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ORIGINAL PAPER

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

Ju Yeon Song · Eun Sook Kim · Dae Wi Kim · Susan E. Jensen · Kye Joon Lee

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Abstract Three open reading frames denoted as *orf21*, *orf22*, and *orf23* were identified from downstream of the currently recognized gene cluster for clavulanic acid biosynthesis in *Streptomyces clavuligerus* ATCC 27064. The new *orf*s were annotated after *in silico* analysis as genes encoding a putative sigma factor, a sensor kinase, and a response regulator. The roles of the individual genes were explored by disruption of the corresponding *orf*s, and the morphological and antibiotic production phenotypes of the resulting mutants were compared. In *orf21* and *orf22* mutants, no growth or morphological differences were noted, but modest reduction of cephamycin C (*orf21*), or both cephamycin C and clavulanic acid production (*orf22*) compared with wild-type, were observed. In *orf23* mutant, cell growth and sporulation was retarded, and clavulanic acid and cephamycin C production were reduced to 40 and 47% of wild-type levels, respectively. Conversely, overexpression of *orf23* caused precocious hyperproduction of spores on solid medium, and antibiotic production was increased above the levels seen in plasmid control cultures. Transcriptional analyses were also carried out on *orf23* and showed that mutation had little effect on transcription of genes associated with the early stages of cephamycin C or clavulanic acid production but transcription of *claR,* which regulates the late stages of clavulanic acid production, was reduced in *orf23* mutants. These observations suggest that the *orf23* product may enable *S. clavuligerus* to respond to environmental changes by altering cell growth and differentiation. In addition, the effects of ORF23 on growth might indirectly regulate the biosynthesis of secondary metabolites such as clavulanic acid and cephamycin C.

Keywords *Streptomyces clavuligerus* · Clavulanic acid biosynthetic gene cluster · Clavulanic acid · Cephamycin C · Response regulator

J. Y. Song · E. S. Kim · D. W. Kim · K. J. Lee (✉)
School of Biological Sciences, Seoul National University, Seoul 151-747, South Korea
e-mail: lkj12345@snu.ac.kr

S. E. Jensen
Department of Biological Sciences, University of Alberta, Edmonton, AB T6G 2E9, Canada

Introduction

Streptomyces clavuligerus produces industrially important metabolites including cephamycin C (CM) and clavulanic acid (CA). CA is an effective inhibitor of β-lactamases and it has been used for the treatment of infectious diseases caused by various pathogenic microorganisms resistant to β-lactam antibiotics [5]. The genes involved in the biosynthesis of CA and CM have been identified. They are grouped into separate clusters in the chromosome, but the two clusters lie adjacent to one another to form a supercluster [36].

Biosynthesis of CA proceeds from the precursors L-arginine and 3-phosphoglyceraldehyde via the enzymes encoded by genes in the CA biosynthetic gene cluster [17]. The enzymes involved in early steps of CA biosynthesis, carboxyethylarginine synthase (CeaS), β-lactam synthetase (Bls), clavaminate synthase (Cas), and proclavaminic acid amidinohydrolase (Pah), have been proved to be essential for the biosynthesis of CA by disruption of the specific genes [13, 28]. Moreover, each of these is actually catalyzed by two isoenzymes encoded by paralogous genes located in separate regions of the chromosome [33, 34].

CA production has been improved by introducing additional copies of biosynthetic genes into the producer organism using integrative or multi-copy plasmids. Introduction of *ceaS2* on a multi-copy plasmid resulted in both earlier onset and higher levels of CA production [28]. Similarly, increasing the copy number of genes involved in the late steps of CA biosynthesis, such as *orf10* (*cyp*), *orf11* (*fd*), *orf12*, and *orf14,* by their introduction on multi-copy plasmids also showed beneficial effects for CA production [21], although the individual functions of these genes have not yet been elucidated clearly. Disruption of genes related to the formation of undesirable metabolites is another possibility to consider in improving production of CA. A specific deletion of the *lat* gene blocked CM production and resulted in a 2- to 2.5-fold increase of CA production [25].

Elucidation of regulatory genes is also important for controlling metabolite production, although the mechanisms regulating the expression of the individual genes are not fully understood. For instance, CcaR, a member of the SARP (*Streptomyces* antibiotic regulatory protein) family is a protein homologous to transcriptional activators such as ActII-ORF4, RedD and AfsR which regulate production of antibiotics in other *Streptomyces* spp. [26]. When the gene encoding CcaR was deleted from the chromosome of *S. clavuligerus*, production of both CA and CM was lost, proving that it is an essential regulator of production of both metabolites [1, 24]. In contrast, overproduction of CcaR in *S. clavuligerus* resulted in increased production of both CA and CM [26].

CA production was also abolished by the mutation of *claR*, a gene from the clavulanic acid gene cluster which encodes a regulator specifically controlling the expression of genes for the late stages of CA biosynthesis [24, 27]. Similarly, amplification of *claR* on a multicopy plasmid resulted in increases in CA production, while CM production was significantly reduced [27]. In addition to manipulation of genes encoding regulatory genes and known essential biosynthetic enzymes, elucidation of the functions of other genes responsible for unknown but essential activities in CA biosynthesis may suggest other ways to increase production of CA. Putative genes such as the *orf*s from *orf10* to *orf20* are currently of interest in this regard [14, 19, 21].

