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Research article

Improvement of clavulanic acid production in *Streptomyces clavuligerus* F613-1 by using a *claR-neo* reporter strategy

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ABSTRACT

Background: *Streptomyces clavuligerus* was the producer of clavulanic acid, *claR*, a pathway-specific transcriptional regulator in *S. clavuligerus*, positively regulates clavulanic acid biosynthesis. In this study, the promoter-less kanamycin resistance gene *neo* was fused with *claR* to obtain strain NEO from *S. clavuligerus* F613-1. The *claR-neo* fusion strain NEO was mutated using physical and chemical mutagens and then screened under high concentrations of kanamycin for high-yield producers of clavulanic acid.
Results: The reporter gene neo was fused downstream of *claR* and used as an indicator for expression levels of *claR* in strain NEO. After three rounds of continuous treatment and screening, the high-yield clavulanic acid-producing strain M3-19 was obtained. In the shaking flask model, the clavulanic acid titer of M3-19 reached 4.33 g/L, which is an increase of 33% over the titer of 3.26 g/L for the starting strains *S. clavuligerus* F613-1 and NEO.
Conclusions: Our results indicate that neo can be effectively used as a reporter for the expression of late-stage biosynthetic genes when screening for high-yield strains and that this approach has strong potential for improving *Streptomyces* strains of industrial value.

1. Introduction

The improvement of microbial strains plays an important role in reducing production costs during industrial fermentation. The conventional method of "mutation and screening" has been widely used with great success, but it is very time-consuming and labor-intensive. Genetic engineering has been applied to improve the production level of useful compounds, such as through the over-expression of global regulators, pathway-specific regulators, or biosynthetic genes, through the modification of key enzymes, and so on [1,2]. A new strategy was developed that utilizes the co-transcription of a reporter gene and a key biosynthetic gene, thus significantly improving screening efficiency [3,4,5]. Nevertheless, the use of genetic engineering to generate high-yield strains of a particular product has succeeded only in a few cases, and it is still a considerable challenge to improve the production yields of most industrial strains.

Streptomyces clavuligerus was first isolated and screened for β-lactam production [6]; however, it also produces clavulanic acid (CA), which was not recognized until reported by Brown [7]. CA effectively inhibits the activity of β-lactamases of molecular classes A and D, such as cephalosporinases, penicillinases, and broad spectrum β-lactamases, and thus it has been widely used clinically to treat diseases caused by β-lactam resistant bacteria [8]. Whereas, 5S clavams, which are also produced by *S. clavuligerus*, have a 3S, 5S stereochemistry and show only antimicrobial activity, without β-lactamase inhibitory activity [9]. The biosynthetic routes and genes involved in the production of CA and other 5S clavams have been investigated for many years, and gene clusters for the biosynthesis of CA, 5S clavam, and their paralogs have been identified [10,11,12]. In 2010, the draft genome sequence and the sequence of a giant plasmid from *S. clavuligerus* ATCC 27064 were reported [13,14].

Biosynthesis of CA involves early-stage and late-stage synthesis [15], and accordingly, genes participating in CA synthesis are divided into early-stage and late-stage genes. *ccaR* and *claR* are two pathway-specific transcriptional regulators critical for the biosynthesis

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of CA [16,17,18]. CcaR, encoded by a gene in the cephamycin-CA supercluster, positively controls the production of cephamycin C and CA by regulating the expression of *lat*, *cefF*, and *cefD* of the cephamycin C gene cluster, and *ceaS2* and *oppA1*, which are involved in the early-stage synthesis of CA [19,20]. CcaR binds directly to the promoter of *claR*, which positively regulates CA biosynthesis by controlling several genes involved in the late-stage synthesis of CA, including *cad, cyp450,* and *oppA2* [21].

S. clavuligerus F613-1 is an industrial strain with a relatively high CA yield and poor 5S clavam background [22]. Recently, the genome of F613-1 was sequenced [23], providing the first complete genome sequence of *S. clavuligerus*, and we utilized this genomic sequence in our analyses of CA synthesis and regulation in *S. clavuligerus*. In this study, our goal was to improve the CA yield of strain F613-1 using a reporter-guided and iterative mutant selection method. Based on our current understanding of the biosynthesis pathway of CA, genes involved in the early-stage synthesis of CA have paralogs, but genes involved in the late-stage synthesis have only one copy, and therefore we hypothesized that these late-stage genes constitute a bottleneck limiting CA yield. Accordingly, a strategy was developed to enable the co-transcription of the late-stage gene *claR* with the reporter gene *neo*, followed by screening under high selective pressure for strains that yielded elevated levels of CA.

