Jianzhu University, Jinan, China

OPEN ACCESS

Edited by:

Marie-Joelle Virole,
Centre National de la Recherche
Scientifique (CNRS), France

Reviewed by:

Rigoberto Rios-Esteva,
Universidad de Antioquia, Colombia
Hung Viet Trinh,
Henry M. Jackson Foundation,
United States

*Correspondence:

Chuanqing Zhong
zhongchuanqing@sdjz.edu.cn
Guangxiang Cao
caozhong0402@163.com

Specialty section:

This article was submitted to
Microbial Physiology and Metabolism,
a section of the journal
Frontiers in Microbiology

Received: 23 September 2018

Accepted: 29 January 2019

Published: 14 February 2019

Citation:

Fu J, Qin R, Zong G, Liu C,
Kang N, Zhong C and Cao G (2019)
The CagRS Two-Component System
Regulates Clavulanic Acid Metabolism
via Multiple Pathways
in *Streptomyces clavuligerus* F613-1.
Front. Microbiol. 10:244.
doi: 10.3389/fmicb.2019.00244

Streptomyces clavuligerus F613-1 produces a clinically important β -lactamase inhibitor, clavulanic acid (CA). Although the biosynthesis pathway of CA has essentially been elucidated, the global regulatory mechanisms of CA biosynthesis remain unclear. The paired genes *cagS* and *cagR*, which are annotated, respectively, as *orf22* and *orf23* in *S. clavuligerus* ATCC 27064, encode a bacterial two-component regulatory system (TCS) and were found next to the CA biosynthetic gene cluster of *S. clavuligerus* F613-1. To further elucidate the regulatory mechanism of CA biosynthesis, the CagRS TCS was deleted from *S. clavuligerus* F613-1. Deletion of *cagRS* resulted in decreased production of CA, but the strain phenotype was not otherwise affected. Both transcriptome and ChIP-seq data revealed that, in addition to CA biosynthesis, the CagRS TCS mainly regulates genes involved in primary metabolism, such as glyceraldehyde 3-phosphate (G3P) metabolism and arginine biosynthesis. Notably, both G3P and arginine are precursors of CA. Electrophoretic mobility shift assays demonstrated that the response regulator CagR could bind to the intergenic regions of *argG*, *argC*, *oat1*, *oat2*, *ceaS1*, and *clnR* *in vitro*, suggesting that CagR can directly regulate genes involved in arginine and CA biosynthesis. This study indicated that CagRS is a pleiotropic regulator that can directly affect the biosynthesis of CA and indirectly affect CA production by regulating the metabolism of arginine and G3P. Our findings provide new insights into the regulation of CA biosynthetic pathways and provide an innovative approach for future metabolic engineering efforts for CA production in *S. clavuligerus*.

Keywords: *Streptomyces clavuligerus*, clavulanic acid, CagRS, arginine, glyceraldehyde 3-phosphate, primary metabolism

INTRODUCTION

Clavulanic acid (CA), a broad-spectrum inhibitor of beta-lactamase, is widely used clinically in combination with penicillin and cephalosporin, as it can effectively improve the antibacterial effect of β -lactam antibiotics (Saudagar et al., 2008). The unique three-dimensional structure (3R, 5R) of CA enables it to irreversibly combine with serine hydroxyl from the active center of β -lactamase, thereby inactivating this β -lactam resistance mechanism (Liras and Rodriguez-Garcia, 2000).

Streptomyces clavuligerus was first isolated and screened for the production of CA (Reading and Cole, 1977), and although *S. jumonjinensis* and *S. katsurahamanus* were also found to produce CA, *S. clavuligerus* is the major production strain for CA (Brown et al., 1976; Jensen and Paradkar, 1999). In addition to CA, *S. clavuligerus* also produces the β -lactam antibiotic cephamycin C and several compounds with a clavam structure and (3S, 5S) stereochemistry; these compounds are considered to be the main by-products of CA fermentation.

Three clusters of genes involved in CA biosynthesis have been isolated in *S. clavuligerus*: the CA biosynthetic gene cluster, the clavam gene cluster, and the paralog gene cluster (Figure 1C). Both the CA biosynthetic gene cluster and the clavam gene cluster are located on the chromosome of *S. clavuligerus*, whereas the paralog gene cluster is located on the pSCL4 plasmid (Jensen et al., 2000; Liras et al., 2008; Song et al., 2010). The CA biosynthetic gene cluster was initially isolated by hybridization with the *cas2* gene, encoding clavamate synthase, and includes the six biosynthetic enzyme-encoding genes *bls2*, *pah2*, *ceaS2*, *cas2*, *car* (also known as *cad*) and *gcaS* (Baggaley et al., 1997; Arulanantham et al., 2006); two genes, *pbpA* and *pbp2*, encoding penicillin-binding proteins (Ishida et al., 2006); the *oat2* gene encoding ornithine acetyltransferase (Hodgson et al., 1995); two genes, *oppA1* and *oppA2*, encoding oligopeptide permeases (Lorenzana et al., 2004; Mackenzie et al., 2010); gene *claR*, which encodes a LysR-type regulatory protein (Liras and Rodriguez-Garcia, 2000); and the *cyp450-fd*, *orf12*, *orf13*, *orf14*, and *orf16* genes, which are required for CA biosynthesis but are of unknown function.

The clavam gene cluster was isolated using the *cas1* gene in *S. clavuligerus*, a duplicate gene encoding a clavamate synthase isoenzyme, as probe (Mosher et al., 1999). This gene cluster contains genes *cvm1* to *cvm13*, *cvmG*, *cvmH*, *cvmP*, and *cas1*, although only *cas1* is required for CA biosynthesis (Liras et al., 2008). Additionally, although *cas1* is a homolog of *cas2* of the CA biosynthetic gene cluster, the two genes are regulated by different mechanisms (Paradkar and Jensen, 1995).

The paralog gene cluster contains the genes *ceaS1*, *bls1*, *pah1*, *oat1*, *cvm6p*, and *cvm7p* (Jensen et al., 2000, 2004a), and the first four of these genes may have been duplicated from genes of the CA biosynthetic gene cluster (Tahlan et al., 2004a,b). *ceaS1* is a homolog of *ceaS2*, with 73% similarity; *bls1* is a homolog of *bls2*, with 60% similarity; and *oat1* is a homolog of *oat2*, with 63% similarity. *pah1* encodes a protein that functions similarly to *Pah2*, and the two proteins have a sequence similarity of 72%. It has been reported that deletion of *pah1* resulted in significantly reduced production of CA as well as of 5S-clavam (Jensen et al., 2004b).

The CA biosynthesis is regulated by several mechanisms in *S. clavuligerus*, including the two pathway-specific regulatory factors *Clar* and *CcaR*. The *Clar*-encoding gene, *claR*, is located in the CA biosynthetic gene cluster, and studies have shown that *Clar* can positively regulate CA biosynthesis (Paradkar et al., 1998; Perez-Redondo et al., 1998; Gomez-Escribano et al., 2006; Martinez-Burgo et al., 2015). The *CcaR*-encoding gene, *ccaR*, is located in the cephamycin C gene cluster, and *CcaR* is reported to positively regulate the expression of genes involved in the early

stage of CA biosynthesis, such as *ceaS2*, *bls2*, *pah2*, and *cas2* (Santamarta et al., 2002, 2011; Alvarez-Alvarez et al., 2014). In addition, *CcaR* also binds the promoter of *claR* (Santamarta et al., 2011; Kurt et al., 2013), indicating that *Clar* and *CcaR* may jointly form a regulatory system to regulate the biosynthesis of CA (Kurt et al., 2013; Kwong et al., 2013).

In addition to the CA pathway-specific regulatory factors, many other factors regulate CA biosynthesis. Deletion of the γ -butyrolactone receptor protein *Brp* can result in increased CA production, and *Brp* negatively regulates the biosynthesis of CA through inhibiting *ccaR* (Santamarta et al., 2005). *BldG* is an upstream regulatory factor that can regulate CA biosynthesis by regulating *ccaR* expression (Bignell et al., 2005), and the sigma factor encoded by *orf21* can bind to the *ccaR* promoter region and thereby also influence CA biosynthesis (Jnawali et al., 2011). In addition, CA biosynthesis was reported to be negatively regulated by *ccaR* and *claR* when amino acids are scarce (Gomez-Escribano et al., 2008).

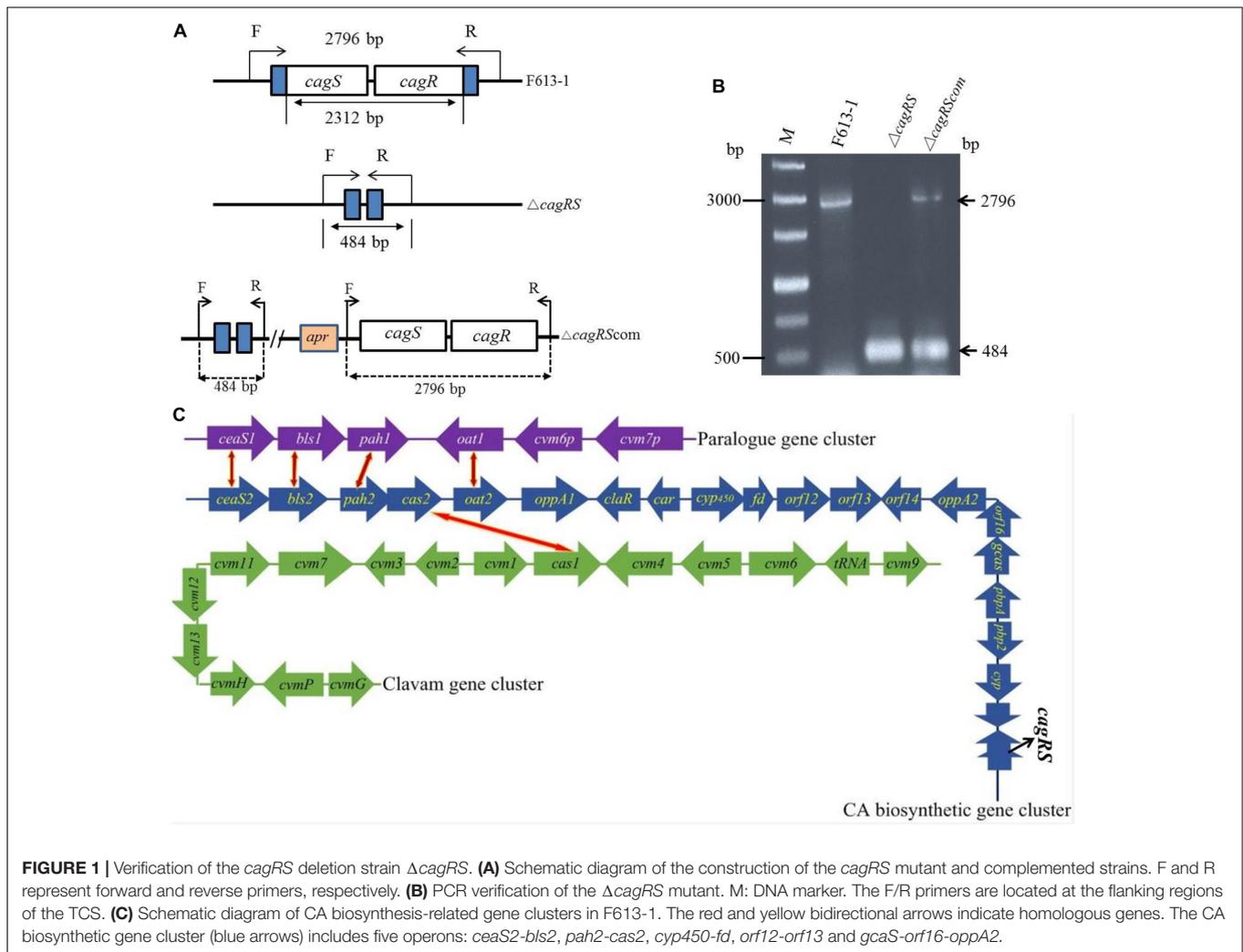
Enhancement of CA production is a very important goal for the commercial pharmaceutical market. Generally, there are two main ways to increase CA production (Paradkar et al., 2001; Jnawali et al., 2010): (1) optimize the medium and conditions for CA fermentation, and (2) clarify and optimize the biosynthetic and regulatory mechanisms of CA production. The biosynthesis pathway of CA and its related by-product (clavam) has been partially elucidated (Liras et al., 2008). However, the mechanisms regulating CA biosynthesis have not been fully delineated, and there are no reports about the global regulation of CA biosynthesis. Therefore, in addition to manipulating genes encoding regulatory factors (such as *claR*, *ccaR*) and known essential biosynthetic enzymes (such as *bls2*, *pah2*, *ceaS2*, *cas2*, *car*, and *gcaS*), elucidation of the functions of other genes responsible for as yet unknown but essential roles in CA biosynthesis may suggest other ways to increase production of CA.

Two-component systems (TCSs) are normally organized as pairs on bacterial genomes, with co-transcription of the response regulator and histidine kinase genes. TCSs not only respond to changes in environmental factors, but in *Streptomyces*, also influence development and secondary metabolism such as antibiotic production (Mendes et al., 2007). *S. clavuligerus* F613-1 is an industrial CA producer strain, and we have previously reported the complete genome sequence of this strain (Cao et al., 2016). In this study, we identified TCS *CagRS*, which is annotated as *orf22/orf23* in *S. clavuligerus* 27064 (Song et al., 2009) and which is close to the CA biosynthetic gene cluster in F613-1. We investigated the effects of TCS *CagRS* on CA production in *S. clavuligerus* F613-1, and our results provide insights into new approaches for improving CA yield.

