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# Deciphering and engineering of the final step halogenase for improved chlortetracycline biosynthesis in industrial *Streptomyces aureofaciens* ☆

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ABSTRACT

Chlortetracycline (CTC) is an important member from antibiotics tetracycline (TC) family, which inhibits protein synthesis in bacteria and is widely involved in clinical therapy, animal feeds and aquaculture. Previous works have reported intricately the biosynthesis of CTC from the intermediates in random mutants of *Streptomyces aureofaciens* and the crucial chlorination remained unclear. We have developed the genetic manipulation in an industrial producer, in which about 15.0 g/l CTC predominated along with 1.2 g/l TC, and discovered that chlorination by *ctcP* (an $FADH_2$-dependent halogenase gene) is the last inefficient step during CTC biosynthesis. Firstly, the Δ*ctcP* strain accumulated about 18.9 g/l "clean" TC without KBr addition and abolished the production of CTC. Subsequently, CtcP was identified to exhibit a substrate stereo-specificity to absolute TC (4*S*) rather than TC (4*R*), with low $k_{cat}$ of $0.51 \pm 0.01$ $min^{-1}$, while it could halogenate several TC analogs. Accordingly, we devised a strategy for overexpression of *ctcP* in *S. aureofaciens* and improved CTC production to a final titer of 25.9 g/l. We anticipate that our work will provide a biotechnological potential of enzymatic evolution and strain engineering towards new TC derivatives in microorganisms.



## 1. Introduction

Tetracyclines (TCs) comprise a group of broad spectrum, orally active antibiotics produced by species of *Streptomyces*. Several natural and semisynthetic members of TCs are widely used in human medicine, animal feeds and aquaculture. Chlortetracycline was isolated from *S. aureofaciens* as the first member of TCs by Cyanamid scientist, Benjamin Minge Duggar, in 1948 (Duggar, 1948) and followed by Pfizer's discovery of oxytetracycline (OTC) from *S. rimosus* in 1950. Subsequently, TC was isolated in small quantities from spent broth of both *S. aureufaciens* (Backus et al., 1954) and *S. rimosus* (Perlman et al., 1960). By forming complexes with bivalent metal ions, such as magnesium, TCs target the 30S ribosomal subunit and inhibit bacterial protein synthesis with relatively few side effects (Brodersen et al., 2000; Nelson, 2002; Pioletti et al., 2001). The biomedical success of TC, saving countless lives over the past six decades, has generated plenty of scientific disciplines, such as biochemistry, crystallography, semisynthesis, spectrum, biological model of activity and resistance of TCs ranging from nature products to the third generation semisynthetic tigecycline approved for clinical use in 2005.

Nevertheless, great value of TCs did not make the number of publications concerning the genetics of their producer, *S. aureofaciens*, significant (Petkovic et al., 2006). The main features of the TC biosynthetic pathway were deduced from early feeding studies and extensive studies of mutant strains of *S. aureofaciens* with genetic blocks. In the course of "cross-feeding" experiments, blocked mutants produced diffusible compounds that enabled other blocked mutants to produce the final products (McCormick et al., 1960a). The promiscuity of some of the enzymes involved in the product allowed many of the later steps to proceed even if one step was not achievable. In the case of industrial manufacturing, CTC product could be inhibited in the normal strain of *S. aureofaciens* by supplying cultures with either aminopterin for demeclocycline, or mercaptothiazole/potassium bromide for TC (Fig. 1). A great deal of microbiological research has been made to meet TCs commercial requirement, ranging from random approach by NTG or X-ray to rational metabolic engineering (Olano et al., 2008; Tang et al., 2011; Yu et al., 2012).

Now, molecular microbiological research indicates that TCs are synthesized by type II polyketide synthase. The minimal PKS generates the poly β-ketone backbone, through successive Claisen like decarboxylative condensations of malonyl-CoA extender units (Fu et al., 1994; Vaněk et al., 1971, 1973). The nonaketamide



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backbone undergoes a set of region selective cyclization by cyclases and modifications by tailoring enzymes, such as oxygenase, transferase, as well as halogenase (Vaněk et al., 1971). While the core structure, including the keto-enol configuration across C-11, C-11a, C-12 positions and hydroxyl residue at C10, C12a, is essential for the biological activities, the chlorine atom at C7-position of CTC and demeclocycline, is replaced in the semisynthetic minocycline and continued glycylcycline derivatives (Doan et al., 2006; McCormick et al., 1960b).