2. Materials and methods

2.1. Plasmids and bacterial strains

All strains and plasmids used in this research are listed in Table 1. *S. clavuligerus* F613-1 is an industrial strain [22] and was used as the starting strain in this study.

2.2. Primers

Primers used in the construction of the reporter vector pJTU28N and in real-time PCR analysis are listed in Table 2.

2.3. Cultivation media

The following media were used to cultivate *S. clavuligerus*: i) BSCA medium (pH 7.5) containing 1.5% malt extract, 0.3% tryptone, 0.4% glucose, and 2.0% agar powder; ii) seed medium (pH 7.1) containing 1.5% soybean powder, 1.0% maize starch, 0.6% yeast extract, 0.07% dipotassium hydrogen phosphate, and 0.8% MOPS; iii) fermentation medium (pH 7.1) containing 1.7% soybean powder, 2.2% soybean protein extract, 3.0% maize starch, 0.15% potassium chloride, 0.1% magnesium chloride hexahydrate, 0.2% dipotassium hydrogen phosphate, 0.04% calcium chloride dihydrate, and 0.8% MOPS.

Table 1
Plasmids and bacterial strains used in this study.

Plasmids or strains	Description	Reference
Plasmids		
pCR-Blunt	General cloning vector	Invitrogen
pSET152	*E. coli–Streptomyces* shuttle vector capable of integrating into *attB* site	[24]
pSET152-NC	Vector containing *neo*, *claR*	This work
pJTU1278	*E. coli-Streptomyces* shuttle vector, *tsr* [a] *bla*[a] *oriT*	[29]
pJTU28N	Reporter vector containing *neo*, *tsr*[a] *bla*[a] *oriT*	This work
Strains		
S. clavuligerus F613-1	Industrial CA producer	[22]
S. clavuligerus NEO	Co-transcribed *claR* and *neo* strain of *S. clavuligerus* containing pJTU28N	This work
S. clavuligerus M1-30	Mutant obtained in the first cycle of mutagenic screening	This work
S. clavuligerus M2-21	Mutant obtained in the second cycle of mutagenic screening	This work
S. clavuligerus M3-19	Mutant obtained in the third cycle of mutagenic screening	This work
E. coli DH5a	General cloning host	Invitrogen
E. coli MA18	*E. coli* containing pJTU1728 vector, *bla*[a]	This work
E. coli ET12567/ pUZ8002	Strain used for conjugation between *E. coli* and *Streptomyces* spp.	[24]

[a] *tsr* means thiostrepton resistant gene, *bla* means ampicillin resistant gene.

Table 2
Primers used in this study (RefSeq: CP016559.1).