As part of an ongoing investigation of the genomics of *S. clavuligerus* ATCC 27064, we have identified three putative *orf*s from downstream of the currently recognized CA cluster. Since the putative genes lie beyond *orf20*, they are denoted as *orf21*, *orf22*, and *orf23*. These newly found *orf*s have been annotated by *in silico* analysis and their roles in CA production were explored by comparing the phenotypes of mutants disrupted the *orf*s with that of the wild-type.

Materials and methods

Microorganisms and plasmids used

All microorganisms, plasmids, and primers used in this work are listed in Table 1. *S. clavuligerus* ATCC 27064, the wild-type strain, and mutants obtained in the present study were maintained on ISP4 agar medium (Difco). Strains of *E. coli* carrying plasmids were grown in Luria-Bertani (LB) medium containing antibiotics as appropriate (50 μg/ml apramycin, 100 μg/ml ampicillin, 25 μg/ml chloramphenicol, 50 μg/ml kanamycin). For maintenance over extended periods, spores of strains of *S. clavuligerus* and cells of *E. coli* were suspended in 20% (w/v) glycerol and stored at −70°C.

General gene manipulation

General methods for the manipulation of DNA in *S. clavuligerus* and *E. coli* were as described previously [18, 29]. Genomic DNA was extracted from mycelium of *S. clavuligerus* ATCC 27064 harvested from submerged cultures grown on TSBS [25] medium at 30°C for 30 h. Agar blocks containing the cells were treated with lysozyme and proteinase K and then the agar blocks were subjected to pulsed field gel eletrophoresis (PFGE) to deplete native plasmids [33]. The plasmid-depleted genomic DNA was extracted by phenol-chloroform after β-agarase treatment to release the DNA from the agar blocks, and physically sheared with a Hamilton syringe to give an average fragment size of 35–40 kb. The DNA fragments were then fractionated by PFGE gel, and fragments ranging from 35 to 40 kb were recovered from the PFG, ligated into pWEB::TNC and then packaged using packaging extract (pWEB::TNC cosmid cloning Kit, Epicentre). The packaged mixture was introduced into *E. coli* EPI100 by transfection, and clones growing on LB medium containing chloramphenicol (25 μg/ml) were selected to give a library of genomic DNA from *S. clavuligerus* ATCC 27064.

Selection of cosmids, DNA sequence, and *orf* analysis

Cosmids with inserts from the CA gene cluster were selected by PCR-based screening using primers based on various identified genes from the CA gene cluster. Cosmid DNA containing the CA gene cluster was subjected to DNA sequence analysis by the Sanger sequencing method using an ABI 3730xl sequencer (Applied Biosystems). DNA sequences were assembled into contigs using Vector NTI software (Invitrogen), and *orf*s were predicted using Frameplot 2.3.2 (http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl). The functions of the putative *orf*s were predicted

Table 1 Microorganisms, cosmids, plasmids and primers used in this study

Microorganisms, plasmids, cosmids, primers	Description[a]	Reference or source
Microorganisms		
S. clavuligerus ATCC 27064	Parent strain	American Type Culture Collection (ATCC) Centre
S. clavuligerus SMF5711	*Δorf21::aac(3)IV*	This study
S. clavuligerus SMF5712	*Δorf22::aac(3)IV*	This study
S. clavuligerus SMF5713	*Δorf23::aac(3)IV*	This study
S. clavuligerus SMF5714	*S. clavuligerus* harboring pSMF5400	This study
S. clavuligerus SMF5715	*S. clavuligerus* harboring pSMF5705	This study
E. coli EPI100	F- mcrA *Δ*(*mrr-hsd*RMS-*mcr*BC) f80d*lac*Z*Δ*M15 D*lac*X74 *rec*A1 *end*A1 *araΔ*139 *Δ*(*ara, leu*)7697 *gal*U *gal*K l- *rps*L *nup*G	Epicentre
E. coli BW25113/pIJ790	*lacI*^{q} *rrnB*T14 *ΔlacZ*WJ16 *hsdR514 ΔaraBAD*AH33 *ΔrhaBAD*LD78	[10]
E. coli ET12567/pUZ8002	*dam- dcm- for methylation –deficient DNA manipulation Δ(mcrA)183 Δ(mcrCB-hsdSMR –mrr)173*	[10]
Plasmids and cosmids		
pWEB::TNC	Cosmid vector; Chl^{r}, Amp^{r}	Epicentre
SCL8B09	Cosmid containing *orf21, orf22* and *orf23*; Chl^{r}, Amp^{r}	This study
8B09*::orf23::aac(3)IV*	8B09 cosmid replaced *orf23* by Apr^{r}	This study
pSMF5400	The derivative plasmid of pWHM3 vector containing *ermE*p; Amp^{r}, Apr^{r}	[32]
pSMF5705	The derivative plasmid of pWHM3 vector containing *ermE*p and *orf23*; Amp^{r}, Apr^{r}	This study
Primers	Oligonucleotide sequences (5′ → 3′)	Usage
ceaS_F	TCA CTA CGG TGT GCT CTT CG	Cosmid screening
ceaS_R	TGG TGA CCG ATG TTC TGG TA	Cosmid screening
ORF13_F	GGC CTC GAC TAC TAC CCT CC	Cosmid screening
ORF13_R	GAA CTG GAT CAC ACC GAA GC	Cosmid screening
ORF19_F	ATG AGC AAT ATC CCG GAG AC	Cosmid screening
ORF19_R	ATC TGG AGG TGC CAG AGC	Cosmid screening
orf21_F_tar	TGT GGT GTG GGG CGG CGA GGC TGA AGG GAA ACG GCC GTG ATT CCG GGG ATC CGT CGA CC	Deletion of *orf21*
orf21_R_tar	GGC GGT GGC GCC CGC GCG GGC GGG GAC GGG GAC GGC CTA TGT AGG CTG GAG CTG CTT C	Deletion of *orf21*
orf22_F_tar	GGT GTC AGC GGT GCC GGT GGT GTC GGT GGT CTC GTA CGG ATT CCG GGG ATC CGT CGA CC	Deletion of *orf22*
orf22_R_tar	CGG GCC CCG CAC CCG TAC CGT CCC CGG GCT GGG CGA CTA TGT AGG CTG GAG CTG CTT C	Deletion of *orf22*
orf23_F_tar	CAG CAG ATA GCC CGC GCC GCG CCG GGT GTG GAT CAT CGG TGT AGG CTG GAG CTG CTT C	Deletion of *orf23*
orf23_R_tar	GCC GGA CTC TCC CGG CCG GAC GGC ACC CCG CTG CGG CTG ATT CCG GGG ATC CGT CGA CC	Deletion of *orf23*
orf23_PstI_F	TGG TCT GCA GTT GAT GAC CCG GAT GG	Overexpression of *orf23*
orf23_EcoRI_R	CGT CGA ATT CTC ATG TCC CCT CCG CG	Overexpression of *orf23*