MATERIALS AND METHODS

Plasmids and Bacterial Strains

All strains and plasmids used in this study are listed in **Supplementary Table S1**. *S. clavuligerus* F613-1 is an industrial strain (Jin et al., 2015) and was used as the parental



strain in this study. Cloning procedures were performed in *Escherichia coli* DH5a, protein expression was performed using *E. coli* BL21(DE3), and *E. coli* ET12567/pUZ8002 was used for intergeneric conjugative transfer of plasmid DNA into *S. clavuligerus* (Kieser et al., 2000).

Primers

All primers used in the construction of the $\Delta cagRS$ deletion mutant strain and complemented strain, construction of a CagR-3 × FLAG-complemented *S. clavuligerus* strain, confirmation of conjugants, and in EMSAs and real-time PCR analysis are listed in **Supplementary Table S2**.

Culture Conditions

Escherichia coli strains were grown at 37°C in LB medium or on solid LB plates supplemented with ampicillin (100 μg/mL), kanamycin (25 μg/mL), or chloramphenicol (25 μg/mL) when required.

Culturing of *S. clavuligerus* F613-1 and the strains derived from it was performed as described previously (Qin et al., 2017). *S. clavuligerus* transformants were cultured on MS plates

(2.0% soybean powder, 2% glycerol, and 2.0% agar powder, pH 7.3) supplemented with nalidixic acid (25 mg/mL) and thiostrepton (15 mg/mL) to select pJTU1278-derived plasmids, and the disrupted mutants were selected with antibiotic-free MS solid media or with apramycin (15 mg/mL) to select pSET152-derived plasmids.

Streptomyces clavuligerus F613-1 and its derived strains were grown at 25°C with a relative humidity of 50-60% on BSCA plates (1.5% malt extract, 0.3% tryptone, 0.4% glucose, and 2.0% agar powder, pH 7.5) for 8 days for collection of spores. For bioassay analysis, F613-1 and derived strains were grown at 25°C with a relative humidity of 50-60% on BSCA plates for 3-9 days; on MSF plates (2.0% soybean powder, 2% mannitol, and 2.0% agar powder, pH 7.3) for 5-11 days; on MM solid media (0.05% L-asparagine, 0.05% dipotassium hydrogen phosphate, 0.02% MgSO₄·7H₂O, 0.001% FeSO₄·7H₂O, 1% dextrose monohydrate, and 2.0% agar powder, pH 7.2) for 9-15 days; or on ISP4 solid media (1% soluble starch, 0.1% dipotassium hydrogen phosphate, 0.1% MgSO₄·7H₂O, 0.1% NaCl, 0.2% ammonium sulfate, 0.2% CaCO₃, 0.0001% FeSO₄·7H₂O, 0.0001% MnCl₂·7H₂O, and 2.0% agar powder, pH 7.2) for 7-13 days.

For the liquid-state fermentation, spores (2×10^6) of the *S. clavuligerus* strains were inoculated into 100 mL SCZ seed medium [2.0% soybean powder, 1.2% maize starch, 0.5% yeast extract, 0.08% dipotassium hydrogen phosphate, and 1.1% (v/v) glycerol trioleate, pH 7.1), and then cultured at 25°C, with shaking on an orbital shaker at 200 rpm for 48 h to obtain seed cultures. Next, 5 mL seed cultures were transferred to 100 mL SCF fermentation medium (2.7% soybean powder, 2.2% soybean protein extract, 3.0% maltodextrin, 0.15% potassium chloride, 0.1% magnesium chloride hexahydrate, 0.2% dipotassium hydrogen phosphate, 0.04% calcium chloride dihydrate, 0.008% ferric chloride hexahydrate, 0.001% zinc chloride, 0.018% sodium chloride, and 4.2% MOPS, pH 7.1) supplemented with or without 1.6% (v/v) glycerol trioleate and grown at 25°C, with shaking on an orbital shaker at 200 rpm. For HPLC analysis of CA, 1 mL samples of fermentation liquid were collected at 24, 72, 120, 168, and 216 h and centrifuged at 5000 rpm to collect the supernatant. In addition, the biomass was measured before the analysis of CA production, using 1 g samples of fermentation liquid centrifuged at 5000 rpm to collect the mycelium.

DNA Manipulation

Genomic DNA from *S. clavuligerus* was isolated using the Kirby mix procedure (Kieser et al., 2000). Plasmids were extracted from *E. coli* using plasmid mini-prep columns (BioTeke, China) according to the manufacturer's protocol. Restriction endonuclease digestions of plasmid DNA were carried out according to the manufacturer's recommendations. DNA ligation was performed using Solution I (TaKaRa, Japan) according to the manufacturer's recommendations.

Construction and Complementation of a *cagRS* Null Mutant

The *cagRS* genes were knocked out through homologous recombination using a strategy similar to that described (Gust et al., 2003; Zhang et al., 2014). The DNA fragment serving as the left arm of *cagRS* was amplified by PCR using primers *cagRS* L-F/R and *S. clavuligerus* F613-1 genomic DNA as template, and then the amplified PCR products were cloned into the general cloning vector pEasy-Blunt-Simple to obtain the recombinant plasmid pEBS-*cagRS* L. The DNA fragment serving as the right arm of *cagRS* was amplified by PCR using primers *cagRS* R-F/R, and then the amplified PCR products were cloned into the general cloning vector pEasy-Blunt-Simple to obtain the recombinant plasmid pEBS-*cagRS* R. After verification by DNA sequencing, both pEBS-*cagRS* L and pEBS-*cagRS* R were digested with *Hind*III and *Bam*HI, and the digested right arm of *cagRS* was cloned into the digested recombinant plasmid pEBS-*cagRS* L to obtain the recombinant plasmid pEBS-*cagRS* L/R. After verification by DNA sequencing, the *cagRS* L/R fragment was excised from pEBS-*cagRS* L/R using *Spe*I and *Bam*HI and then cloned into plasmid pJTU1278 (also digested with *Spe*I and *Bam*HI) to obtain the recombinant plasmid pJTU-*cagRS*.

Deletion of *cagRS* was performed by double recombination between the *S. clavuligerus* F613-1 genome and the pJTU1278-derived plasmid pJTU-*cagRS*, resulting in the knockout of the *cagRS* genes (Figure 1). Conjugation was performed using *S. clavuligerus* F613-1 and *E. coli* ET12567/pUZ8002 as described (Sambrook et al., 1989; Kieser et al., 2000). The Δ *cagRS* mutant strain was confirmed by PCR.

For complementation, *cagRS* was amplified with the primers CagRS com-F and CagRS com-R, generating a fragment carrying the coding sequence and the *cagRS* promoter, and cloned into *Nde*I/*Xba*I-cut pSET152 to create pSET-*cagRS*. The plasmids pSET152 and pSET-*cagRS* were introduced individually into the Δ *cagRS* mutant by conjugation.

Construction of a CagR-3 × Flag-Complemented *S. clavuligerus* Strain

To engineer an *S. clavuligerus* strain expressing CagR with a C-terminal, triple-Flag tag (DYKDHDGDYKDHDIDYKDDDDK), the pSET152-derived construct pSET-*cagR*Flag was created via assembly of multiple DNA fragments using the Gibson Assembly Cloning Kit (New England BioLabs). To avoid folding of the 3 × Flag tag into the inside of the CagR protein, the [Gly₄Ser]₃ linker (GGGGSGGGGSGGGGS) (Bush et al., 2013) was inserted between the 3 × Flag tag and the coding region of *cagR*. The flow chart for construction of the recombinant plasmid pSET-*cagR*Flag is shown in Supplementary Figure S1. The recombinant plasmid pSET-*cagR*Flag was confirmed by DNA sequencing, and then pSET-*cagR*Flag was introduced into the Δ *cagRS* mutant by conjugation, and its ability to restore CA production was assessed during liquid-state fermentation.

Bioassay and HPLC Analysis of CA Production

The concentration of CA was analyzed by bioassay analysis and HPLC analysis. The indicator *E. coli* strain MA18 was spread on LB solid medium supplemented with 100 μg/mL ampicillin (Jones et al., 2005). Briefly, for the bioassay analysis, *S. clavuligerus* strains were grown on BSCA plates for 3–9 days, on MSF solid media for 5–11 days, on MM solid media for 9–15 days, or on ISP4 solid media for 7–13 days, and then the corresponding agar blocks were excavated with a 6 mm punch and transferred onto the LB agar plates. The diameter of the inhibition zone was gauged after overnight culturing at 37°C. The concentration of CA during the liquid-state fermentation was detected by HPLC with an Inertsil ODS-3 4.6 mm × 150 mm, 5 μm column (Jin et al., 2015; Qin et al., 2017), using clavulanate lithium (provided by Lunan Pharmaceutical Co.) as the standard for quantification. For bioassay and HPLC analysis of CA production, the experiments were conducted in triplicate. Statistical analysis was performed using SPSS Statistics V19.0 software.

Transcriptome Sequencing and Analysis

For transcriptome sequencing and analysis, mycelium of *S. clavuligerus* F613-1 and its derivative Δ *cagRS* were harvested

from fermentation liquid at 72 h and rapidly frozen in liquid nitrogen. Total RNA of F613-1 and $\Delta cagRS$ was purified using an RNA extraction kit (SBSBIO, Beijing China) according to the manufacturer's protocol. The extracted total RNA samples were then treated with RNase-free DNase I (Invitrogen) twice according to the recommended protocols to remove the residual chromosomal DNA. Two sets of RNA for both strains were prepared separately. The integrity of total RNA was determined using a NanoDrop One C (Thermo Fisher Scientific), and the RNA Integrity Number value of each sample met the standard required for preparing a cDNA library. The cDNA libraries were prepared according to the manufacturer's instructions (Illumina). Briefly, the ribosomal RNA in 1–4 μg total RNA was removed using Ribo-zero rRNA Removal solution (Illumina), leaving only mRNA. The mRNA was then fragmented, to an average fragment length of about 200 nt, and reverse transcribed into single-stranded cDNA using random hexamer priming. When the second cDNA strand was synthesized, the dTTP was replaced by dUTP. The cDNA fragments with a single 'A' base overhang at their 3'-ends were obtained after end-repair and 3'-adenylation. Adapters were then ligated to the ends of the cDNA fragments. Fifteen rounds of PCR amplification were performed to enrich the adapter-modified cDNA library using primers complementary to the ends of the adapters, and PCR products were purified using Ampure XP beads (Agencourt). The ready-to-sequence Illumina library was quantified as previously described (Zhang et al., 2017).

The transcriptome was sequenced using a HiSeq 3000 sequencer (Illumina) at RibBio Corporation (Shenzhen, China). A paired-end, 2×150 bp sequencing strategy was used, and more than 1 Gb data was obtained for each sample. The sequencing generated 6 files of reads corresponding to F613-1 and $\Delta cagRS$, with three replicates for each. After filtering the raw data, removing the linker sequences, low-quality reads and the residual rRNA sequences, the remaining data were marked as the effective reads. The effective reads could be obtained using `fastx_clean`¹, a home-made software package based on the FASTX toolkit² and SortMeRNA (excludes the ribosomal RNA-like reads) (Kopylova et al., 2012). The effective reads were used for subsequent genome comparisons, and the distribution of reads compared to the genome was statistically analyzed. For $\Delta cagRS$, 93.58% of effective reads were distributed in ORFs (open reading frames), and 6.42% in intergenic regions. For F613-1, 95.18% of effective reads were distributed in exonic regions, and 4.82% in intergenic regions. The expression level of each gene was normalized by the number of reads per kilobase of transcriptome per million mapped reads (RPKM). The differentially expressed genes were selected using the Audics program with parameters of $|\log_2\text{FoldChange}| > 1$ and $q\text{-value} < 0.001$. The transcriptome data has been deposited in the Gene Expression Omnibus database³. The accession number is GSE119208.

¹<http://www.genoscope.cns.fr/fastxtend>

²http://hannonlab.cshl.edu/fastx_toolkit/

³<http://www.ncbi.nlm.nih.gov/geo>

Real-Time Quantitative PCR Analysis

Total RNA isolation and real-time quantitative PCR (RT-qPCR) procedures were performed as described previously (Fu et al., 2017). Mycelium of *S. clavuligerus* F613-1 and $\Delta cagRS$ were harvested from fermentation liquid at 24, 72, 120, 168, and 216 h, rapidly frozen in liquid nitrogen and then total RNA was extracted using an RNA extraction kit (SBSBIO, Beijing China) according to the manufacturer's protocol. Total RNA samples were treated with Turbo DNA-free reagents (Ambion, United States) to remove the residual chromosomal DNA. The cDNAs were synthesized using random hexamer primers (pdN6, Amersham Pharmacia Biotech, England), M-MLV reverse transcriptase (Invitrogen, England) and dNTPs (Roche, Switzerland). Real-time PCR assays were performed on the Roche LightCycler 480 using SYBR Green Mix (ToYoBO, Osaka, Japan). Relative quantities of cDNA were normalized to the amounts of 16S rRNA. For RT-qPCR assays, experiments were conducted in triplicate. Statistical analysis was performed using SPSS Statistics V19.0 software.