Primers	Sequence of primers (5′ → 3′)
For recombinant plasmid construction	
claR L-F	TGGGCTGCAGGTCGACTCTAGAACTACGCCTACACCTGGAA C(2038820-2038839)[a,b]
claR L-R	AGTTCTTCTGAGCGGGCCGCGACCGGGTCCGGA(2040434-2040413)
claR Mid-F	TCGCGGCCCGCTCAGAAGAACTCGTCAAGAAG
claR Mid-R	CGGATGTCCGGCATGATTGAACAAGATGGATTGC
claR R-F	*TGTTCAATCAT*GCCGGACATCCGGGCCCCGGCC(2040438-2040454)
claR R-R	TATCGCGCGCGGCCGCGGATCCGCGAGGAGAATCCGAAGAG C(2041928-2041909)
For validation of co-transcribed strain	
claR V-F	TCGGCGCGAAGTGAGCA(2040332-2040348)
claR V-R	CCACTGGCCGATCCGGC(2040486-2040470)
For real-time PCR	
oppA1 rt-F	GACGAGGGCTTCGACGCCGG(2038705-2038724)
oppA1 rt-R	CAGACCCGGCCGTCCTCGGAG(2038961-2038941)
claR rt-F	GCACGGCACCGTGGAGC(2041704-2041688)
claR rt-R	GCGTCCACGGCCTCGGA(2041510-2041526)
cad rt-F	GAAAGTCGCGCTCATCA(2042782-2042766)
cad rt-R	TCCACCCCCTGCCGGTCGG(2042588-2042606)
cyp450 rt-F	CTCAGTCCGACCAGGTG(2043225-2043241)
cyp450 rt-R	GCTCGTCACCGCGGTGA(2043424-2043408)
orf12 rt-F	ACAAGGAGCGATGATGA(2044868-2044884)
orf12 rt-R	GATGAAGCGCGCGGCCAC(2045045-2045028)
orf14 rt-F	AACGACGACGAAACGGTC(2048199-2048182)
orf14 rt-R	GTCGGGCAGGGCCAGCCG(2048005-2048022)
gcaS rt-F	GTCAACTGGAGCCTGTGT(2052601-2052584)
gcaS rt-R	GTGAGCAGCCAGACCTCA(2052405-2052422)
ceas2 rt-F	AGGCCGCGTCGATTCTCTTCG(2031762-2031782)
ceas2 rt-R	AGAGGTTGGTCATACCGGGGC(2031917-2031897)
bls2 rt-F	TGCCGCTGTACACCTGTGTGG(2033798-2033818)
bls2 rt-R	CGCGGGCACCTGGTAGACAC(2033943-2033924)
pah2 rt-F	ACGGCGCAGAGCCATCTGTC(2035385-2035404)
pah2 rt-R	TTGGTGTCGGAGTGCGCGTC(2035539-2035520)
cas2 rt-F	CTCCGAGCTTCCCGAGGTGC(2036104-2036123)
cas2 rt-R	CGCGCAGCAGCAGATAACCG(2036252-2036233)
oat2 rt-F	CGACTTCACCGTCCTCGCCT(2037412-2037431)
oat2 rt-R	GGTCGCGACATTCGCGTTGC(2037571-2037552)
16S rt-F	CTCGTGTCGTGAGATGTTGG(4098059-4098078)
16S rt-R	ACCTCACGGTTTCGCAGCTC(4098270-4098251)

[a] Numbers represent the location of each primer in the genome of strain F613-1.
[b] The indicated restriction enzyme sites are underlined and were used for cloning purposes.

2.4. Culture conditions for S. clavuligerus

S. clavuligerus F613-1 and its derived strains were grown at 25°C with a relative humidity of 50–60% on BSCA plates for 5 d for bioassay analysis or 8 d for collection of spores. For the liquid-state fermentation, spores on 1 cm^2 pieces of agar were collected and inoculated into 100 mL seed medium, and then cultured at 25°C, with shaking on an orbital shaker at 250 rpm for 48 h to obtain seed cultures. Next, 1 mL seed cultures were transferred to 100 mL fermentation medium and grown at 25°C, with shaking on an orbital shaker at 250 rpm for 120 h to analyze CA production. A 1 mL sample of fermentation liquid was centrifuged at 5000 rpm to collect the mycelium. The biomass was measured before the analysis of CA production.