[a] Amp^{r}, ampicillin resistance; Apr^{r}, apramycin resistance (*aac(3)IV*), Chl^{r}, chloramphenicol resistance

by BLAST analysis (http://www.ncbi.nlm.nih.gov). Domains in the putative genes were analyzed by searching against the Conserved Domain (CD) database (http://www.ncbi.nlm.nih.gov) using Pfam search (http://www.sanger.ac.uk).

Disruption of targeted genes and determination of phenotypes

The new *orf*s (*orf21*, *orf22*, and *orf23*) were disrupted by replacing the genes with the apramycin resistance gene

(*aac(3)IV*) using PCR-targeting mutagenesis [10]. The *aac(3)IV* cassette was amplified by PCR using pIJ773 as template and primer sets specific for each of the genes (see Table 1), and the resulting PCR products were purified using a gel extraction kit (Qiagen). In order to induce the replacement of the targeted genes with the apramycin resistance gene cassette by λ RED recombination, cosmid SCL8B09, carrying all three *orf*s, was introduced into *E. coli* BW25113/pIJ790, and the resulting transformant (*E. coli* BW25113/pIJ790/SCL8B09) was cultured in LB medium containing L-arabinose at 30°C to an OD_{600} of 0.6. The purified *aac(3)IV* cassettes were then individually introduced to *E. coli* BW25113/pIJ790/SCL8B09 and incubated at 37°C for 16 h. Candidate strains in which the targeted genes were disrupted were selected by culturing on LB agar plates containing apramycin (50 µg/ml). Gene replacements in the mutated cosmids were confirmed by digested pattern analysis with restriction enzymes. Confirmed mutant cosmids were transformed into *E. coli* ET12567/pUZ8002 (methylation negative strain) [10], and then conjugated into spores of *S. clavuligerus* ATCC 27064. Exconjugants were recovered by culturing on AS-1 agar plates supplemented with 10 mM $MgCl_2$ at 30°C for 16 h and then overlaying the plates with apramycin (25 µg/ml) and nalidixic acid (20 µg/ml). Colonies that developed on the plates were transferred to MYM agar plates containing apramycin (25 µg/ml) and incubated at 30°C for 5 days [34]. *orf21* was disrupted by replacement of the entire sequence (606 bp) with *aac(3)IV* (1,377 bp), yielding mutant SMF5711 (*orf21::aac(3)IV*). *orf22* was disrupted by replacement of part of the gene (1,090 bp of 1,716 bp) with *aac(3)IV*, yielding mutant SMF5712 (*orf22::aac(3)IV*), and *orf23* was disrupted by replacement of part of the gene (624 bp of 816 bp) with *aac(3)IV* yielding mutant SMF5713. Mutations in the targeted genes were confirmed by Southern analysis using probes labeled with a DIG DNA labeling kit (Roche).

Phenotypes of the mutants were compared with that of the parent strain on MYM [34] and ISP4 agar media (Difco). Spores were inoculated on plates and incubated at 30°C.

Overexpression of *orf23* in *S. clavuligerus*

The multicopy plasmid, pSMF5705 harboring *ermE*p and *orf23* was constructed to allow overexpression of *orf23* [32]. The *orf23* gene was obtained by PCR amplification from SCL8B09 using primers orf23_PstI_F and orf23_EcoRI_R. The *orf23* PCR product, digested with *Pst*I and *Eco*RI, was inserted into pSMF5400, a derivative of plasmid pWHM3 [32], which had previously been modified by insertion of *ermE*p, the highly active promoter of the *ermE* gene. pSMF5400, harboring only *ermE*p, was used as a control plasmid. pSMF5400 and pSMF5705 were transferred by conjugation from *E. coli* ET12567/pUZ8002 to *S. clavuligerus* ATCC 27064 to give strains SMF5714 and SMF5715.