Chromatin Immunoprecipitation and DNA Sequencing Assay (ChIP-Seq Assay)

Chromatin immunoprecipitation assay was performed as previously described (Bush et al., 2013). *S. clavuligerus* CagR-Flag ($\Delta cagRS:: cagR\text{-}[\text{Gly}_4\text{Ser}]_3\text{-}3^*\text{Flag-cagS}$) was grown in four 50-mL SCF at 25°C for 72 h. Formaldehyde was added to cultures at a final concentration of 1% (vol/vol) for 30 min. Glycine was then added to a final concentration of 125 mM to stop the cross-linking. The samples were left at room temperature for 5 min and washed twice in PBS (phosphate-buffered saline, pH 7.4) buffer. The pellets were resuspended in 5 mL of ChIP-lysis buffer [10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 15 mg/mL lysozyme] and incubated at 37°C for 30 min or until lysed. Then, 5 mL ChIP-IP buffer (50 mM Tris-Cl, pH 8.0, 250 mM NaCl, 0.8% [vol/vol] Triton X-100 and 1 mM PMSF) was added, and the samples were chilled on ice. Sonication was performed at a high-power setting for 40 cycles (10 s on and 10 s off) using an ultrasonic processor (FS-250N, Shengxi Ultrasonic Instrument Co., Ltd., Shanghai) to shear chromosomal DNA into fragments ranging from 200 to 500 bp on average. The samples were centrifuged at 12,000 rpm at 4°C for 10 min to collect the supernatant, after which 50 μL of each was set aside for total DNA extraction (input). Next, 40 μL of IgG-agarose (Sigma-Aldrich, A0919) was washed according to the manufacturer's instructions and added to the remaining lysates. The mixtures were then incubated on a rotating wheel at 4°C for 2 h, and then the samples were centrifuged at 5,500 g at 4°C for 30 s to collect the supernatants. Next, the 40 μL anti-FLAG M2 Affinity Gel (Sigma-Aldrich, A2220) was washed according to the manufacturer's instructions and added to the above supernatants. The mixtures were then incubated on a rotating wheel at 4°C overnight. The samples were centrifuged at 5,500 g at 4°C for 30 s, and the pellets were washed twice with $0.5 \times$ ChIP-IP buffer and then twice with $1 \times$ ChIP-IP buffer and transferred

to new tubes after the first washing step. The pellets and 50 μ L of total cell extracts (set aside earlier) were eluted overnight at 65°C in 100 μ L of ChIP-IP elution buffer (50 mM Tris-HCl, pH 7.6, 10 mM EDTA and 1% SDS) to reverse the cross-links. The samples were centrifuged at 12,000 rpm for 5 min to remove the beads. The pellets were extracted with 50 μ L of TE buffer (10 mM Tris-Cl, pH 7.4 and 1 mM EDTA) and incubated with 0.2 mg/mL proteinase K (Sigma) and 20 μ g/mL RNase A (Sigma) for 2 h at 55°C. The samples were extracted twice with phenol-chloroform and once with chloroform and further purified using Bioteke columns (Bioteke, Beijing). DNA was quantified using a Nano-Drop spectrophotometer (Thermo Scientific).

ChIP-seq libraries were prepared and sequenced on a HiSeq 2500 sequencer (Illumina, Novogene) by Novogene Science and Technology Co., Ltd. BigWig files were generated from the alignment for visualization purposes (Kent et al., 2010). MACS2 (Model-based Analysis of ChIP-seq) was used to identify peaks using a p -value ≤ 0.005 (Zhang et al., 2008). To identify possible binding motifs of the CagR DNA-binding response regulator, the ChIP peak sequences were analyzed by Dreme software (Bailey and Elkan, 1994; Bailey, 2011).

Overexpression and Purification of His-Tagged CagR

The *cagR* gene of *S. clavuligerus* F613-1 was amplified using primer pairs *cagR*His-F/R (Supplementary Table S2) and then inserted into the pMD18T vector to generate the intermediate recombinant plasmid pMD18T-*cagR*. After confirmation by DNA sequencing, *cagR* from pMD18T-*cagR* was cloned into the pET-15b expression vector, producing the recombinant plasmid pET-*cagR*. Finally, pET-*cagR* was introduced into *E. coli* BL21(DE3) for protein expression. His-tagged CagR protein was induced and purified as previously described (Fu et al., 2017). The purity of His-tagged CagR protein was determined on a 10% SDS-PAGE gel.

Electrophoretic Mobility Shift Assays (EMSAs)

PCR was used to amplify 150–300 bp fragments from the intergenic regions of genes using genomic DNA of F613-1 as template (primers are listed in Supplementary Table S2). The amplified DNA fragments were labeled at the 3'-end with biotin-11-UTP using the Biotin 3' End DNA Labeling kit (Thermo Scientific), according to the manufacturer's instruction. Non-specific cold probes (PolydI/dC) were added to control reaction mixtures as competitors. EMSAs were carried out as described previously (Zhang et al., 2015).

RESULTS

Deletion of *cagRS* Results in Decreased Production of CA

Streptomyces clavuligerus F613-1 is an industrial CA producer, and its complete genome sequence has been reported (Cao et al., 2016). The putative TCS CagRS is located near the CA

biosynthetic gene cluster in F613-1 (Figure 1C). To characterize the function of this TCS, a *cagRS* null mutant of F613-1 was constructed, and a complemented strain, Δ *cagRS*com, was also constructed by expressing *cagRS* *in trans* in the ϕ C31 integration site in Δ *cagRS* (Figure 1A). Deletion and complementation were confirmed by PCR (Figure 1B).

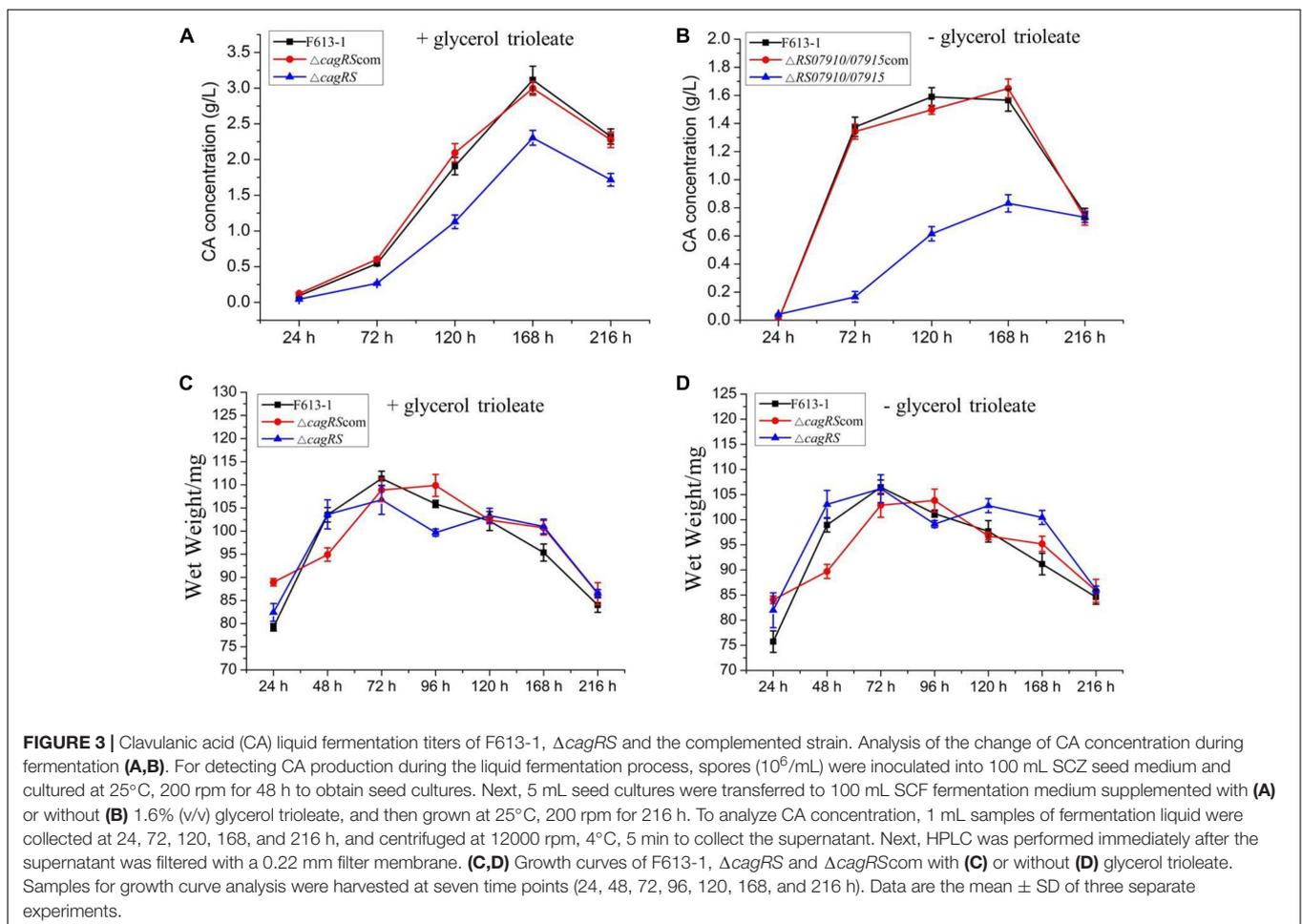
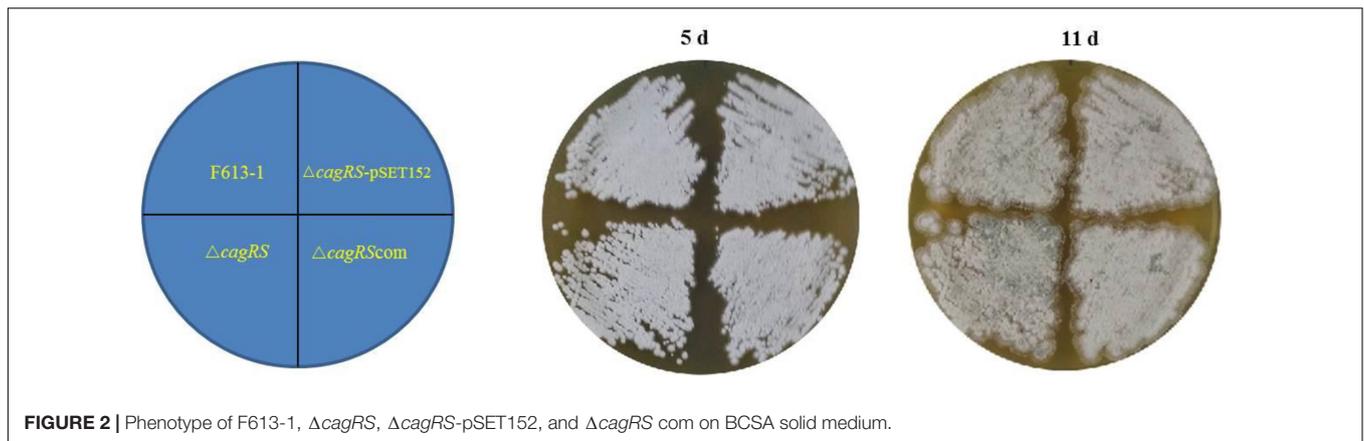
On different media (BSCA, MSE, MM, and ISP4), no growth or phenotypic differences were noted for Δ *cagRS* compared with the parental strain F613-1 (Figure 2). Interestingly, a bioassay revealed that deletion of *cagRS* resulted in a modest reduction in CA biosynthesis, as indicated by the smaller diameter of the inhibition zone produced by Δ *cagRS* compared to that of the wild-type strain F613-1 (data not shown).

To examine the effect of CagRS on CA production in more detail, an HPLC assay was performed to quantitatively analyze the production of CA in Δ *cagRS* and F613-1. No differences were detected in the biomasses of Δ *cagRS* and F613-1 (Figures 3C,D). However, the CA concentration produced by Δ *cagRS* in fermentation medium supplemented with glycerol trioleate was decreased by 50.8% ($t = 41, 63, P < 0.05$) at 72 h, 40.9% ($t = 69, 103, P < 0.05$) at 120 h, 26.0% ($t = 122, 190, P < 0.05$) at 168 h, and by 26.1% ($t = 109, 125, P < 0.05$) at 216 h, when compared with production by F613-1 (Figure 3A). It was reported that the addition of glycerol trioleate could enhance CA production in *S. clavuligerus* NRRL 3585 (Kim et al., 2009). In this study, when compared with F613-1 levels, the CA concentrations produced by Δ *cagRS* in fermentation medium without glycerol trioleate were decreased by 87.9% ($t = 32, 75, P < 0.05$) at 72 h, 61.3% ($t = 50, 101, P < 0.05$) at 120 h, and 46.9% ($t = 58, 99, P < 0.05$) at 168 h (Figure 3B). The results revealed that CA production in Δ *cagRS* is decreased when compared with F613-1 whether or not glycerol trioleate is present in the fermentation medium, consistent with the bioassay results. Additionally, CA production by Δ *cagRS* dropped more markedly in the absence of glycerol trioleate supplementation, indicating that *cagRS* may affect metabolic processing of the CA direct precursor glyceraldehyde 3-phosphate (G3P).