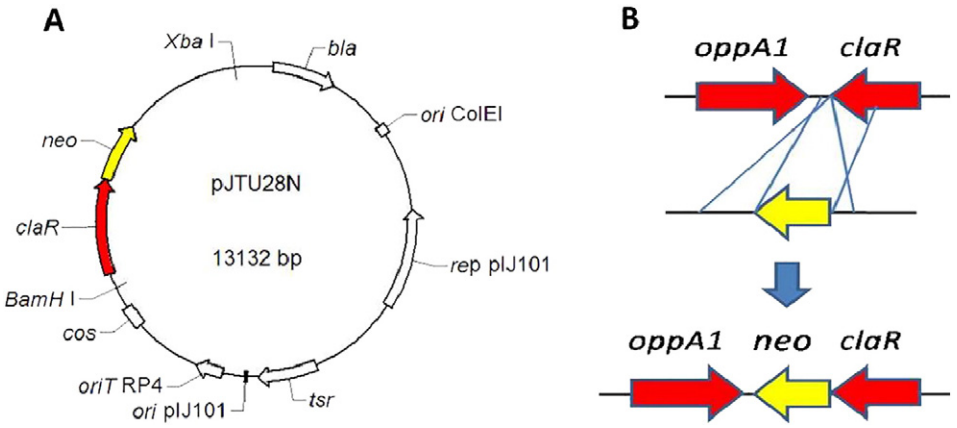

Fig. 1. Schematic diagrams of the *claR-neo* gene fusion design. (A) Plasmid profile of pJTU28N, which contains the fused genes; (B) *claR* and *neo* gene fusion scheme. Three fragments, including the whole *claR* gene, the *neo* gene without its native promoter, and a 1.5 kb fragment downstream of the *claR* gene, were ligated together to form the *claR-neo* fusion gene. *E. coli* DH5α cells were used to screen the recombinant plasmid pSET152-NC for proper cloning and orientation of the fragments. pSET152-NC was digested with *Bam*HI and *Xba*I to get *claR-neo* fusion gene, and ligated with pJTU1278 to yield the recombinant plasmid pJTU28N.

2.5. Plasmids, DNA and RNA extraction

Plasmids from *Escherichia coli* were extracted with plasmid mini-prep columns (BioTeke) according to the manufacturer's protocol. Genomic DNA was isolated from *S. clavuligerus* F613-1 and its derivatives using the Kirby mix procedure [24]. RNA was isolated from *S. clavuligerus* NEO and M3-19 with the RNA extraction kit (SBSBIO) according to the manufacturer's protocol. Briefly, for RNA extraction, mycelium was recovered by centrifugation (5000 rpm, 10 min), and the mycelium pellet was washed and collected by filtering through filter paper to remove impurities in the broth, and ground in liquid nitrogen before dispensing into Redzol reagent. Total RNA samples were treated with Turbo DNA-free reagents (Ambion) to remove chromosomal DNA.

2.6. Construction of the co-transcribed claR-neo strain

The recombinant plasmid pJTU28N was constructed using the Gibson Assembly Master Mix (BioLabs). Using the *S. clavuligerus* F613-1 genome as template, fragment 1, which contained the whole *claR* gene, was amplified by PCR with primers claR R-F and claR R-R (contains a *Bam*HI site) (primer pairs are listed in Table 2); fragment 2, which contained *neo* without its native promoter, was amplified with primers claR Mid-F and claR Mid-R using pCR-Blunt as template; fragment 3, which contained the region downstream of *claR* gene, was amplified with primers claR L-F (contains an *Xba*I site) and claR L-R; and then the pSET152 plasmid was digested with *Xba*I and *Bam*HI to get the fourth fragment, which would serve as the backbone vector. Finally, the four fragments were joined together using the Gibson Assembly Master Mix in vitro, and the assembled construct was transformed into *E. coli* DH5α cells to screen the recombinant plasmid pSET152-NC for proper *claR-neo* fusion gene cloning. Next, the *claR-neo* fusion gene was cut from pSET152-NC using *Xba*I and *Bam*HI, and ligated into pJTU1278 to yield the recombinant plasmid pJTU28N (Fig. 1A). All constructs were verified by DNA sequencing.

Subsequently, pJTU28N was transformed into *E. coli* ET12567 containing pUZ8002 and conjugated into *S. clavuligerus* F613-1 by intergeneric conjugation, as described by Kieser et al. [24]. The positive conjugants were selected using 10 μg/mL kanamycin, and the recombinant strain *S. clavuligerus* NEO was confirmed by colony PCR with primers claR V-F and claR V-R. A schematic diagram of the design of the fusion gene is shown in Fig. 1B.

2.7. Mutagenic screening of the co-transcribed claR-neo strain

A combination of ^{60}Co, nitrosoguanidine (NTG), and UV radiation was used in the three-step mutagenesis. The dosage of each treatment causing 90% lethality was chosen as the mutagenic dosage. First, mature spores were collected to prepare suspensions of 10^6 CFU/mL, which were then induced by ^{60}Co, NTG, and UV radiation. The spore suspensions were then diluted and spread onto BSCA agar plates with various concentrations of kanamycin. Finally, single colonies were picked and screened to identify positive mutants.