Kinetic analysis of submerged cultures and analytical methods

Spores of the parent strain and disruption mutants were pregerminated by growth in 30 ml of 2xYT medium for 6 h [18]. The pregerminated spores were harvested and transferred into 100 ml of glycerol-arginine (GA) medium [15] in 500 ml baffled culture flasks and incubated at 30°C on a rotary shaking incubator. All strains were grown in duplicate cultures and the independent experiments were repeated twice.

Mycelial growth was estimated by measuring OD_{595} as described [20]. Glycerol concentrations and CA production were quantified by methods described previously and CM production was measured by a bioassay specific for β-lactam antibiotics [15, 24].

Assessment of transcripts of CA genes by semi-quantitative RT-PCR

In order to prepare RNA for analysis of transcripts, mycelium from the parent and SMF5713 strains which were grown in 100 ml of GA medium, was harvested and resuspended in RNA protect bacterial reagent (Qiagen) for 1 h at 30°C. RNA was extracted from the mycelial suspension using phenol-chloroform and then purified using the RNeasy midi kit (Qiagen) according to the manufacturer's instructions. Purified RNA was used for RT-PCR after PCR amplification to check for DNA contamination. The cDNA was synthesized using Superscript II RT (Invitrogen) and then double-stranded DNA was amplified using 2xDyemix DNA polymerase (Enzynomics, Korea). Semi-quantitative RT-PCR was performed using a 95°C denaturation (5 min) step followed by 25 cycles of 94°C (40 s), 57°C (40 s), 72°C (30 s) and finally a post-polymerization step of 72°C (5 min). For transcriptional analysis of CA and CM biosynthetic genes, primers for *ceaS2*, *cas2*, *bls2*, *pah2*, *ccaR*, *claR* and *lat* were used [32]. The abundance of amplified PCR products was estimated by Multi Gauge version 3.0 (Fuji Film) and *hrdB* was amplified to use as an internal control for RNA level.

Results

Identification of three *orf*s downstream of the known CA gene cluster

Total DNA of *S. clavuligerus* was shown to harbor three linear plasmids, 430, 120, and 11.2 kb, as has been reported

previously [22]. Genomic DNA was depleted of plasmids by PFGE and genomic DNA fragments of 35–40 kb were then cloned into the cosmid vector pWEB::TNC. Consequently, 768 clones harboring DNA fragments from the genome of *S. clavuligerus* were obtained. Cosmids SCL3F11 and SCL8B09, identified by PCR screening as harboring the CA gene cluster, were subjected to DNA sequence determination. Both cosmids contained genes from the CA biosynthetic gene cluster, but cosmid SCL8B09 was of particular interest because its insert included three additional *orf*s from downstream of the CA cluster (Fig. 1). Since the three *orf*s are located beyond *orf20* (GenBank Accession No. AY258009), the most distant edge of the clavulanic acid gene cluster described to date, the three *orf*s are now denoted as *orf21*, *orf22*, and *orf23* (GenBank Accession No. EU487003). The start codon of *orf21* is located 808 nucleotides downstream from *orf20* (Fig. 1; Table 2).

In silico annotation of the newly identified *orf*s

orf21 consists of 601 bp with a GC content of 70.8%, and encodes a putative protein of 201 amino acids (Mw 22.77 kDa) and pI of 10.08. The peptide sequence of ORF21, through BLAST analysis, shows significant identity (>47%) and similarity (>60%) to proteins designated as putative RNA polymerase SigL belonging to ECF-subfamily sigma factors (Table 2). This similarity extends to include conserved domains found in regions 2 and 4 of sigma-70 type sigma factors. Region 2 of sigma-70 interacts with the -10 promoter element and is highly conserved in most sigma factors, and region 4 is involved in binding to the -35 promoter element via a helix-turn-helix motif [6]. Therefore, the results of domain analysis further supported the identification of ORF21 as a SigL type RNA polymerase sigma factor (Protein cluster No. PRK09645).

orf22 consists of 1,716 bp with a GC content of 76.3%. It encodes a putative protein of 573 amino acids (Mw 59.75 kDa) and a pI of 6.38, which shows similarity to other proteins denoted as two component system sensor kinases or histidine kinases (Table 2). Through CD analysis (http://www.ncbi.nlm.nih.gov), a histidine residue (H283) was identified as the phosphorylation site within the conserved HisKA domain of ORF22, which represents the most likely site of autophosphorylation and phosphate transfer to an Asp residue in the corresponding response regulator [35]. Other potential dimer interface sites were also identified. A HATPase_c domain, characteristic of the histidine kinase-like family of ATPases is also evident in ORF22, extending from amino acid residues 388–511. Within this domain, potential ATP binding sites, a Mg^{2+} binding site, and two potential G–X–G motifs located in loops defining the top and bottom of the ATP binding pocket were found [9, 23]. Additionally, Pfam analysis identified a HAMP domain at amino acid 184–262, upstream of the HisKA domain in ORF22. HAMP is a highly conserved domain in prokaryotic signaling proteins such as histidine kinases, methyl-accepting proteins and adenylyl cyclases [2]. The presence of these conserved HAMP, HisKA, and HAPase_c domains in ORF22 all support its designation as the sensor kinase element of a two component system.