Δ *cagRS* was almost fully complemented by a wild-type copy of *cagRS* introduced into the ϕ C31 integration site (Figures 2, 3), suggesting that the TCS CagRS contributes to regulation of CA production, although it does not affect the phenotype of *S. clavuligerus* F613-1.

Transcriptome Analysis Revealed That *cagRS* Regulates CA Production via Multiple Pathways

To gain further insights into the mechanisms by which CagRS regulates CA biosynthesis, transcriptome analysis was performed. Transcriptome sequencing revealed that 5943 genes were expressed in F613-1 under the test conditions, and 5843 genes were expressed in Δ *cagRS*. Statistical analysis showed that 2036 genes showed significant differences in expression (\log_2 FC > 1.0) between Δ *cagRS* and F613-1 during fermentation, with 1286 of these genes upregulated in Δ *cagRS*. The differentially expressed genes were further analyzed by Gene Ontology



(GO) term enrichment analysis⁴ and KEGG pathway analysis⁵. Significant enrichment in GO terms was found mainly in the categories of “metabolic process,” “oxidation-reduction process,” and “regulation of transcription” (**Supplementary Figure S3A**). KEGG pathway analysis revealed that many of the differentially

expressed genes are involved in fatty acid biosynthesis, pyruvate metabolism, arginine biosynthesis and carbon metabolism (**Supplementary Figure S3B**). RT-qPCR was also performed to validate the transcriptome results, and the results for the tested genes were in general agreement with the transcriptome results except for *oat2* (**Table 1**).

Arginine and G3P are two direct precursors of CA (Townsend and Meng-Fei, 1985; Khaleeli et al., 1999), and therefore, arginine

⁴<http://amigo.geneontology.org>

⁵<https://www.genome.jp/kegg/pathway.html>

and G3P metabolism may affect CA production. As shown in **Table 1**, the expression levels of all of the genes in the arginine biosynthetic gene cluster increased significantly in $\Delta cagRS$ compared with levels in F613-1, and the expression levels of many genes involved in G3P metabolism also changed notably in $\Delta cagRS$ compared with F613-1, suggesting that CagRS regulates arginine and G3P metabolism. In addition, the expression levels of *ceaS2*, *oat2*, *car*, and *orf14*, genes related to CA biosynthesis, were significantly changed in $\Delta cagRS$ when compared with F613-1 levels, suggesting that CagRS regulates the CA biosynthetic

gene cluster. The above transcriptome data indicate that the TCS CagRS may affect CA production both directly, through the CA biosynthetic gene cluster, and indirectly, by affecting arginine and G3P metabolism.

ChIP-Seq Analysis of the *in vivo* Targets of the Response Regulator CagR

In order to further determine which genes are directly regulated *in vivo* by CagRS in *S. clavuligerus* F613-1, ChIP-seq analysis

TABLE 1 | Differential expression of genes associated with G3P and arginine metabolism and CA biosynthesis in $\Delta cagRS$.

Gene ID in ATCC 27064	Gene ID in F613-1	Fold change transcriptome ^c	Fold change RT-qPCR ^d	Product	Pathway	p-value
Genes involved in G3P and arginine metabolism						
SCLAV_1867 ^a (<i>gpmA1</i>)	BB341_RS18990 ^b	0.17	0.39	Phosphoglycerate mutase	Glycolysis	0
SCLAV_1059(<i>fucA</i>)	BB341_RS22720	2.00	3.73	Class II aldolase family protein	Glycolysis	6.66E-16
SCLAV_2648	BB341_RS15270	2.06	4.12	Class II fructose-bisphosphate aldolase	Glycolysis	1.11E-15
SCLAV_0289	BB341_RS26335	0.49	0.33	ROK family protein	Glycolysis	1.30E-39
SCLAV_3958	BB341_RS21415	0.46	0.24	Glucokinase	Glycolysis	1.66E-70
SCLAV_1613(<i>aceE</i>)	BB341_RS13605	0.33	0.45	Pyruvate dehydrogenase E1	Glycolysis	0
SCLAV_4928(<i>poxB</i>)	BB341_RS21120	0.46	0.53	Pyruvate dehydrogenase	Glycolysis	0
SCLAV_5509	BB341_RS01000 (<i>gap2</i>)	0.29	0.37	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	0
SCLAV_0879	BB341_RS23575	0.29	0.31	Glycerol-3-phosphate dehydrogenase/oxidase	Glycerol metabolism	9.96E-184
SCLAV_0228	BB341_RS26625	51.98	9.94	Acyl-CoA dehydrogenase	Fatty acid degradation	6.66E-16
SCLAV_2974(<i>paaH</i>)	BB341_RS03665(<i>paaH</i>)	2.99	2.08	3-hydroxybutyryl-CoA dehydrogenase	Fatty acid degradation	6.66E-16
SCLAV_4820	BB341_RS04820	11.89	8.69	Acyl-CoA dehydrogenase	Fatty acid degradation	4.44E-16
SCLAV_4816(<i>echA5</i>)	BB341_RS04840	3.10	2.16	Enoyl-CoA hydratase	Fatty acid degradation	2.22E-16
SCLAV_0801(<i>argC</i>)	BB341_RS23945 (<i>argC</i>)	9.61	2.12	<i>N</i> -acetyl-gamma-glutamyl-phosphate reductase	Arginine synthesis	1.11E-15
SCLAV_0800(<i>argJ</i>)	BB341_RS23950 (<i>argJ</i>)	7.33	6.45	Bifunctional ornithine	Arginine synthesis	1.11E-15
SCLAV_0799(<i>argB</i>)	BB341_RS23955 (<i>argB</i>)	6.74	5.48	Acetylglutamate kinase	Arginine synthesis	1.11E-15
SCLAV_0798(<i>argD</i>)	BB341_RS23960 (<i>argD</i>)	5.33	5.67	Acetylornithine aminotransferase	Arginine synthesis	1.11E-15
SCLAV_0797	BB341_RS23965 (<i>argR</i>)	5.04	5.49	Arginine repressor	Arginine synthesis	1.11E-15
SCLAV_0796	BB341_RS23970 (<i>argG</i>)	3.47	2.63	Argininosuccinate synthase	Arginine synthesis	1.11E-15
SCLAV_0795	BB341_RS23975 (<i>argH</i>)	4.70	3.47	Argininosuccinate lyase	Arginine synthesis	1.11E-15
Genes involved in CA biosynthesis						
SCLAV_4185	BB341_RS07870 (<i>orf14</i>)	0.40	0.34	GNAT family acetyltransferase	Biosynthesis of CA	0
SCLAV_4190	BB341_RS07845 (<i>car</i>)	0.35	0.48	Oxidoreductase	Biosynthesis of CA	0
SCLAV_4193(<i>oat2</i>)	BB341_RS07830 (<i>oat2</i>)	2.81	0.89	Ornithine acetyltransferase	Biosynthesis of CA	1.11E-15
SCLAV_4197	BB341_RS07810 (<i>ceaS2</i>)	0.40	0.7	<i>N</i> (2)-(2-carboxyethyl)arginine synthase	Biosynthesis of CA	0
SCLAV_0471(<i>avaA2</i>)	BB341_RS25520(<i>avaA2</i>)	2.60	5.57	Gamma-butyrolactone biosynthesis protein	Regulation of CA	2.17E-13

^alocus_tag in *Streptomyces clavuligerus* ATCC 27064. ^blocus_tag in *Streptomyces clavuligerus* F613-1. ^cResults show fold change in $\Delta cagRS$ compared with F613-1 as determined by transcriptome analysis. ^dResults show fold change $\Delta cagRS$ compared with F613-1 as determined by RT-qPCR. Red lettering indicates down-regulated genes. Green lettering indicates up-regulated genes.

was conducted. Strain CagR-Flag was first constructed, which lacks CagRS at its native locus but expresses CagR and a C-terminal, triple Flag-tagged version of CagR. No growth or phenotypic differences were noted for strain CagR-Flag when compared with F613-1 and $\Delta cagRS$, and the CA concentration of strain CagR-Flag was similar to F613-1 levels (**Supplementary Figure S2A**). Furthermore, CagR-[Gly4Ser]-3 \times Flag was readily detected using anti-Flag antibody in western blot assays, without visible cross-reaction with any other protein, and the highest peak of CagR-[Gly4Ser]-3 \times Flag expression emerged at 72 h during fermentation (**Supplementary Figure S2B**), suggesting optimal conditions for clean ChIP-seq experiments. Therefore, the mycelium of strain CagR-Flag was collected at 72 h during fermentation, and the ChIP-seq assay was conducted with Anti-FLAG M2 affinity gel. In addition, the total (non-immunoprecipitated) input DNA was used as a negative control.

The average DNA fragment sizes for the input and anti-Flag ChIP libraries were 294 and 253 bp, respectively. The input library had 12.74 million reads, and the Flag antibody ChIP library had 17.26 million reads. Over 95% of the reads were mapped to the *S. clavuligerus* F613-1 genome. The locations of the enriched peaks identified by the MACS2 program in the *S. clavuligerus* F613-1 genome are presented in a **Supplemental Table (Additional File S2A)**. All 162 enriched regions were mapped to previously annotated genes in *S. clavuligerus* F613-1. Of those 162 CagR ChIP-seq targets, 22 targets are transcriptional regulators, and 41 targets are hypothetical proteins. In addition, eight targets of CagR are potentially involved in CA biosynthesis (**Table 2** and **Figure 4B**): two genes (*cyp450* and *claR*) located in the CA biosynthetic gene cluster; the *BB341_RS25520* (*avaA2*) gene, as it encodes a gamma-butyrolactone biosynthesis protein and the γ -butyrolactone signaling system affects CA biosynthesis (Santamarta et al., 2005); *pgk*, encoding phosphoglycerate kinase; *BB341_RS07030*, *BB341_RS26625* and *BB341_RS03665* (*paaH*), which are involved in fatty acid degradation; and *BB341_RS20995* (*glnA3*), a gene encoding glutamine synthetase, which is involved in arginine biosynthesis. Moreover, transcriptome analysis revealed that the expression level of the *BB341_RS25520* (*avaA2*), *BB341_RS26625* and *BB341_RS03665* (*paaH*) genes increased significantly in $\Delta cagRS$ compared with F613-1 (**Table 1**).

Statistical analysis showed that the 162 enriched peaks were concentrated near transcriptional start sites, suggesting that CagR is a transcriptional regulatory protein (**Figure 4A**). Using the motif discovery algorithm DREME (Discriminative Regular Expression Motif Elicitation) (Bailey, 2011), the most significantly enriched motif within the peak regions was CGCNGCCG (P -value of $3.3e-10$ and an E -value of $3.9e-009$) (**Figure 4C**). Approximately 78% of the potential CagR targets had a CGCNGCCG motif correlating closely with the position of the ChIP-seq peak. Bioinformatics analysis found that the CGCNGCCG motif is also present in almost all homologous genes, i.e., the potential CagR target genes, in *S. clavuligerus* ATCC 27064. Of the 162 CagR ChIP-seq targets, approximately 35% of the associated genes showed a greater than twofold change in expression when comparing $\Delta cagRS$ to F613-1 in transcriptome analysis (**Additional File S2B**). Strikingly, 21% of the genes identified by ChIP-seq as CagR targets were upregulated

significantly, and 16% were downregulated significantly, in $\Delta cagRS$. These findings suggest that CagR is bifunctional, working almost equally as an activator and as a repressor to control differentiation in *S. clavuligerus*.

cagRS Regulates the Biosynthesis of Arginine, a Direct Precursor of CA

Arginine is one of the direct precursors for CA biosynthesis (Liras et al., 2008). Seven genes (*argH*, *argG*, *argR*, *argD*, *argB*, *argJ*, and *argC*) involved in the biosynthesis of arginine are located in the arginine biosynthetic gene cluster, in which *argR*, *argD*, and *argB* form an operon (**Figure 5A**). Transcriptome and RT-qPCR analyses revealed that the expression of the gene cluster increased significantly in $\Delta cagRS$ compared with F613-1 (**Table 1**). As deletion of CagRS was associated with activation of these seven consecutive genes, we speculated that CagR may interact directly with one or more of the intergenic regions in the arginine gene cluster. Therefore, we amplified five intergenic regions, i.e., the intergenic regions of *argB*, *argC*, *argJ*, *argH*, and *argG*, to use as probes in EMSAs. Obvious shifting was only observed with the intergenic region probes for *argG* and *argC* (**Figure 5B** and **Supplementary Figure S5**), and notably, these two intergenic regions contain sequences identical (*argG*) or highly similar (*argC*) to the conserved CGCNGCCG motif sequence (**Figure 5C**), consistent with the binding of CagR to these intergenic regions. However, we did not detect interactions between CagR and the intergenic region probes for the *argB-D-R* operon, *argJ* or *argH*, although the transcription levels of these genes also increased significantly in the $\Delta cagRS$ strain.

cagRS May Regulate G3P Metabolic Processes

G3P is another direct primary metabolic precursor of CA (Khaleeli et al., 1999; Liras et al., 2008), and glyceraldehyde-3-phosphate dehydrogenases (GAPDHs) are responsible for catalyzing the formation of G3P into 1,3-diphosphoglycerate. Two genes (*gap1* and *gap2*) encoding distinct GAPDHs have been characterized in *S. clavuligerus* 27064 (Li and Townsend, 2006). In this study, the expression level of *gap2* in $\Delta cagRS$ was reduced significantly compared to levels in F613-1 (**Table 1**), whereas *gap1* showed no significant differences in expression between the two strains. *BB341_RS23575*, which encodes glycerol-3-phosphate dehydrogenase, an enzyme involved in converting glycerol into G3P, was also significantly down-regulated in the mutant.