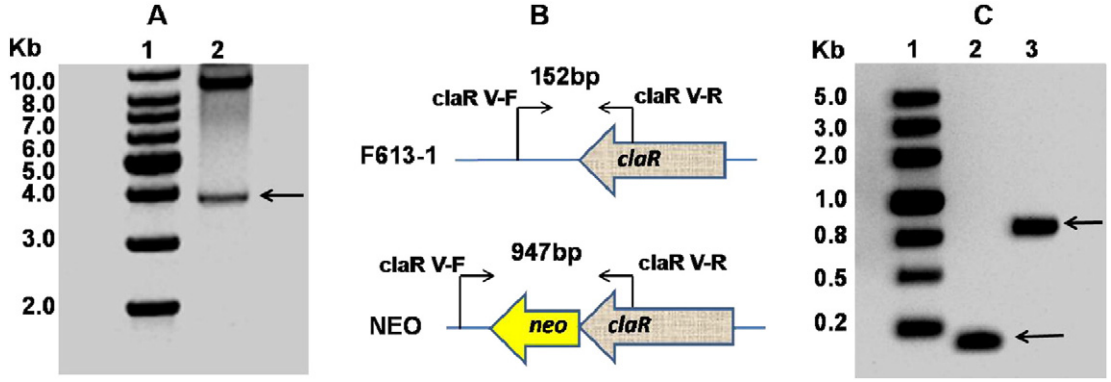

Fig. 2. Verification of the *claR-neo* fusion strain. (A) Double-enzyme digestion of pJTU28N; 1, marker; 2, pJTU28N digested by *Xba*I and *Bam*HI. (B) Diagram showing principle of PCR verification of the double cross-over in strain NEO. (C) PCR verification of strain NEO; 1, marker; 2, strain F613-1; 3, strain NEO.

Table 3
Correlation between dosage of mutagen and lethality rate for strain NEO.

^{60}Co		NTG		UV	
Dosage (Gy)	Lethality rate (%)	Dosage (m/v%)	Lethality rate (%)	Dosage (min)	Lethality rate (%)
400	65.5	0.03	87.34	5	51.32
800	90.12	0.06	91.25	10	67.25
1200	95.63	0.09	98.63	15	89.21
1600	99.82	0.12	100	20	97.32

2.8. Bioassay and HPLC analysis of CA production

The concentration of CA during solid-state fermentation was analyzed by bioassay analysis. *E. coli* MA18 was used as the indicator and was spread on LB agar plates supplemented with 100 μg/mL ampicillin. Briefly, for the bioassay, *S. clavuligerus* were grown on BSCA plates for 5 d, and then BSCA 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 [25]. 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 [22], using clavulanate lithium (provided by Lunan Pharmaceutical Co.) as the standard for quantification.

2.9. Real-time PCR analysis

Total RNA was annealed with random hexamer primers (pdN6, Amersham Pharmacia Biotech), incubated at 65°C for 5 min, and then reverse transcription was carried out at 42°C for 60 min with M-MLV reverse transcriptase (Invitrogen) and dNTPs (Roche). Real-time PCR assays were performed on the LightCycler 480 (Roche) using SYBR Premix Ex Taq kits (TaKaRa). The primers designed for detecting the *oppA1*, *claR*, *cad*, *cyp450*, *orf12*, *orf14*, and *gcaS* genes are shown in Table 2. The cDNA was amplified, and the melting curves of the PCR products and their specificity were determined under the recommended thermal cycler conditions compatible with SYBR Premix Ex Taq, using methods described previously [26]. Relative quantities of cDNA were normalized to the amounts of 16S rRNA. Strain NEO was used as a control.

3. Results and discussion

3.1. Construction of a claR-neo fusion strain of S. clavuligerus

In order to fuse *neo* with *claR*, both *claR* and *neo* were amplified by PCR, joined, and cloned into pSET152; the resulting recombinant plasmids were sequenced to verify correct fusion. Finally the fragment that contained the fused genes was digested with *Xba*I and *Bam*HI, purified, and ligated into pJTU1278 to obtain the recombinant plasmid pJTU28N (Fig. 2A). pJTU28N was then transferred into *S. clavuligerus* F613-1 through conjugation. Single cross-over mutants were screened by plating on BSCA medium containing thiostrepton, and double cross-over mutants were then selected on BSCA medium containing kanamycin. Recombinant strains were confirmed by colony PCR using the primers claR V-F and claR V-R (Fig. 2B–C). The *claR-neo* fusion strain was named strain NEO.