orf23 consists of 816 bp with a GC content of 72.8%, and encodes a putative protein of 271 aa (Mw 29.67 kDa) and a pI of 7.06. ORF23 showed a high degree of similarity (>89%) to proteins annotated as response regulators (Table 2) with REC and trans-reg_C domains apparent in the N-terminus and C-terminus, respectively. REC domains

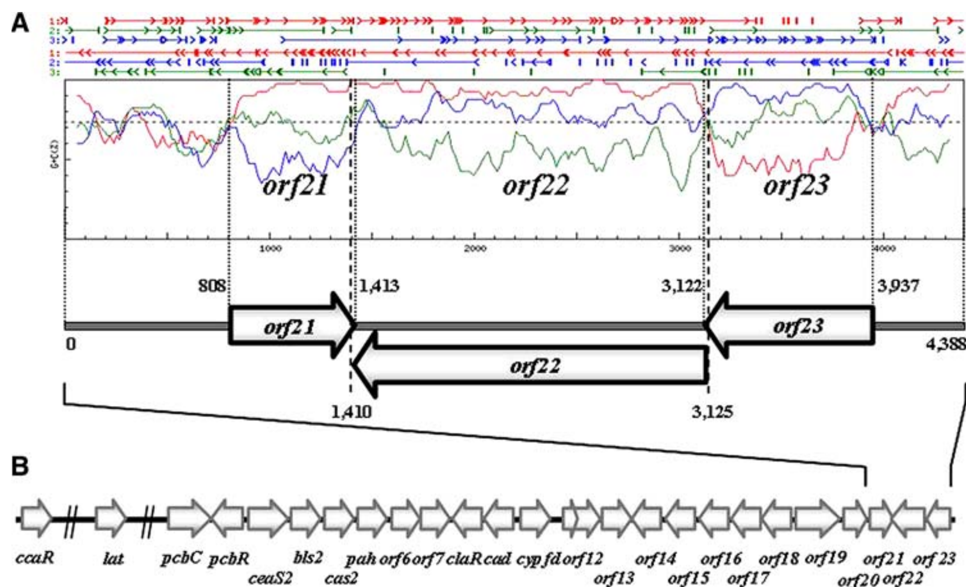

Fig. 1 Frame analysis of the *orf21*, *orf22* and *orf23* region in the newly sequenced DNA. **a** Three *orf*s identified by Frameplot analysis. **b** Diagrammatic representation of *orf21*, *orf22* and *orf23* located in newly sequenced DNA

Table 2 Predicted functions of putative *orf21, orf22* and *orf23* encoded proteins

*orf*s[a]	Size (bp)	Start and stop codon/GC (%)	Amino acid[b]/ Mw (kDa)[c]/pI[d]	Proteins with high similarity			
				Proteins	Accession no. (GenBank)	Identity (%)	Similarity (%)
orf21(808–1413)	606	GTG, TAG/70.8	201/22.77/10.08	Putative RNA polymerase sigma factor SigL (SAV5123, 198 aa)[e] of *Streptomyces avermitilis* MA-4680	NP_826300	47	60
				Putative RNA polymerase sigma factor SigL (SCO2954, 194 aa) *of Streptomyces coelicolor* A3(2)	NP_627178	46	59
orf22(3125-1410)	1,716	ATG, TAG/76.3	573/59.75/6.38	Histidine kinase (Sare2278, 477 aa) of *Salinispora arenicola* CNS-205	YP_001537128	58	68
				Two component system histidine kinase (SCO4021, 524 aa) of *Streptomyces coelicolor* A3(2)	NP_628203	48	59
orf23(3937-3122)	816	TTG, TGA/72.8	271/29.67/7.06	Two component transcriptional regulator (Krad3036, 273 aa) of *Kineococcus radiotolerans* SRS30216	YP_001362764	78	89
				Two component system response regulator (SCO4020, 271 aa) of *Streptomyces coelicolor* A3(2)	NP_628202	75	84

[a] The numbers in brackets show start and stop coordinates of the *orf*s sequenced relative to the end of *orf20*

[b] Number of amino acids

[c, d] Molecular weight (Mw) and pI value were computed by Compute pI/Mw tool (http://www.expasy.org)

[e] The brackets contains the protein ID and number of amino acids (aa) of the proteins with high similarity