In addition, transcriptome analysis and RT-qPCR revealed that many genes involved in glycolysis, such as *BB341_RS18990*, *BB341_RS26335*, *BB341_RS21415*, *BB341_RS13605*, and *BB341_RS21120*, were also significantly down-regulated in $\Delta cagRS$ compared with F613-1 (**Table 1**). Furthermore, ChIP-seq assays revealed that *BB341_RS22330* (*pgk*), encoding the phosphoglycerate kinase involved in glycolysis, may be directly regulated by CagR *in vivo* (**Table 2** and **Figure 4B**). Both glycolysis and the glycerol-converting process affect G3P concentration, as summarized in **Figure 8A**, and our transcriptional data revealed that G3P concentration may be decreased in $\Delta cagRS$ when compared with F613-1.

TABLE 2 | Clavulanic acid (CA) biosynthesis-associated genes identified as CagR targets by ChIP-seq.

Gene ID in ATCC 27064	Gene ID in F613-1	Biological function	Pathway	–Log ₁₀ (p-value)
SCLAV_4189(<i>cyp450</i>)	BB341_RS07850 (<i>cyp450</i>)	Cytochrome P450	Biosynthesis of CA	3.05595
SCLAV_4191(<i>claR</i>)	BB341_RS07840 (<i>claR</i>)	Transcriptional regulator	CA biosynthesis regulation	4.0205
SCLAV_0471(<i>avaA2</i>)	BB341_RS25520(<i>avaA2</i>)	Gamma-butyrolactone biosynthesis protein	CA biosynthesis regulation	2.53812
SCLAV_1147 (<i>pgk</i>)	BB341_RS22330 (<i>pgk</i>)	Phosphoglycerate kinase	Glycolysis	3.0029
SCLAV_4367	BB341_RS07030	Enoyl-CoA hydratase	Fatty acid degradation	2.33484
SCLAV_0228	BB341_RS26625	Acyl-CoA dehydrogenase	Fatty acid degradation	3.83473
SCLAV_2974(<i>paaH</i>)	BB341_RS03665(<i>paaH</i>)	3-hydroxybutyryl-CoA dehydrogenase	Fatty acid degradation	4.78544
SCLAV_1431(<i>glnA3</i>)	BB341_RS20995(<i>glnA3</i>)	Glutamine synthetase	Arginine synthesis	2.80523

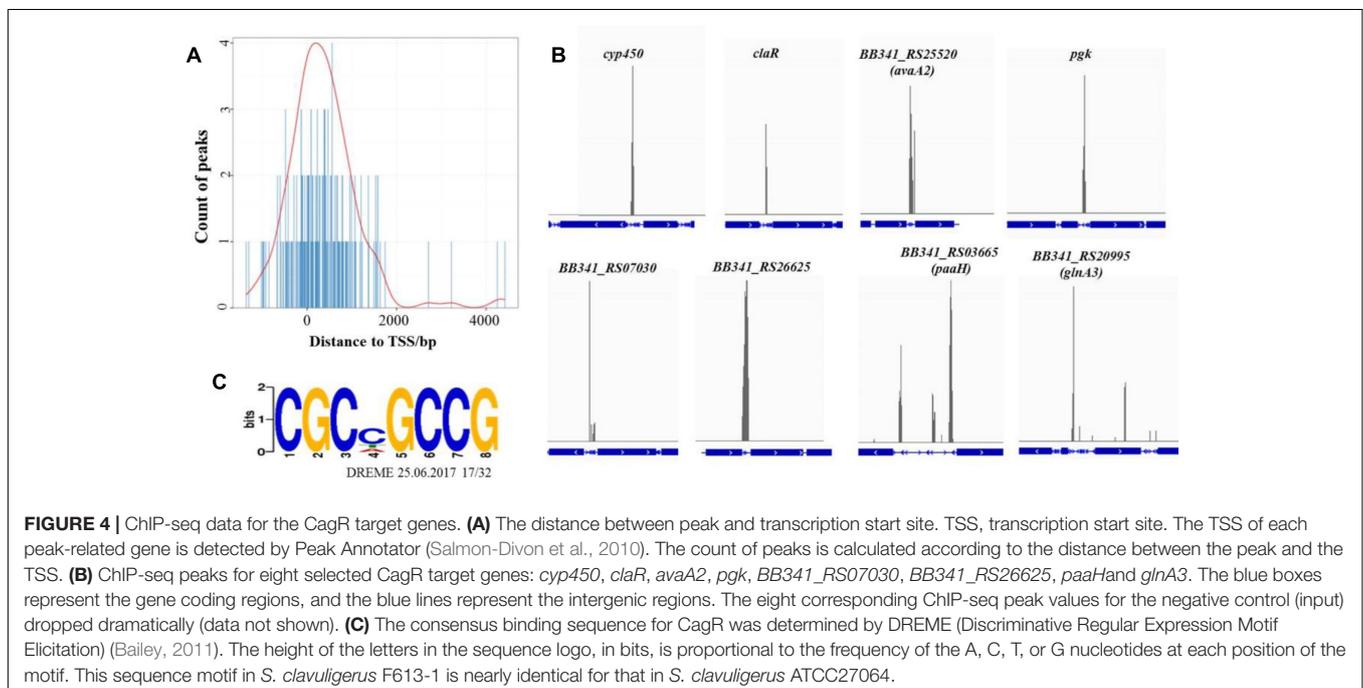
The genes listed in this table are limited to those associated with CA biosynthesis.

Glycerol trioleate, which dissociates into glycerol and oleic acid, was reported to enhance CA production in *S. clavuligerus* (Kim et al., 2009), with the produced glycerol converted into G3P for CA production. We found that, although CA production decreased significantly in the absence of glycerol trioleate, CA concentration increased from 72 h to 168 h continuously in Δ *cagRS*, indicating that *cagRS* may affect primary metabolic processing of the direct CA precursor G3P.

cagRS Modulates Expression of the CA Biosynthetic Gene Cluster

As noted previously, gene clusters related to CA biosynthesis include the CA biosynthetic gene cluster, the paralog gene cluster and the clavam gene cluster (Figure 1C). Both the transcriptome and ChIP-seq analyses indicated that CagRS might affect the genes involved in CA biosynthesis (Tables 1, 2), so we speculated that CagR may interact directly with

one or more of the promoters in the CA biosynthesis-related gene clusters. To investigate the targets of CagR, we amplified 14 promoter fragments (Figure 6A), which covered the upstream regions for 20 transcripts localizing to these gene clusters, to use as probes in EMSAs. Only the *cas1* gene in the clavam gene cluster is involved in CA biosynthesis, but when incubated with purified His-tagged CagR, EMSA assays showed that CagR does not bind the intergenic region of *cas1* (Figure 6A). *ceaS1*, *bls1*, *pah1*, and *oat1* in the paralog gene cluster are also involved in CA biosynthesis, and EMSA assays showed that CagR does not bind the intergenic region of the *bls1* and *pah1* genes, but CagR could bind the intergenic region of the *ceaS1* and *oat1* genes (Figure 6A and Supplementary Figure S5). For genes in the CA biosynthetic gene cluster, EMSA assays showed that CagR could bind the intergenic region of *claR* and *oat2* genes (Figure 6A and Supplementary Figure S5). DNA sequence alignment also showed that promoter regions of *claR*, *ceaS1*, *oat1*, and *oat2* contain sequences



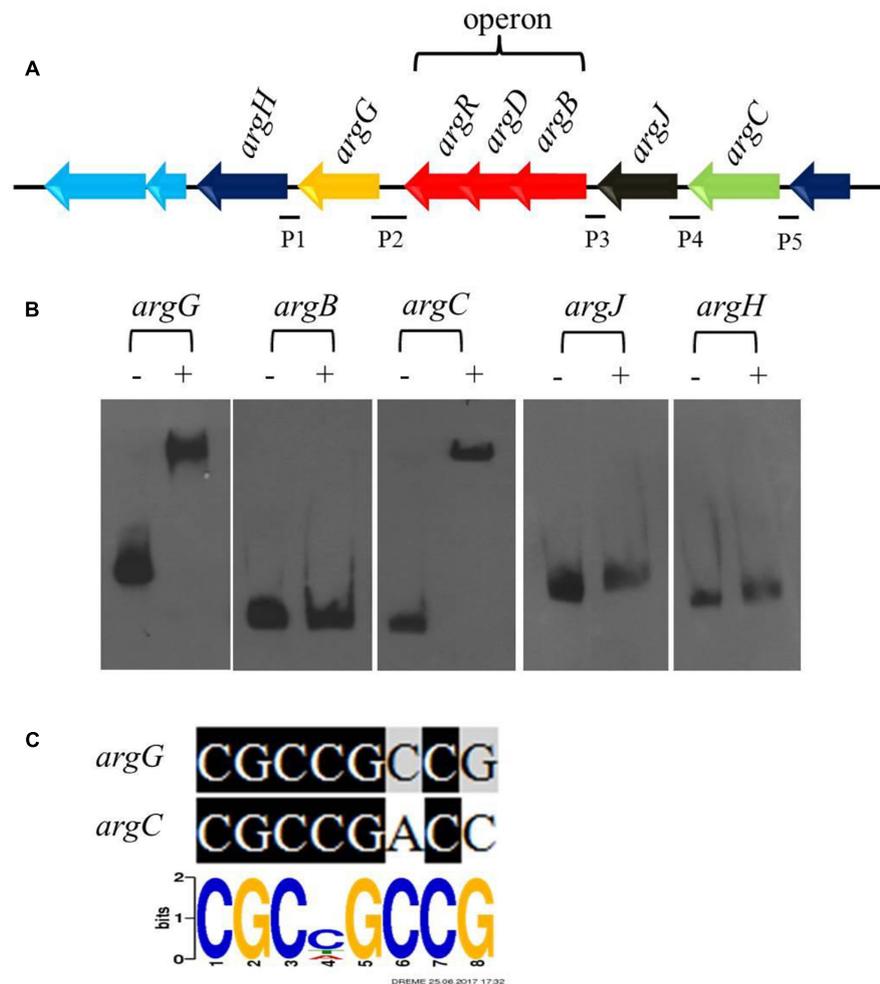


FIGURE 5 | CagRS regulates the biosynthesis of arginine—the direct precursor of CA. **(A)** Schematic diagram of the arginine biosynthetic gene cluster. P1–P5 are different promoter regions of the arginine gene cluster: P1, 211 bp of the *argH* upstream region; P2, 299 bp of the *argG* upstream region; P3, 154 bp of the *argB-D-R* upstream region; P4, 271 bp of the *argJ* upstream region; P5, 160 bp of the *argC* upstream region. **(B)** The binding of CagR to the promoter regions of *argC* and *argG*. EMSAs of *argG*, *argB*, *argC*, *argJ*, and *argH* with purified His-tagged CagR. The promoter fragments were labeled with biotin-11-UTP using the Biotin 3' End DNA Labeling kit. The above probes were incubated either with no protein (–) or 3.0 μ g CagR (+). The appropriate amount of polydI/dC (1.0 mg) was used as competitor. **(C)** Comparison of *argG* and *argC* promoter sequences with the conserved CGCNGCCG motif for CagR binding.

with strong similarity to the conserved CGCNGCCG motif (**Figure 6B**) in F613-1.