Recently, Tang's group have elucidated more details of the OTC biosynthetic pathway through heterologous expression in *S. ceolicolor* and biochemical analysis (Pickens and Tang, 2009; Pickens and Tang, 2010; Wang et al., 2012; Zhang et al., 2006, 2008). For CTC, Nakano et al. cloned three genes responsible for the CTC biosynthesis and deduced the penultimate reduction of the C5a–C11a in 6-demethylchlortetracycline producer, *S. aureofaciens* NRRL 3203 (Dairi et al., 1995; Nakano et al., 2004, 2000). Later on, the *ctc* gene cluster appeared in the US patent (Ryan et al., 1999). As a matter of fact, the CTC biosynthetic pathway was intricately elucidated, since the genetic differences between *S. aureofaciens* wild type and random mutants remained unknown.

Herein we developed the genetic manipulation in an industrial strain of *S. aureofaciens*, which attracted many attempts in the past decades. As chlorine modification on the TCs core structure is very special in the biosynthesis of CTC, we first aimed to functionally assign in the *ctc* gene cluster the two-component halogenase, CtcP, formally known as Cts4 with an ignorance of N-terminal 103 amino acids (Dairi et al., 1995). Surprisingly, the *ctcP* disruptive mutant yielded "clean" TC (without adding potassium bromide) up to an industrial level, comparable to the parent's CTC yield. Further reconstituted *in vitro*, CtcP showed substrate specificity to TC (4*S*) with proof of inefficiency. Overexpression of *ctcP* in *S. aureofaciens* resulted in remarkable CTC yield improvement. We highlight that *ctcP* is responsible for the last rate-limited step during CTC biosynthesis, and anticipate that our results will inspire the directed evolution of halogense as well as provide a platform for the research of biosynthesis of TCs and their new derivatives.

## 2. Materials and methods

### 2.1. Bacterial strains, culture conditions and general methods

Industrial *S. aureofaciens* F3 was provided by Jinhe Biotech. Co., Ltd. (Hohhot, China). The strains of *Streptomyces* were cultured on YM medium (See SI) 6–7 days for spores. *Escherichia coli* DH10B, BW25113 harboring pKD46, and ET12567/pUZ8002 were used for cloning, λ-Red-mediated recombination and conjugation between *E. coli* and *Streptomyces* spp., respectively, according to standard procedures (Kieser et al., 2000).

*Escherichia coli* BL21 (DE3)/pLysE and pET28a derivatives were used for protein expression. When antibiotic selection of transformants was necessary, 50 μg/ml of apramycin, 50 μg/ml of spectinomycin or 30 μg/ml of kanamycin were used. A complete list of the strains and plasmids used in this study is presented in Table S1. PCR amplification was carried out using either Taq DNA polymerase or KOD DNA polymerase if necessary with genomic DNA or fosmid as a template and specific primers, as listed in Table S2. Any other recombinant DNA techniques for *E. coli* and *Streptomyces* were as described (Kieser et al., 2000; Russell, 2000).

### 2.2. Cloning and analysis of the ctc gene cluster from industrial S. aureofaciens

The complete genomic DNA library of industrial *S. aureofaciens* F3 was constructed followed the protocol of CopyControl™ Fosmid Library Production Kit (Epicentre). Fosmid 11D1 harboring the whole *ctc* gene cluster was identified by PCR screening with primers chl3F/R according to the halogenase gene sequence (Table S2). Shotgun sequencing resulted in two contigs and combined primer walking technique closed a 20 bp gap. The *ctc* gene cluster was analyzed using Frameplot 4.0 (http://nocardia.nih.go.jp/fp4/) and the roles of all proteins were assigned with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi, Table S3). The halogenase gene and the flavin reductase gene were identified and submitted to protein alignment with their homologous ones with Clustal W.