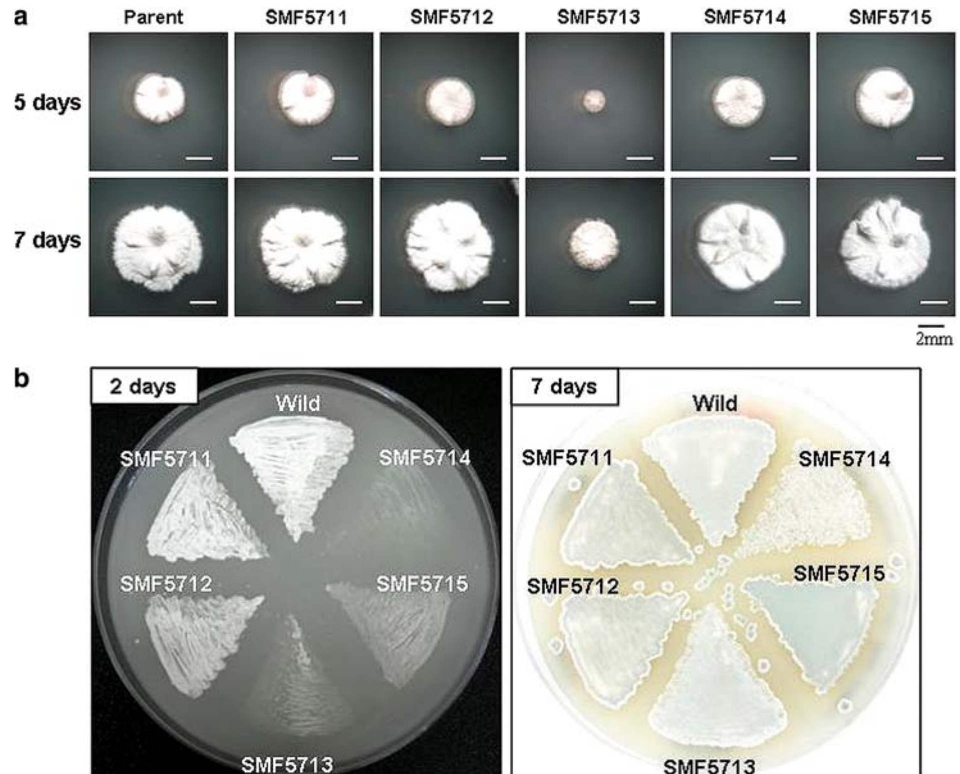

Fig. 2 Morphological phenotypes of *S. clavuligerus* wild-type and modified strains growing on MYM (**a**) and ISP4 (**b**) agar plates

act as receivers of the signal, and possess phosphoacceptor sites that are phosphorylated by their cognate histidine kinases [31]. CD search of the REC domain of ORF23 identified potential phosphorylation sites. In addition, putative intermolecular recognition sites and residues involved in the dimerization interface were detected as conserved motifs [31], and a trans_reg_C type effector domain typical of response regulators, which causes cellular response by binding to DNA or RNA polymerase [16], and conserved residues involved in DNA binding were all evident. ORF23 was also noted to have a domain structure very similar to that of OmpR (osmolarity response regulator) as determined by CD analysis.

Effects of genetic modification on morphology and batch culture kinetics in shake flask

Mutants SMF5711 (*orf21::aac(3)IV*), SMF5712 (*orf22::aac(3)IV*), and SMF5713 (*orf23::aac(3)IV*) were constructed using PCR-targeted mutagenesis whereas strain SMF5715 was created by introduction of *orf23* into the wild-type parent on a multicopy plasmid. The phenotypes of the mutant and modified strains were compared with the wild-type and plasmid control (SMF5714) strains by culturing on MYM and ISP4 agar media at 30°C. Mutants SMF5711 and SMF5712 showed no obvious differences when compared to the wild-type on both media. In contrast, the SMF5713 mutant showed delayed growth and produced smaller colonies compared to the wild-type strain on MYM solid medium (Fig. 2a). Similar results were seen on ISP4, a medium which supports sporulation in *S. clavuligerus*; strain SMF5713 showed retarded mycelial growth and spore formation compared to the wild-type (Fig. 2b).

Strain SMF5715, which overexpresses *orf23* from a multicopy plasmid, showed no difference in growth compared to the wild-type or the plasmid control strains when growing on MYM. In contrast, SMF5715 showed delayed growth initially on ISP4 but ultimately the strain grew well. Moreover, SMF5715 sporulated prematurely and abundantly, when compared with both the wild-type and plasmid control SMF5714 strains (Fig. 2b).

Culture kinetics for glycerol utilization, mycelium growth, and CA production of the wild-type and modified strains in liquid GA medium batch cultures were also compared (Figs. 3, 4). Growth patterns of mutants SMF5711 and SMF5712 resembled that of the parent strain characterized by a rapid increase followed by an equally rapid decline in cell mass (Fig. 3a). Glycerol utilization in the parent and mutant strains was also shown to follow a similar pattern (Fig. 3b). With regard to CA production, SMF5711 resembled the wild-type strain whereas SMF5712 showed CA production reduced slightly to 76% of wild-type levels (Fig. 3c). CM production was also decreased somewhat in strains SMF5711 and SMF5712, to 70 and 59%, respectively (Fig. 3d).