Antibiotic susceptibility tests showed that all colonies of strain NEO survived in medium with 10 μg/mL kanamycin, whereas strain F613-1 could not grow in the presence of this concentration of kanamycin. Additionally, 10% of colonies of strain NEO survived in medium with 75 μg/mL kanamycin. Results showed that strain NEO is kanamycin resistant whereas the original strain F613-1 is sensitive to kanamycin.

3.2. Mutagenic screening of S. clavuligerus NEO

We adopted a three-step mutagenic screening approach to improve the CA yield of strain NEO. We first determined the relationship between mutagenic dosage and lethality rate (Table 3), and the treatment that resulted in a lethality rate of about 90% was chosen. Next, we used a series of three different treatments: in the first round, spores were treated with 800 grays (Gy) of ^{60}Co and were plated on BSCA plates containing 75 μg/mL kanamycin; in the second round, spores were treated with 0.06% NTG for 1 h and then plated on BSCA plates containing 200 μg/mL kanamycin; and in the third round, spores were exposed to 8 W of UV radiation for 15 min followed by plating on BSCA plates containing 400 μg/mL kanamycin (Fig. 3). The ability of mutants to yield high levels of CA underwent preliminary screening on BSCA plates and was further screened using shaking flasks.

During the three rounds of mutagenesis, 300, 420, and 380 mutants were selected for preliminary screening, respectively. Our bioassay data showed that 291, 377, and 250 of those mutants produced larger inhibition zones compared with strain F613-1, resulting in positive mutagenesis rates of 97.0%, 89.8%, and 65.8%, respectively. The top 36, 30, and 30 mutants with larger inhibition zones were selected and were further evaluated using the shaking flask fermentation model, and HPLC analysis showed that the positive mutagenic rates were 88.9%, 76.7%, and 70.0%, respectively. Strains M1-30, M2-21, and M3-19 were obtained from the first, second, and third round of mutagenesis, respectively. The CA titers of M1-30, M2-21, and M3-19 were increased by 12.0% ($P < 0.05$), 24.8% ($P < 0.05$) and 32.8% ($P < 0.05$), respectively, compared with the starter strain F613-1. The CA titers of F613-1 and NEO were about 3.30 g/L in the flask model (Table 4), whereas the CA titer of F613-1 reached 4.87 g/L in the fermentation tank [22]. No differences were detected in the biomass of the mutants and the starting strain. These data showed that both the positive mutagenic rates and the screening efficiency were improved when using the fused *neo* as a reporter for CA titer.

3.3. Genetic stability of the high-yield strain M3-19

Physical and chemical mutagens are often used to improve production of bioactive metabolites [27]. However, these mutagens can easily lead to unpredictable genetic mutations; therefore, the mutagenic strain induced by physical or chemical mutagens usually has poor genetic stability. In addition, insertion sequences (IS), transposons that promote rearrangements and chromosomal deletions are usually associated with genetic instability [28]. Therefore, the genetic stability of the high-yield strain M3-19 was evaluated by continuous subculture for five generations and three replicates each. The fermentation titers of the continuous subcultures were 4.33 ±

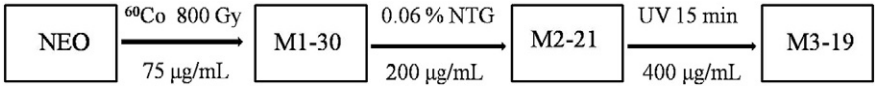

Fig. 3. Flowchart of the mutagenesis procedure for obtaining high-yield CA strains. The spores, approximately 1 × 10^9 CFU/dish, were treated with 800 Gray ^{60}Co, diluted and plated on medium with 75 μg/mL kanamycin in the first round; 0.06% NTG for 1 h and plating with 200 μg/mL kanamycin in the second round; 8 W power UV for 15 min at a 30 cm distance, and plating with 400 μg/mL kanamycin in the third round. The mutated spores used were diluted in series from 10^{-1} to 10^{-6}. The best dilution was chosen according to the number of colonies growing on the plates, and was the dilution that yielded 30–300 CFU.