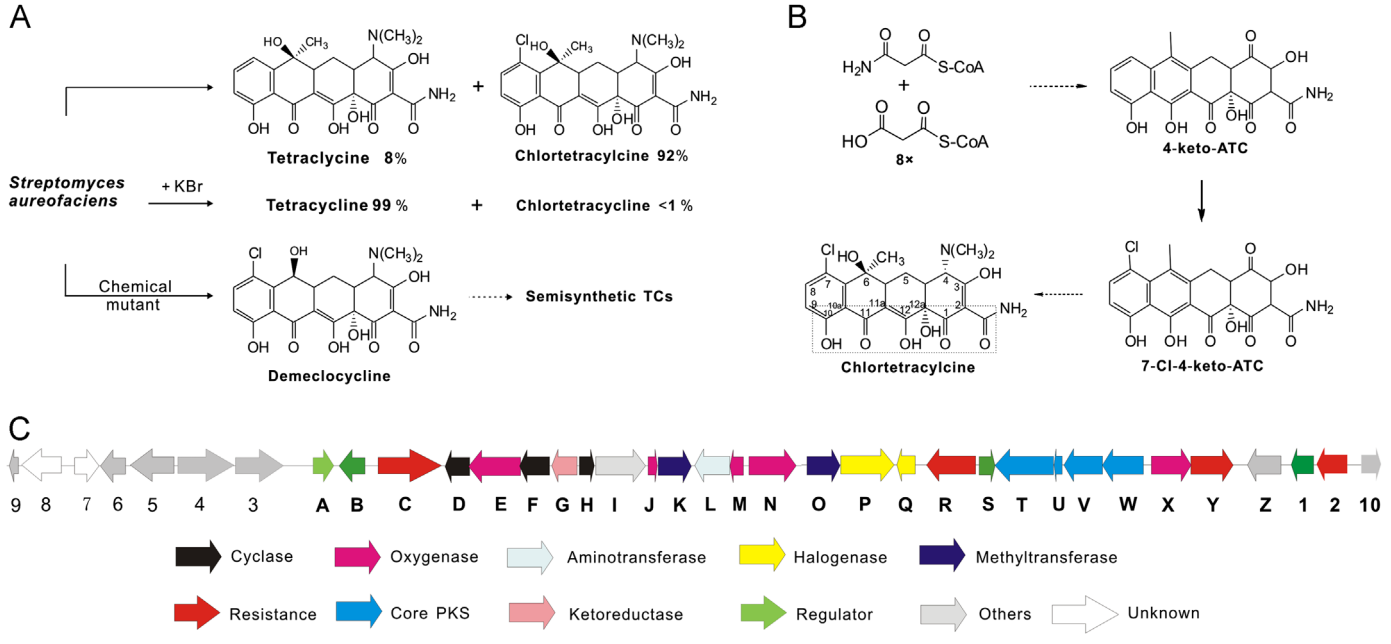



**Fig. 1.** Industrial products from *S. aureofaciens* and the biosynthesis of TCs. (A) *S. aureofaciens* provides the commercial antibiotics CTC and TC, and also demeclocycline which manufactured by multistep chemical transformations to minocycline and tigecycline. (B) Published CTC biosynthesis and proposed halogenation of 4-keto-anhydrotetracycline (4-keto-ATC). (C) Genetic architecture of the complete CTC biosynthetic gene cluster from *S. aureofaciens*.

### 2.3. Fosmid-based gene inactivation and complementation in S. aureofaciens

Fosmid 11D1 contained the whole *ctc* gene cluster was first introduced into *E. coli* BW 25113, which harboring the inducible λ-Red recombinase. Targeted gene was mutated on 11D1 by PCR targeting strategy (Kieser et al., 2000) and then the whole fosmid was introduced into *S. aureofaciens* by conjugation between *E. coli* ET12567 and *Streptomyces*. In the PCR-targeted fosmid 11D1, the 1668 bp complete coding DNA sequence (CDS) of the halogenase gene, *ctcP*, was replaced by a 1484 bp *oriT+aadA* cassette using primers targPF/R. The halogenase inactivated *Streptomyces* strains were confirmed by PCR using primers designed 100–200 bp away from both terminals of the halogenase gene.

For the complementation of the halogenase, a 2070 bp fragment containing both promoter and CDS of *ctcP* was amplified from the genomic DNA with primers P1/P2. The PCR product was double digested with XbaI/EcoRI and ligated to the corresponding sites of pSET152, generating plasmid pJTU4302. At the same time, the complete CDS of both *ctcP* and *ctcQ* were amplified and inserted into pIB139 under the control of the consecutive promoter *PermE**, resulting plasmid pJTU4303 and pJTU4304, respectively. The HindIII-digested 1.9 kb fragment of pJTU4303 was treated with Klewnow I to fill-in of 5′-overhangs and inserted into the EcoRV site of pJTU4304, producing plasmid pJTU4305. All of the plasmids were integrated into the chromosome of the *ctcP* deficient strain by conjugation and the insert plasmids were investigated by PCR with primers M13 (−47/−48).