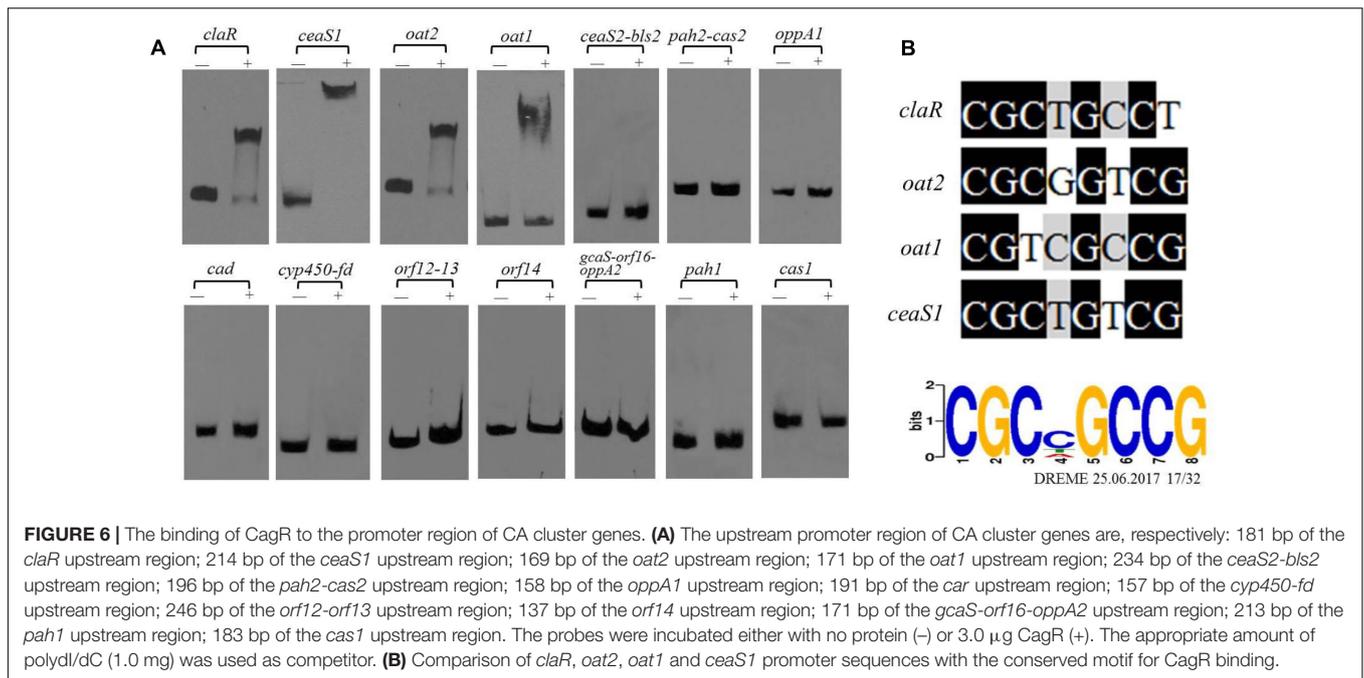
The above data indicated that CagRS is directly associated with CA synthesis, so the relative expression levels of genes in the CA biosynthetic gene cluster, as well as of homologous genes needed for CA biosynthesis in the paralog and clavam gene clusters, were analyzed by RT-qPCR assays. The mycelium of F613-1 and Δ *cagRS* cultured at 24, 72, 120, 168, and 216 h was harvested, and the relative expression levels of genes involved in CA biosynthesis during the CA fermentation process were monitored. As shown in **Figure 7**, the expression level of *claR*, *oat2*, *oppA1*, *oppA2*, *car*, *cyp450*, *orf12*, *orf14*, *orf16*, and *gcaS* in the CA biosynthetic gene cluster increased during the fermentation process and peaked at 168 h; the expression level of *oat1* and *pah1* in the paralog gene cluster increased during the fermentation process and peaked at 168 h; and the expression level of *cas1* in the clavam gene cluster

also increased during the fermentation process and peaked at 168 h. When compared with F613-1 levels, the expression levels of almost all of the 20 tested genes were decreased in Δ *cagRS* before the 120 h time point. This trend in gene expression was similar to the trend in the CA fermentation levels (**Figure 3**).

DISCUSSION

cagRS Is a Global Regulatory TCS

Two-component regulatory systems constitute a family of proteins that mediate adaptation to changing environments by modifying the phosphorylated state of a pair of proteins: a sensor histidine kinase and a response regulator. TCSs are reported to be involved in a variety of bacterial cellular responses, such as biofilm development, morphological development, chemotaxis,



sporulation, photosynthesis, osmoregulation, antibiotic production, and pathogenicity (Ogura and Tanaka, 2002; Bijlsma and Groisman, 2003; Hutchings et al., 2004; Mikkelsen et al., 2011). TCSs are very abundant in *Streptomyces* species and are reported to affect antibiotic production such as pimaricin biosynthesis (Mendes et al., 2007). The TCS *orf22/orf23* of *S. clavuligerus* ATCC 27064 was reported to have high similarity to the TCS SCO4020/4021 of *S. coelicolor* A3(2) and to affect CA production in *S. clavuligerus* ATCC 27064; *orf22/orf23* is located downstream of the CA gene cluster, and an *orf23* deletion mutant reduced CA production, cell growth and sporulation in strain ATCC 27064 (Song et al., 2009). However, Jnawali et al. (2008) reported that *orf23* deletion reduced CA production but had no effect on cell growth or morphological development. Beyond that, the mechanisms by which *orf22/orf23* affects CA production have not been further investigated (Song et al., 2009). The TCS CagRS in *S. clavuligerus* F613-1, which was annotated as *orf22/orf23* in ATCC 27064, is also located next to the CA biosynthetic gene cluster, and we found that deletion of CagRS significantly reduced CA production but did not affect the phenotype. We also found that single-gene deletion mutants of CagRS also significantly reduced CA production but did not affect the phenotype (Supplementary Figure S4). Our results with the *cagRS* double-gene deletion mutant were similar to those obtained with the previously described *orf23* deletion mutant (Jnawali et al., 2008).

In our ChIP-seq experiments, enriched peaks were concentrated near transcriptional start sites, indicating that the response regulator CagR is indeed a transcriptional regulatory protein. Both the transcriptome and ChIP-seq data revealed that the TCS CagRS mainly regulates genes involved in fatty acid degradation, G3P and arginine metabolism, and CA production. Given the phenotype of the mutant and our

transcriptome and ChIP-seq results, we conclude that CagRS is a global regulatory TCS and that this system regulates aspects of both primary metabolism (such as G3P and arginine metabolism) and secondary metabolism such as CA production. However, this TCS has little or no effect on phenotype or spore development under the test conditions.

***cagRS* Negatively Regulates Arginine Biosynthesis**

As a direct precursor of CA biosynthesis, arginine is very important for CA production (Brian et al., 1993; Khaleeli et al., 1999). The arginine biosynthetic gene cluster of *S. clavuligerus* was characterized by Rodriguez-Garcia et al. (2000), and the schematic diagram of this cluster in *S. clavuligerus* F613-1 is shown in Figure 5A. In our study, transcriptome data revealed that deletion of *cagRS* resulted in the increased expression of the arginine biosynthetic gene cluster, and these findings were largely supported by RT-qPCR data for *argB*, *argC*, *argJ*, *argH*, and *argG*. Additionally, EMSAs revealed that CagR could bind to the promoter regions for *argG* and *argC*. Overall, these data suggest that CagRS modulates the biosynthesis of arginine in a negative manner.

***cagRS* May Positively Regulate G3P Metabolism**

Glycerol, glycerol trioleate and other oils were reported to enhance CA production in *S. clavuligerus* (Baggaley et al., 1997; Ives and Bushell, 1997; Kim et al., 2009). The glycerol trioleate in the fermentation medium was absorbed into the cell and then was enzymatically dissociated into glycerol and oleic acid, with the produced glycerol converted into G3P (through primary metabolic pathways) for CA production. The produced G3P can

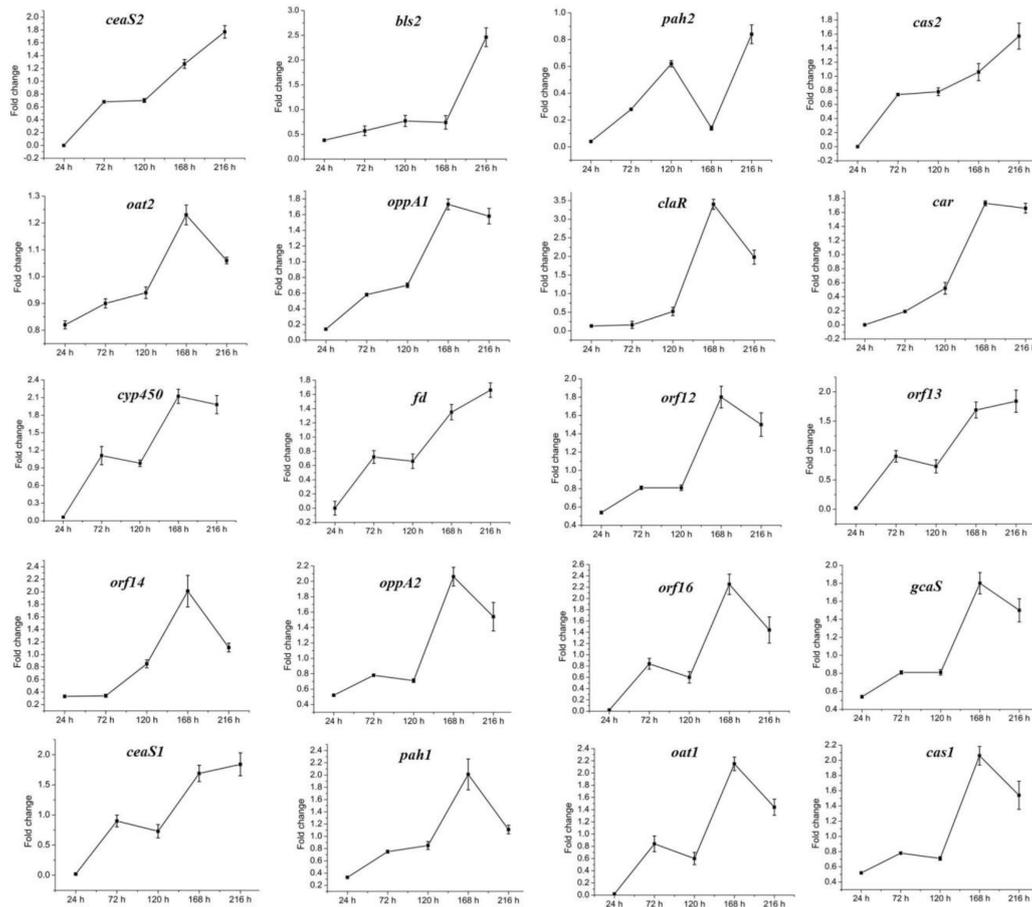


FIGURE 7 | Expression of CA cluster genes by RT-qPCR in F613-1 and $\Delta cagRS$. Results were normalized for *16S rDNA* gene content and are shown as fold change over the F613-1 control, which was given a value of 1. Fold change: expression level in $\Delta cagRS$ compared with F613-1. Data are the mean \pm SD of three separate experiments.

also be converted into 1,3-bisphosphoglycerate by GAPDH in the glycolytic pathway and then enters the tricarboxylic acid cycle through pyruvate. Tricarboxylic acid cycle-intermediate accumulation is also reported to be associated with CA biosynthesis in *S. clavuligerus* (Ramirez-Malule et al., 2018). In this study, we found that conversion of glycerol into G3P may be inhibited in the $\Delta cagRS$ mutant strain because the gene (*BB341_RS23575*) that encodes glycerol-3-phosphate dehydrogenase was significantly down-regulated. Two genes (*gap1* and *gap2*), whose protein products are distinct GAPDHs, were characterized in *S. clavuligerus* 27064, and whereas the *gap1* mutant had twice the normal production levels of CA, the *gap2* mutant produced a level of CA similar to that of the wild-type strain (Li and Townsend, 2006), indicating that *gap1* plays a major role in converting G3P in *S. clavuligerus*. In $\Delta cagRS$, the expression level of *gap2* was reduced significantly compared with F613-1 levels; however, the expression level of *gap1* was similar in the two strains, suggesting that the rate for conversion of G3P into acetyl-CoA in $\Delta cagRS$ would not differ greatly from that of F613-1. Additionally, our transcriptome and RT-qPCR analysis revealed that many genes involved in

G3P and glycerol metabolism were significantly down-regulated in $\Delta cagRS$ compared with F613-1 (Table 1 and Figure 8A). Based on these data, we predict that the G3P concentration was therefore reduced, leading to the reduced CA production. Data from CA fermentation confirmed our hypothesis, as the amount of CA peaked at 168 h during the fermentation process whether supplemented with glycerol trioleate or not, but the CA concentration dropped significantly without glycerol trioleate.

cagRS Positively Regulates the CA Biosynthetic Gene Cluster

Clavulanic acid is a potent β -lactamase inhibitor produced by *S. clavuligerus* F613-1 (Qin et al., 2017), a strain that also synthesizes cephamycin C (Reading and Cole, 1977) and a few other clavam-based structures. The biosynthesis pathway of CA and its related by-product (clavam) has been largely elucidated (Liras et al., 2008). However, there have been no reports on the global regulation of CA biosynthesis. In this study, deletion of *CagRS* resulted in markedly decreased production of CA, consistent with the reduced expression of multiple genes involved