Table 4
Fermentation titers of M1-30, M2-21, and M3-19 and other strains (unit: g/L).

Strains	Flask1	Flask2	Flask3	Average titer	Relative titer	Deviation standard
F613-1	3.3	3.23	3.25	3.26	1	0.03
NEO	3.24	3.31	3.28	3.28	1.01	0.04
M1-30	3.66	3.72	3.56	3.65	1.12	0.07
M2-21	4.1	3.89	4.21	4.07	1.25	0.13
M3-19	4.41	4.32	4.25	4.33	1.33	0.07

Notes: F613-1 was used as the referral strain for determining relative titers and was given the value of 1. Standard deviations were analyzed from three separate experiments.

0.08 g/L, 4.25 ± 0.23 g/L, 4.29 ± 0.21 g/L, 4.32 ± 0.13 g/L, and 4.37 ± 0.18 g/L, respectively. Statistical analysis revealed no significant differences in the CA titer among subcultures, suggesting that M3-19 is genetically stable.

3.4. Gene expression analysis of the high-yield strain M3-19

In order to clarify the molecular mechanism underlying the high-level production of CA by strain M3-19, expression levels of the 11 late-stage synthetic genes and 5 early-stage synthetic genes of CA (Fig. 4A) were analyzed by real-time PCR, including genes in the operons *cyp450-fd*, *orf12-orf13*, and *oppA2-gcaS* [15]. We first analyzed the transcriptional levels of *claR* during different fermentation stages and found that *claR* expression peaked at 96 h in both the M3-19 and NEO strains, but the transcriptional level of *claR* in M3-19 was approximately 31.4-fold ($P < 0.05$) higher than in NEO (Fig. 4B). To investigate if genes regulated by ClaR were also affected, we compared the expression of the late-stage genes in strains M3-19 and NEO at 96 h. Results indicated that the transcriptional level of *cad* in M3-19 was 93.9-fold ($P < 0.05$) higher than in NEO at 96 h, whereas expression levels of *cyp450*, *orf12,* and *gcaS* were, respectively, 16.1-fold ($P < 0.05$), 6.9-fold ($P < 0.05$), and 10.3-fold ($P < 0.05$) higher than in NEO. However, the two late-stage genes *oppA1* and *orf14* and the five early-stage genes were not obviously up-regulated in M3-19 (Fig. 4C and Fig. 4D). Based on these results, we assume that *ccaR* was not affected during the mutagenesis steps, because CcaR positively controls the early-stage genes [20]. In contrast, *claR* encodes a LysR-type regulator and controls the expression of genes involved in the late steps of the CA pathway [21], and our data suggest that the higher expression of *claR* in strain M3-19 may be up-regulating *cad*, *cyp450-fd*, *orf12-orf13*, and *oppA2-gcaS*, resulting in the increased yield of CA.

4. Conclusion

In this study, we constructed a recombinant *S. clavuligerus* strain NEO, in which *neo* was fused downstream of *claR* and was used as an indicator for expression levels of *claR*, the critical regulatory gene for CA biosynthesis in *S. clavuligerus*. Our strategy involved first treating strain NEO with a chemical or physical mutagen and then