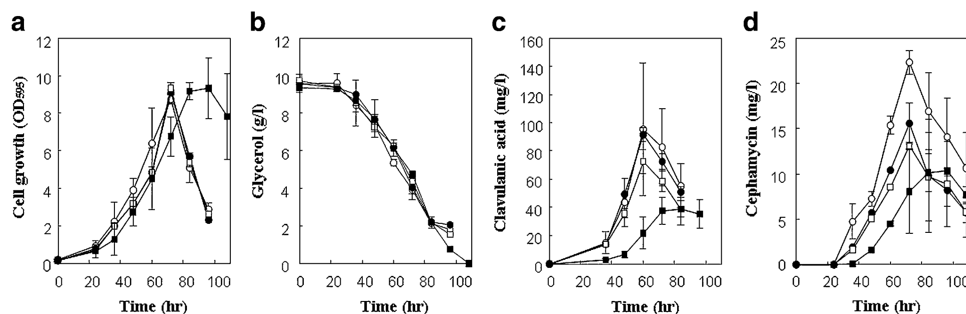

Fig. 3 Kinetic analysis of shake flask batch cultures of *S. clavuligerus* wild-type and *orf21*, *orf22* and *orf23* mutant strains. **a** Cell growth, **b** glycerol utilization, **c** production of clavulanic acid, and **d** production of cephamycin C; Genotypes: parent *S. clavuligerus* ATCC 27064 (*open circles*), strain SMF5711 (*Δorf21::aac(3)IV*; *closed circles*), strain SMF5712 (*Δorf22::aac(3)IV*; *open square*), and strain SMF5713 (*Δorf23::aac(3)IV*; *closed square*). Culture conditions: 100 ml culture in 500 ml baffled flask, 30°C, 220 rpm grown in GA medium

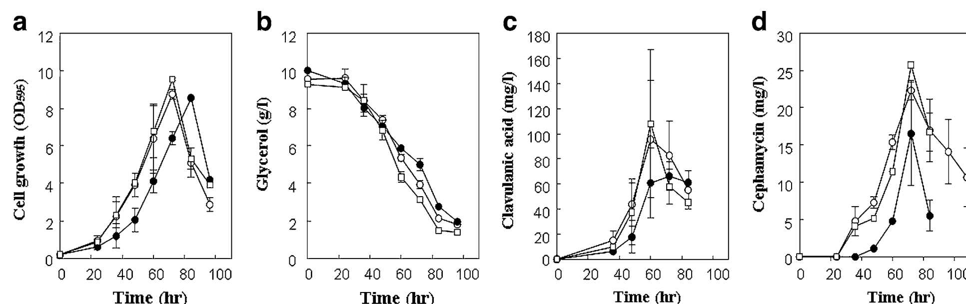

Fig. 4 Kinetic analysis of shake flask batch cultures of *S. clavuligerus* wild-type and ORF23 overproducing strains. **a** Cell growth, **b** glycerol utilization, **c** production of clavulanic acid, and **d** production of cephamycin C; Genotypes: parent *S. clavuligerus* ATCC 27064 (*open circle*), a control strain SMF5714 carrying the *ermE*p in a multicopy plasmid (*closed circle*), strain SMF5715 carrying *ermE*p and *orf23* in a multicopy plasmid (*open square*). Culture conditions: 100 ml culture in 500 ml baffled flask, 30°C, 220 rpm grown in GA medium

The SMF5713 mutant showed a slower growth rate initially, but went on to achieve an extent of growth equivalent to the wild-type by 108 h. The SMF5713 mutant also sustained its cell mass in the stationary phase with much less decline than was seen in the wild-type (Fig. 3a). Despite achieving a similar level of growth overall, CA and CM production in SMF5713 were lower, at 40 and 47% of wild-type, respectively (Fig. 3c, d).

The SMF5715 strain carrying additional copies of *orf23* on a multicopy plasmid, was also grown in submerged culture and CA and CM production was compared with wild-type and plasmid control SMF5714 strains (Fig. 4). The control culture, carrying only *ermE*p on the multicopy vector, showed delayed growth and decreased antibiotic production compared to the wild-type. In the case of the overexpression strain, SMF5715, growth and production of CA and CM, were restored to wild-type levels thereby overcoming the deleterious effects of the multicopy plasmid.

Assessment of transcript levels of CA and CM biosynthetic genes in the *orf23* mutant

Strain SMF5713, the *orf23* defective mutant, showed reduced levels of CA and CM production. In order to determine whether ORF23 regulates expression of CA and CM biosynthetic genes, semi-quantitative RT-PCR was carried out. Among genes involved in CA and CM biosynthesis, *ceaS2*, *cas2*, *bls2*, *pah2*, and *lat,* encoding biosynthetic enzymes specific for the early stages of CA or CM production, were subjected to transcription analysis. Transcription levels of *ccaR* and *claR* were also determined, since these genes encode regulators of CA and CM biosynthesis.

The wild-type and SMF5713 strains were harvested at the peak of CA production in stationary phase and used to prepare total RNA for RT-PCR analysis. When transcription levels of biosynthetic genes involved in antibiotic production were evaluated, the wild-type and SMF5713 strains showed similar patterns for most of the genes (Fig. 5a). The regulatory gene, *ccaR,* also was similarly transcribed in both wild-type and ORF23 defective mutant strains. On the other hand, *claR* transcription levels in the *orf23* mutant strain were lower than those of the wild-type strain as determined by semi-quantitative RT-PCR (Fig. 5b). These RT-PCR results suggest that ORF23 was not involved in transcription of early biosynthetic enzymes for either CA or CM production, but may affect the transcription level of *claR,* a regulator of the late steps of CA production.