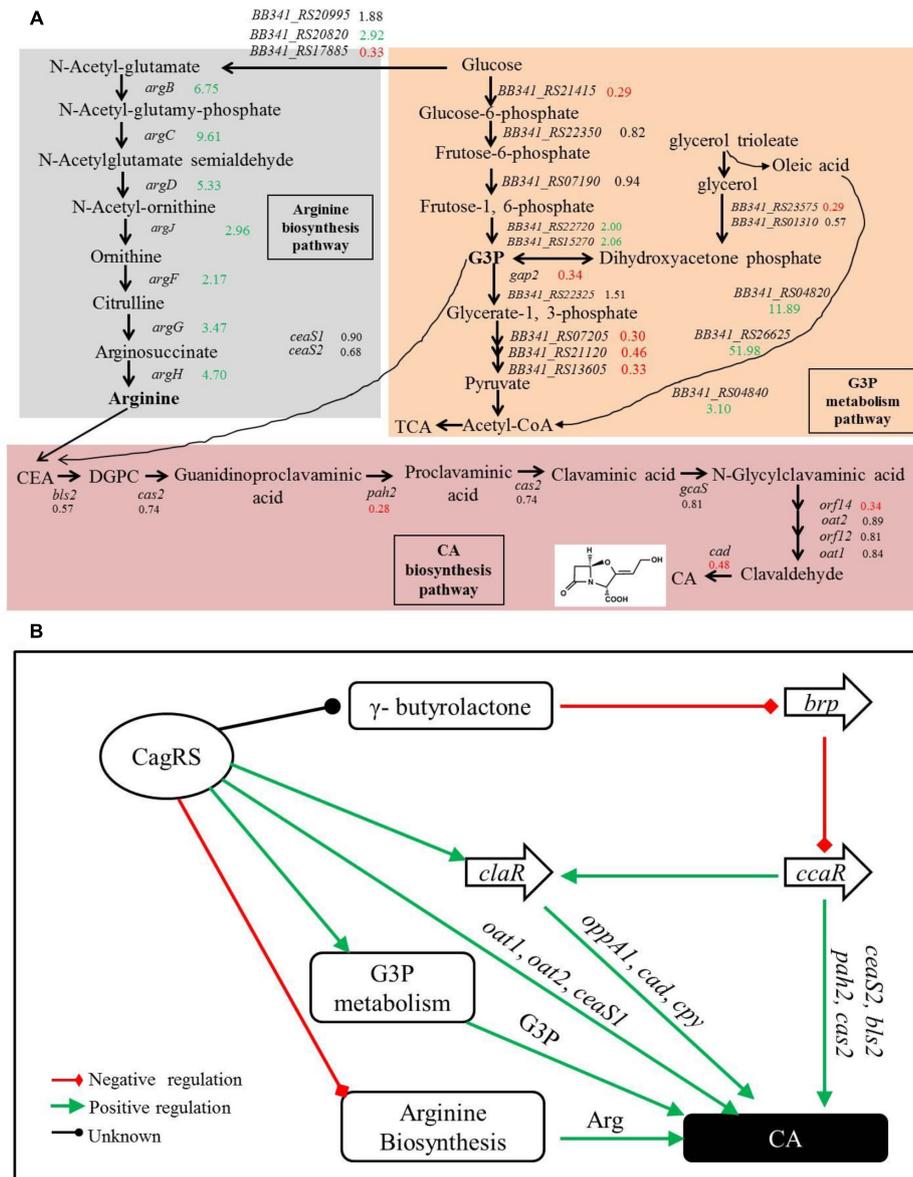


FIGURE 8 | Clavulanic acid biosynthesis is regulated by the TCS CagRS. **(A)** Schematic overview of the expression profiles of genes involved in CA, G3P and the arginine metabolism pathway. CEA, *N*²-(2-carboxyethyl)-arginine; DGPC, deoxyguanidino-proclavaminic acid; TCA, tricarboxylic acid cycle. The numbers are the ratios of the comparative expression levels in Δ *cagRS* compared with the control F613-1, and the data are from the transcriptome and the RT-qPCR assays. Red indicates downregulation, green indicates upregulation, and black indicates no notable change. **(B)** Model of the TCS CagRS regulatory network of CA biosynthesis.

in CA biosynthesis at early time points, including *pah2*, *bls2*, *claR*, *car* (these four genes located in CA biosynthetic gene cluster), and *oat1* (in paralog gene cluster), compared with levels in F613-1. *Clar* is a pathway-specific regulatory factor of CA biosynthesis and positively regulates the expression of *ceaS2* (Gomez-Escribano et al., 2006); the patterns of expression of the late CA synthetic genes, such as *orf12*, *orf14*, *oppA1*, *oppA2*, *orf16*, *gcaS* and *car*, were similar to *claR* expression patterns, indicating that *cagRS* may indirectly regulate these genes through *claR*. The above data indicate that CagRS positively regulates the expression of the CA biosynthetic gene cluster.

Our EMSAs revealed that CagR can interact with the promoters of *ceaS1*, *oat1*, and *oat2*. Both EMSA and ChIP-seq assays also revealed that CagR can interact with the *claR* promoter, indicating that CagR can directly regulate *claR*, thereby affecting the expression of *Clar* target genes in the CA biosynthetic pathway. Given the above data, we propose that CagRS modulates CA biosynthesis through *claR*, *ceaS1*, *oat1*, and *oat2*. *CcaR* is another pathway-specific regulatory factor involved in the biosynthesis of CA and was reported to positively regulate the expression of early stage CA biosynthetic genes (such as *ceaS2*, *bls2*, *pah2*, and *cas2*) and *claR*

(Santamarta et al., 2002, 2011; Alvarez-Alvarez et al., 2014), suggesting that CagR and CcaR both directly regulate CA synthesis. In addition, the γ -butyrolactone signaling system negatively regulates CA production through inhibiting *ccaR* (Santamarta et al., 2005). Interestingly, our ChIP-seq assays revealed that CagR can bind the promoter of the *BB341_RS25520* (*avaA2*) gene, which encodes a gamma-butyrolactone biosynthesis protein, suggesting that CagR can also regulate CA production through the γ -butyrolactone signaling system-CcaR pathway (Figure 8B).

In conclusion, we found that CagR can modulate expression of the CA biosynthetic gene cluster and also affect genes involved in the metabolism of G3P and arginine, two direct precursors of CA. Our results provide new insights into the global regulation of CA biosynthesis and provide an important resource for future metabolic engineering efforts for CA production in *S. clavuligerus*.

AUTHOR CONTRIBUTIONS

GC designed the work. RQ executed the experiments. JF analyzed the data and prepared the manuscript draft and revised the manuscript. GZ and CL carried out the interpretation of data, drawing up figures and statistical analysis. NK helped in data

analysis. CZ contributed to the experimental design, manuscript preparation, and submission. All authors read and approved the final manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (No. 31700065), Shandong Provincial Natural Science Foundation (No. ZR2017BC040), and the Innovation Project of the Shandong Academy of Medical Sciences (No. 201604).

ACKNOWLEDGMENTS

We thank Dr. Susan T. Howard for critical reading of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.00244/full#supplementary-material>

REFERENCES

- Alvarez-Alvarez, R., Rodriguez-Garcia, A., Santamarta, I., Perez-Redondo, R., Prieto-Dominguez, A., Martinez-Burgo, Y., et al. (2014). Transcriptomic analysis of *Streptomyces clavuligerus* *deltaccar:tsr*: effects of the cephamycin C-clavulanic acid cluster regulator CcaR on global regulation. *Microb. Biotechnol.* 7, 221–231. doi: 10.1111/1751-7915.12109
- Arulanantham, H., Kershaw, N. J., Hewitson, K. S., Hughes, C. E., Thirkettle, J. E., and Schofield, C. J. (2006). ORF17 from the clavulanic acid biosynthesis gene cluster catalyzes the ATP-dependent formation of N-glycyl-clavaminic acid. *J. Biol. Chem.* 281, 279–287. doi: 10.1074/jbc.M507711200
- Baggaley, K. H., Brown, A. G., and Schofield, C. J. (1997). Chemistry and biosynthesis of clavulanic acid and other clavams. *Nat. Prod. Rep.* 14, 309–333. doi: 10.1039/np9971400309
- Bailey, T. L. (2011). DREME: motif discovery in transcription factor ChIP-seq data. *Bioinformatics* 27, 1653–1659. doi: 10.1093/bioinformatics/btr261
- Bailey, T. L., and Elkan, C. (1994). Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* 2, 28–36.
- Bignell, D. R., Tahlan, K., Colvin, K. R., Jensen, S. E., and Leskiw, B. K. (2005). Expression of *ccaR*, encoding the positive activator of cephamycin C and clavulanic acid production in *Streptomyces clavuligerus*, is dependent on *bldG*. *Antimicrob. Agents Chemother.* 49, 1529–1541. doi: 10.1128/AAC.49.4.1529-1541.2005
- Bijlsma, J. J., and Groisman, E. A. (2003). Making informed decisions: regulatory interactions between two-component systems. *Trends Microbiol.* 11, 359–366. doi: 10.1016/S0966-842X(03)00176-8
- Brian, P. V., Christopher, R. B., Andrew, D., Jennifer, M., and Stephen, W. E. (1993). Evidence that arginine is a later metabolic intermediate than ornithine in the biosynthesis of clavulanic acid by *Streptomyces clavuligerus*. *J. Chem. Soc. Chem. Commun.* 15, 1210–1211. doi: 10.1039/C39930001210
- Brown, A. G., Butterworth, D., Cole, M., Hanscomb, G., Hood, J. D., Reading, C., et al. (1976). Naturally-occurring beta-lactamase inhibitors with antibacterial activity. *J. Antibiot.* 29, 668–669. doi: 10.7164/antibiotics.29.668
- Bush, M. J., Bibb, M. J., Chandra, G., Findlay, K. C., and Buttner, M. J. (2013). Genes required for aerial growth, cell division, and chromosome segregation are targets of WhiA before sporulation in *Streptomyces venezuelae*. *mBio* 4, e684–13. doi: 10.1128/mBio.00684-13
- Cao, G., Zhong, C., Zong, G., Fu, J., Liu, Z., Zhang, G., et al. (2016). Complete genome sequence of *Streptomyces clavuligerus* F613-1, an industrial producer of clavulanic acid. *Genome Announc.* 4, e1020–16. doi: 10.1128/genomeA.01020-16
- Fu, J., Zong, G., Zhang, P., Zhao, Z., Ma, J., Pang, X., et al. (2017). XdhR negatively regulates actinorhodin biosynthesis in *Streptomyces coelicolor* M145. *FEMS Microbiol. Lett.* 364:fnx226. doi: 10.1093/femsle/fnx226
- Gomez-Escribano, J. P., Liras, P., Pisabarro, A., and Martin, J. F. (2006). An *rplKDelta29-PALG-32* mutation leads to reduced expression of the regulatory genes *ccaR* and *claR* and very low transcription of the *ceaS2* gene for clavulanic acid biosynthesis in *Streptomyces clavuligerus*. *Mol. Microbiol.* 61, 758–770. doi: 10.1111/j.1365-2958.2006.05266.x
- Gomez-Escribano, J. P., Martin, J. F., Hesketh, A., Bibb, M. J., and Liras, P. (2008). *Streptomyces clavuligerus* *relA*-null mutants overproduce clavulanic acid and cephamycin C: negative regulation of secondary metabolism by (p)ppGpp. *Microbiology* 154, 744–755. doi: 10.1099/mic.0.2007/011890-0
- Gust, B., Challis, G. L., Fowler, K., Kieser, T., and Chater, K. F. (2003). PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. *Proc. Natl. Acad. Sci. U.S.A.* 100, 1541–1546. doi: 10.1073/pnas.0337542100
- Hodgson, J. E., Fosberry, A. P., Rawlinson, N. S., Ross, H. N., Neal, R. J., Arnell, J. C., et al. (1995). Clavulanic acid biosynthesis in *Streptomyces clavuligerus*: gene cloning and characterization. *Gene* 166, 49–55. doi: 10.1016/0378-1119(95)00560-9
- Hutchings, M. I., Hoskisson, P. A., Chandra, G., and Buttner, M. J. (2004). Sensing and responding to diverse extracellular signals? Analysis of the sensor kinases and response regulators of *Streptomyces coelicolor* A3(2). *Microbiology* 150, 2795–2806. doi: 10.1099/mic.0.27181-0
- Ishida, K., Hung, T. V., Liou, K., Lee, H. C., Shin, C. H., and Sohng, J. K. (2006). Characterization of *pbpA* and *pbp2* encoding penicillin-binding proteins located on the downstream of clavulanic acid gene cluster in *Streptomyces clavuligerus*. *Biotechnol. Lett.* 28, 409–417. doi: 10.1007/s10529-005-6071-5