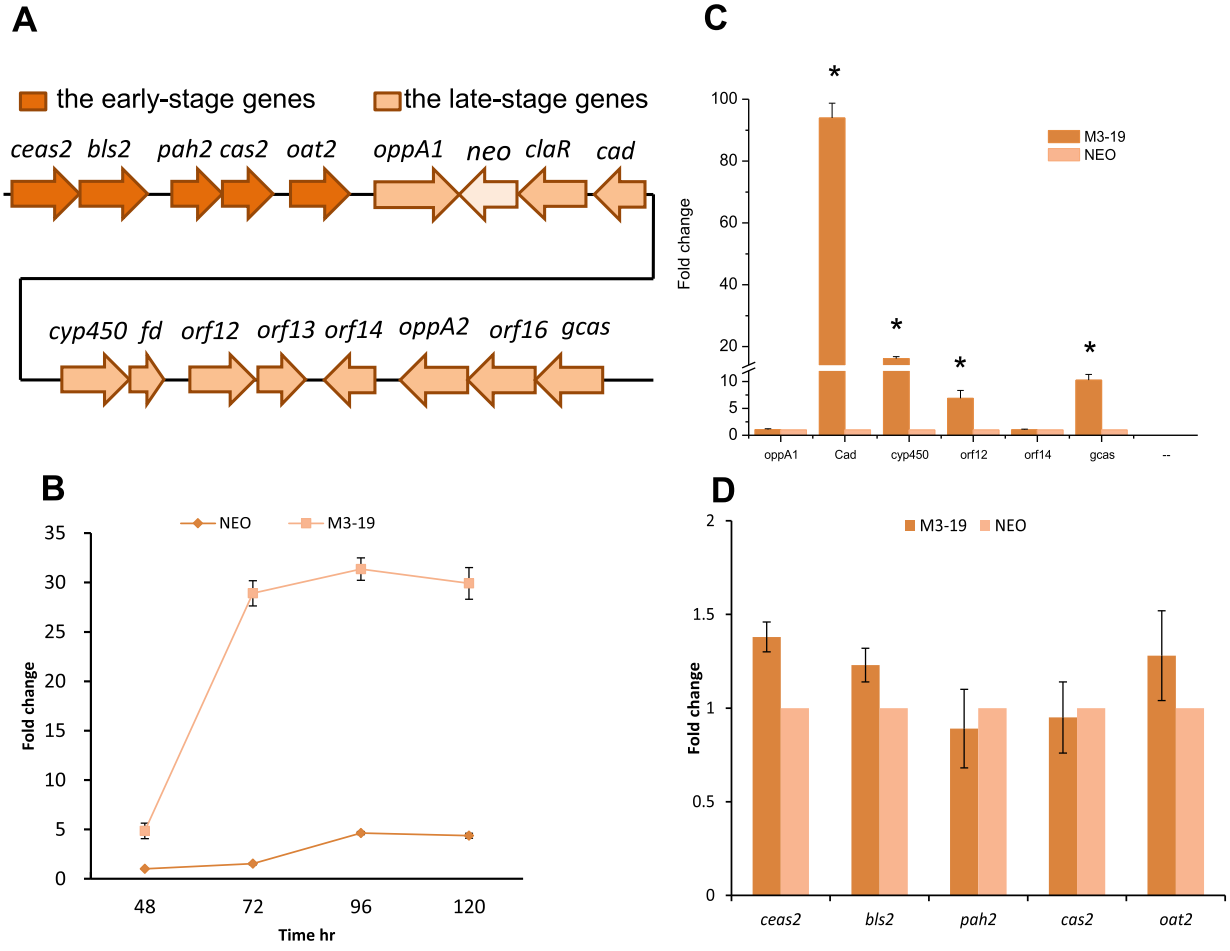

Fig. 4. The expression of CA synthetic genes in strains M3-19 and NEO detected by real-time PCR. (A) The early-stage and late-stage genes in the CA biosynthetic gene cluster. (B) The expression level of *claR* was detected at different time points during fermentation. (C) The relative expression of late-stage synthetic genes was evaluated at 96 h. (D) The relative expression of early-stage synthetic genes was evaluated at 96 h. For (C–D) results are shown as fold change in expression level in M3-19 relative to the level in NEO, which was given the value of 1 for each gene. Data are the means ± standard deviations from three separate experiments. *, statistically significant difference ($P < 0.05$) between M3-19 and NEO.

screening under a high concentration of kanamycin. Results showed that our mutagenesis approach was effective and that the mutation rate in each cycle of treatment was greater than 65%. After three cycles of mutation, the CA high-yield strain M3-19 was obtained. Transcriptional analysis indicated that *claR*, which is fused with *neo*, is dramatically over-expressed (more than 30-fold) in strain M3-19 compared to the starting strain NEO during the late growth phase and that biosynthetic genes involved in late-stage synthesis of CA were also up-regulated. In summary, our study indicates that using *neo* as a reporter for *claR* expression was successful for detecting a high-yield CA strain of *S. clavuligerus* and that this approach has potential for developing improved variants of other industrial strains.

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Conflict of interest statement

We declared that we have no conflicts of interest to this work.

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