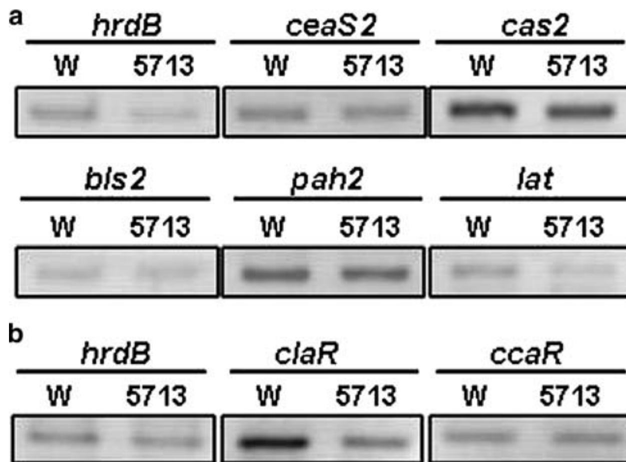

Fig. 5 Assessment of the transcription levels of selected cephamycin C and clavulanic acid biosynthetic genes in wild-type and *orf23* mutant strains of *S. clavuligerus*. **a** RT-PCR analyses of the transcripts from *ceaS2*, *bls2*, *cas2*, *pah2*, *lat* genes involved in CA and CM biosynthesis **b**. RT-PCR analyses of the transcripts from *ccaR* and *claR*, genes encoding regulators of CA and CM biosynthesis. The levels of transcripts from the wild–type (*W*) and strain SMF5713 (*5713*) were compared for each gene using *hrdB* as a control for RNA content

Discussion

We have identified three new *orf*s from downstream of the known CA biosynthetic gene cluster in *S. clavuligerus*. In silico analysis of the products of the *orf*s showed that the *orf21* product is highly similar to sigma factors, while the *orf22* and *orf23* products are predicted elements of a two component regulatory system.

Streptomyces spp. have developed complex cellular and molecular mechanisms to respond to changes in the soil environment, and the existence of more than 60 sigma factors encoded by the genomes of *Streptomyces* spp. provides just one example of this complexity [3, 11]. As further examples of their ability to react to various stimuli, 84 genes encoding sensor kinases and 67 genes encoding response regulators were annotated in the genome of *S. coelicolor* [3, 11]. Most of these two component regulator genes are arranged as cognate pairs, although thirteen orphan response regulators were also characterized as playing roles in development and antibiotic production in *S. coelicolor* [11].

Several examples of the regulation of antibiotic production or development in *Streptomyces* spp. by two component systems have been verified experimentally. The PhoPR system that controls Pho regulon genes was shown to negatively regulate production of secondary metabolites such as actinorhodin (Act) and undecylprodigiosin (Red) in *S. coelicolor* [30]. Deletion of *absA1* and *absA2* caused overproduction of Act and Red explained as Pha phenotype (precocious hyperproduction of antibiotics) [4]. CutRS was shown to exert negative regulation on antibiotic production since gene deletion and overexpression resulted in overproduction and decreased production of Act, respectively, in *S. lividans* and *S. coelicolor* [7]. In addition, AfsQ1 overproduction in *S. lividans* stimulated biosynthesis of secondary metabolites by controlling A-factor production [12].

In the present study, the functions of three *orf*s newly identified in the CM-CA biosynthesis gene cluster were investigated. While mutation of *orf21* and *orf22* showed no major effects on growth or antibiotic production, *orf23* appeared to affect both of these processes. Growth on solid and liquid medium was slowed and sporulation was suppressed by the absence of ORF23 (*orf23* disruptant) whereas overabundance (ORF23 overproducing strain) of ORF23 caused precocious hyperproduction of spores on solid media (Figs. 2, 3). Effects of overabundance of ORF23 on growth in liquid media were more difficult to assess because of the interfering effects of the multicopy plasmid, but when compared to the plasmid control, growth appeared to be enhanced by increased levels of ORF23. Similarly, the ORF23 deficient mutant produced less CA than the wild-type control, whereas overproduction of ORF23 caused CA production to increase relative to that seen in the plasmid control culture.

Transcription levels of the CM and CA biosynthetic genes examined were unaffected in the *orf23* disruptant as judged by semi-quantitative RT-PCR. However, these studies provide only a snapshot of RNA levels at a single time point, that of maximum CA concentration in the culture filtrate, which may not correspond to times of maximum transcript levels. Moreover, the decreased levels of *claR* transcripts seen in the *orf23* mutant suggest that ORF23 may exert its effect on the late steps of CA production whereas the biosynthetic genes that were analyzed by RT-PCR catalyze early biosynthetic steps.

These results suggest that ORF23 may represent the response regulator component of a two component regulatory system capable of positively affecting cell growth and differentiation as well as CA production in response to environmental changes. Since *orf22* presumably encodes the cognate sensor kinase for *orf23*, it is unclear why a corresponding phenotype is not seen for the *orf22* mutant. Perhaps *orf23* can receive inputs from more than one sensor kinase, and so the full phenotype can only be seen with mutation of the response regulator.

Environmental stimuli such as nutrient limitation, growth rate and cell density, as monitored with signaling compounds, are factors controlling the hierarchical regulation of secondary metabolite biosynthesis [8]. Although the regulatory mechanisms controlling the biosynthetic pathway for CA are not fully understood, the findings in the current report indicate that the genes downstream of the CA–CM gene clusters may play a part in the hierarchical

regulation of cell growth, differentiation and CA and CM production under some environmental conditions.

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