- Ives, P. R., and Bushell, M. E. (1997). Manipulation of the physiology of clavulanic acid production in *Streptomyces clavuligerus*. *Microbiology* 143(Pt 11), 3573–3579. doi: 10.1099/00221287-143-11-3573
- Jensen, S. E., Elder, K. J., Aidoo, K. A., and Paradkar, A. S. (2000). Enzymes catalyzing the early steps of clavulanic acid biosynthesis are encoded by two sets of paralogous genes in *Streptomyces clavuligerus*. *Antimicrob. Agents Chemother.* 44, 720–726. doi: 10.1128/AAC.44.3.720-726.2000
- Jensen, S. E., and Paradkar, A. S. (1999). Biosynthesis and molecular genetics of clavulanic acid. *Antonie Van Leeuwenhoek* 75, 125–133. doi: 10.1023/A:1001755724055
- Jensen, S. E., Paradkar, A. S., Mosher, R. H., Anders, C., Beatty, P. H., Brumlik, M. J., et al. (2004a). Five additional genes are involved in clavulanic acid biosynthesis in *Streptomyces clavuligerus*. *Antimicrob. Agents Chemother.* 48, 192–202. doi: 10.1128/AAC.48.1.192-202.2004
- Jensen, S. E., Wong, A., Griffin, A., and Barton, B. (2004b). *Streptomyces clavuligerus* has a second copy of the proclavamate amidinohydrolase gene. *Antimicrob. Agents Chemother.* 48, 514–520.
- Jin, X., Cao, G., Zhang, X., Chen, Y., Wang, L., and Zhong, C. (2015). Studies on the formation and synthetic mechanism of related substance G in potassium clavulanate production. *Braz. J. Pharm. Sci.* 51, 77–83. doi: 10.1590/s1984-82502015000100008
- Jnawali, H. N., Lee, H. C., and Sohng, J. K. (2010). Enhancement of clavulanic acid production by expressing regulatory genes in gap gene deletion mutant of *Streptomyces clavuligerus* NRRL3585. *J. Microbiol. Biotechnol.* 20, 146–152.
- Jnawali, H. N., Liou, K., and Sohng, J. K. (2011). Role of sigma-factor (orf21) in clavulanic acid production in *Streptomyces clavuligerus* NRRL3585. *Microbiol. Res.* 166, 369–379. doi: 10.1016/j.micres.2010.07.005
- Jnawali, H. N., Oh, T. J., Liou, K., Park, B. C., and Sohng, J. K. (2008). A two-component regulatory system involved in clavulanic acid production. *J. Antibiot.* 61, 651–659. doi: 10.1038/ja.2008.92
- Jones, R. N., Craig, W. A., Ambrose, P. G., Dudley, M. N., and Pottumarthy, S. (2005). Reevaluation of *Enterobacteriaceae* MIC/disk diffusion zone diameter regression scattergrams for 9 beta-lactams: adjustments of breakpoints for strains producing extended spectrum beta-lactamases. *Diagn. Microbiol. Infect. Dis.* 52, 235–246. doi: 10.1016/j.diagmicrobio.2005.02.006
- Kent, W. J., Zweig, A. S., Barber, G., Hinrichs, A. S., and Karolchik, D. (2010). Bigwig and bigbed: enabling browsing of large distributed datasets. *Bioinformatics* 26, 2204–2207. doi: 10.1093/bioinformatics/btq351
- Khaleeli, N., Li, R. F., and Townsend, C. A. (1999). Origin of the β -Lactam carbons in clavulanic acid from an unusual thiamine pyrophosphate-mediated reaction. *J. Am. Chem. Soc.* 121, 9223–9224. doi: 10.1021/ja9923134
- Kieser, T., Bibb, M. J., Chater, K. F., Buttner, M. J., and Hopwood, D. A. (2000). *Practical Streptomyces Genetics: a Laboratory Manual*. Norwich: John Innes Foundation.
- Kim, S. J., Kim, J. O., Shin, C. H., Park, H. W., and Kim, C. W. (2009). An approach to strain improvement and enhanced production of clavulanic acid in *Streptomyces clavuligerus*. *Biosci. Biotechnol. Biochem.* 73, 160–164. doi: 10.1271/bbb.80569
- Kopylova, E., Noe, L., and Touzet, H. (2012). SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics* 28, 3211–3217. doi: 10.1093/bioinformatics/bts611
- Kurt, A., Alvarez-Alvarez, R., Liras, P., and Ozcengiz, G. (2013). Role of the cmcH-ccaR intergenic region and ccaR overexpression in cephamycin C biosynthesis in *Streptomyces clavuligerus*. *Appl. Microbiol. Biotechnol.* 97, 5869–5880. doi: 10.1007/s00253-013-4721-4
- Kwong, T., Tahlan, K., Anders, C. L., and Jensen, S. E. (2013). Carboxyethylarginine synthase genes show complex cross-regulation in *Streptomyces clavuligerus*. *Appl. Environ. Microbiol.* 79, 240–249. doi: 10.1128/AEM.02600-12
- Li, R., and Townsend, C. A. (2006). Rational strain improvement for enhanced clavulanic acid production by genetic engineering of the glycolytic pathway in *Streptomyces clavuligerus*. *Metab. Eng.* 8, 240–252. doi: 10.1016/j.ymben.2006.01.003
- Liras, P., Gomez-Escribano, J. P., and Santamarta, I. (2008). Regulatory mechanisms controlling antibiotic production in *Streptomyces clavuligerus*. *J. Ind. Microbiol. Biotechnol.* 35, 667–676. doi: 10.1007/s10295-008-0351-8
- Liras, P., and Rodriguez-Garcia, A. (2000). Clavulanic acid, a beta-lactamase inhibitor: biosynthesis and molecular genetics. *Appl. Microbiol. Biotechnol.* 54, 467–475. doi: 10.1007/s002530000420
- Lorenzana, L. M., Perez-Redondo, R., Santamarta, I., Martin, J. F., and Liras, P. (2004). Two oligopeptide-permease-encoding genes in the clavulanic acid cluster of *Streptomyces clavuligerus* are essential for production of the beta-lactamase inhibitor. *J. Bacteriol.* 186, 3431–3438. doi: 10.1128/JB.186.11.3431-3438.2004
- Mackenzie, A. K., Valegard, K., Iqbal, A., Caines, M. E., Kershaw, N. J., Jensen, S. E., et al. (2010). Crystal structures of an oligopeptide-binding protein from the biosynthetic pathway of the beta-lactamase inhibitor clavulanic acid. *J. Mol. Biol.* 396, 332–344. doi: 10.1016/j.jmb.2009.11.045
- Martinez-Burgo, Y., Alvarez-Alvarez, R., Rodriguez-Garcia, A., and Liras, P. (2015). The pathway-specific regulator ClaR of *Streptomyces clavuligerus* has a global effect on the expression of genes for secondary metabolism and differentiation. *Appl. Environ. Microbiol.* 81, 6637–6648. doi: 10.1128/AEM.00916-15
- Mendes, M. V., Tunca, S., Anton, N., Recio, E., Sola-Landa, A., Aparicio, J. F., et al. (2007). The two-component phoR-phoP system of *Streptomyces natalensis*: inactivation or deletion of phoP reduces the negative phosphate regulation of pimaricin biosynthesis. *Metab. Eng.* 9, 217–227. doi: 10.1016/j.ymben.2006.10.003
- Mikkelsen, H., Sivaneson, M., and Filloux, A. (2011). Key two-component regulatory systems that control biofilm formation in *Pseudomonas aeruginosa*. *Environ. Microbiol.* 13, 1666–1681. doi: 10.1111/j.1462-2920.2011.02495.x
- Mosher, R. H., Paradkar, A. S., Anders, C., Barton, B., and Jensen, S. E. (1999). Genes specific for the biosynthesis of clavam metabolites antipodal to clavulanic acid are clustered with the gene for clavamate synthase 1 in *Streptomyces clavuligerus*. *Antimicrob. Agents Chemother.* 43, 1215–1224. doi: 10.1128/AAC.43.5.1215
- Ogura, M., and Tanaka, T. (2002). Recent progress in *Bacillus subtilis* two-component regulation. *Front. Biosci.* 7:d1815–d1824. doi: 10.2741/ogura
- Paradkar, A. S., Aidoo, K. A., and Jensen, S. E. (1998). A pathway-specific transcriptional activator regulates late steps of clavulanic acid biosynthesis in *Streptomyces clavuligerus*. *Mol. Microbiol.* 27, 831–843. doi: 10.1046/j.1365-2958.1998.00731.x
- Paradkar, A. S., and Jensen, S. E. (1995). Functional analysis of the gene encoding the clavamate synthase 2 isoenzyme involved in clavulanic acid biosynthesis in *Streptomyces clavuligerus*. *J. Bacteriol.* 177, 1307–1314. doi: 10.1128/jb.177.5.1307-1314.1995
- Paradkar, A. S., Mosher, R. H., Anders, C., Griffin, A., Griffin, J., Hughes, C., et al. (2001). Applications of gene replacement technology to *Streptomyces clavuligerus* strain development for clavulanic acid production. *Appl. Environ. Microbiol.* 67, 2292–2297. doi: 10.1128/AEM.67.5.2292-2297.2001
- Perez-Redondo, R., Rodriguez-Garcia, A., Martin, J. F., and Liras, P. (1998). The claR gene of *Streptomyces clavuligerus*, encoding a LysR-type regulatory protein controlling clavulanic acid biosynthesis, is linked to the clavulanate-9-aldehyde reductase (car) gene. *Gene* 211, 311–321. doi: 10.1016/S0378-1119(98)00106-1
- Qin, R., Zhong, C., Zong, G., Fu, J., Pang, X., and Cao, G. (2017). Improvement of clavulanic acid production in *Streptomyces clavuligerus* F613-1 by using a claR - neo reporter strategy. *Electron. J. Biotechnol.* 28, 41–46. doi: 10.1016/j.ejbt.2017.05.002
- Ramirez-Malule, H., Junne, S., Nicolas Cruz-Bournazou, M., Neubauer, P., and Rios-Estapa, R. (2018). *Streptomyces clavuligerus* shows a strong association between TCA cycle intermediate accumulation and clavulanic acid biosynthesis. *Appl. Microbiol. Biotechnol.* 102, 4009–4023. doi: 10.1007/s00253-018-8841-8
- Reading, C., and Cole, M. (1977). Clavulanic acid: a beta-lactamase-inhibiting beta-lactam from *Streptomyces clavuligerus*. *Antimicrob. Agents Chemother.* 11, 852–857. doi: 10.1128/AAC.11.5.852
- Rodriguez-Garcia, A., De La Fuente, A., Perez-Redondo, R., Martin, J. F., and Liras, P. (2000). Characterization and expression of the arginine biosynthesis gene cluster of *Streptomyces clavuligerus*. *J. Mol. Microbiol. Biotechnol.* 2, 543–550. doi: 10.1128/AAC.11.5.852
- Salmon-Divon, M., Dvinge, H., Tammoja, K., and Bertone, P. (2010). PeakAnalyzer: genome-wide annotation of chromatin binding and modification loci. *BMC Bioinformatics* 11:415. doi: 10.1186/1471-2105-11-415
- Sambrook, J., Fritsch, E. F., and Maniatis, J. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd Edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

- Santamarta, I., Lopez-García, M. T., Kurt, A., Nardiz, N., Alvarez-Alvarez, R., Perez-Redondo, R., et al. (2011). Characterization of DNA-binding sequences for CcaR in the cephamycin-clavulanic acid supercluster of *Streptomyces clavuligerus*. *Mol. Microbiol.* 81, 968–981. doi: 10.1111/j.1365-2958.2011.07743.x
- Santamarta, I., Perez-Redondo, R., Lorenzana, L. M., Martin, J. F., and Liras, P. (2005). Different proteins bind to the butyrolactone receptor protein are sequence located upstream of the regulatory ccaR gene of *Streptomyces clavuligerus*. *Mol. Microbiol.* 56, 824–835. doi: 10.1111/j.1365-2958.2005.04581.x
- Santamarta, I., Rodriguez-Garcia, A., Perez-Redondo, R., Martin, J. F., and Liras, P. (2002). CcaR is an autoregulatory protein that binds to the ccaR and cefD-cmcI promoters of the cephamycin C-clavulanic acid cluster in *Streptomyces clavuligerus*. *J. Bacteriol.* 184, 3106–3113. doi: 10.1128/JB.184.11.3106-3113.2002
- Saudagar, P. S., Survase, S. A., and Singhal, R. S. (2008). Clavulanic acid: a review. *Biotechnol. Adv.* 26, 335–351. doi: 10.1016/j.biotechadv.2008.03.002
- Song, J. Y., Jensen, S. E., and Lee, K. J. (2010). Clavulanic acid biosynthesis and genetic manipulation for its overproduction. *Appl. Microbiol. Biotechnol.* 88, 659–669. doi: 10.1007/s00253-010-2801-2
- Song, J. Y., Kim, E. S., Kim, D. W., Jensen, S. E., and Lee, K. J. (2009). A gene located downstream of the clavulanic acid gene cluster in *Streptomyces clavuligerus* ATCC 27064 encodes a putative response regulator that affects clavulanic acid production. *J. Ind. Microbiol. Biotechnol.* 36, 301–311. doi: 10.1007/s10295-008-0499-2
- Tahlan, K., Anders, C., and Jensen, S. E. (2004a). The paralogous pairs of genes involved in clavulanic acid and clavam metabolite biosynthesis are differently regulated in *Streptomyces clavuligerus*. *J. Bacteriol.* 186, 6286–6297. doi: 10.1128/JB.186.18.6286-6297.2004
- Tahlan, K., Park, H. U., Wong, A., Beatty, P. H., and Jensen, S. E. (2004b). Two sets of paralogous genes encode the enzymes involved in the early stages of clavulanic acid and clavam metabolite biosynthesis in *Streptomyces clavuligerus*. *Antimicrob. Agents Chemother.* 48, 930–939. doi: 10.1128/AAC.48.3.930-939.2004
- Townsend, C. A., and Meng-Fei, H. (1985). Biosynthesis of clavulanic acid—origin of the C5 Unit. *J. Am. Chem.* 107, 1065–1066. doi: 10.1021/ja00290a056
- Zhang, P., Wu, H., Chen, X. L., Deng, Z., Bai, L., and Pang, X. (2014). Regulation of the biosynthesis of thiopeptide antibiotic cyclothiazomycin by the transcriptional regulator SHJG8833 in *Streptomyces hygroscopicus* 5008. *Microbiology* 160, 1379–1392. doi: 10.1099/mic.0.076901-0
- Zhang, P., Wu, L., Zhu, Y., Liu, M., Wang, Y., Cao, G., et al. (2017). Deletion of MtrA inhibits cellular development of *Streptomyces coelicolor* and alters expression of developmental regulatory genes. *Front. Microbiol.* 8:2013. doi: 10.3389/fmicb.2017.02013
- Zhang, P., Zhao, Z., Li, H., Chen, X. L., Deng, Z., Bai, L., et al. (2015). Production of the antibiotic FR-008/candicidin in *Streptomyces* sp. FR-008 is co-regulated by two regulators, FscRI and FscRIV, from different transcription factor families. *Microbiology* 161, 539–552. doi: 10.1099/mic.0.000033
- Zhang, Y., Liu, T., Meyer, C. A., Eeckhoutte, J., Johnson, D. S., Bernstein, B. E., et al. (2008). Model-based analysis of ChIP-Seq (MACS). *Genome Biol.* 9:R137. doi: 10.1186/gb-2008-9-9-r137

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Fu, Qin, Zong, Liu, Kang, Zhong and Cao. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.