### 2.4. Production, isolation and analysis of TCs and their derivatives

For the profiles of antibiotic productions of all *Streptomyces* strains, spores were incubated at 30 °C in seed medium (See SI) for about 24 h and transferred with a volume of 10% to fermentation medium (See SI) for another 4–5 days for antibiotic production. Property antibiotics were added within seed medium for selection of the mutants and fermentation procedure was performed in 500 ml flask containing 100 ml liquid medium.

After incubation, the broth was adjusted to pH = 1.5–2.0 with oxalic acid to release the antibiotics from cells. The lysate mixture was centrifuged and the supernatant was collected. HPLC procedure was performed on an Agilent 6530 Accurate-Mass Q-TOF LC/MS system. The mass spectrometer was operated with a 5.0 l/min drying gas flow rate at 300 °C, under 35 psi nebulizer pressure and positive ion mode. Fragments between 100 and 800 m/z were detected. All samples were analyzed on an Agilent TC–C18 column (5 μm, 4.6 mm × 250 mm$^2$). The separation was achieved under a constant flow rate of 1.0 ml/min with 80% buffer A (contained 20 mM oxalic acid and 20 mM triethylamine, pH 2.0) and 20% buffer B (contained authentic acetonitrile). All TCs were monitored at 360 nm and quantified on the basis of peak areas from standard curves using the Mass Hunter software.

### 2.5. Heterologous expression and anaerobic purification of recombinant CtcP/CtcQ

For the heterologous expression of CtcP in *E. coli* BL21(DE3)/pLysE/pJTU4308 (pET28a harboring *ctcP*), the full CDS of *ctcP* was amplified from the genomic DNA using primers ctcPF/R and inserted into the NdeI/HindIII sites of pET28a (Table S2). For the overexpression of the flavin reductase CtcQ, plasmid pJTU4309 (pET28a containing) followed the same procedure using primers ctcQF/R (Table S2). For the expression of C-terminal His-tagged CtcQ, full length of *ctcQ* gene was amplified with primers ctcQ3F/R (Tables S1 and S2) and inserted into the corresponding sites of pET28a digested with NcoI/HindIII, resulting plasmid pJTU4310. The construction of pJTU4340 for the expression of the *E. coli* flavin reductase, *fre*, followed the procedure provided before (Lin et al., 2007). All recombinants were cultured in *E. coli* BL21(DE3) in LB medium containing kanamycin and chloramphenicol and induced by isopropyl-β-D-thiogalactoside (IPTG) for protein production. Strains were first grown at 37 °C to $A_{600}$ = 0.6, induced by 0.8 mM IPTG and then incubated at 16 °C for another 16 h. The purification proceeded in an anaerobic Unilab glove box (MBRAUN, USA) with less than 2 ppm of $O_2$. $His_6$-tagged proteins were absorbed to a 1 ml HiTrap chelating column (GE Healthcare) and eluted at a rate of 1 ml/min with 500 mM imidazole and 500 mM NaCl in 20 mM Tris–HCl (pH 7.9) using the Amersham Biosciences ÄKTA FPLC.

Eluted proteins were further purified by gel-filtration chromatography on Superdex G200 GL10 (GE healthcare). The buffer changed to PBS (pH 7.4) by centrifuge filtration (10.0 kDa cutoff, Millipore) and then stored at −80 °C. The final storage buffer contained 20 mM PBS (pH 7.4) and 10% glycerol. The purity of protein was examined by 12% SDS-PAGE, and the concentration was determined by Bradford assay using bovine serum albumin (BSA) as the standard.

### 2.6. In vitro reconstitution of halogenation step catalyzed by CtcP/Q

Both of CtcP and CtcQ were purified to homogeneity. The halogenation reaction were prepared in a Unilab glove box maintained < 2 ppm $O_2$, as described before (Tseng et al., 2004). The general enzyme assay of CtcP catalyzed at 30 °C for 6 h in a 100 μl system in the presence of 20 mM PBS (pH 7.4), 100 μM FAD, 5 mM NADH, 200 μM PKS substrates, 100 mM NaCl, 100 μM purified CtcP and 5 μM purified CtcQ. The reaction ended by addition of 900 μl MeOH and centrifuged twice to remove the precipitated proteins. The supernatant was evaporated *in vacuo* below 37 °C and dissolved in 100 μl water before subjected to HPLC and LC–MS. Besides TC and OTC purchased commercially, TC (4*S* or 4*R*), 2-acetyl-2-decarboyamino-tetracycline (2-ADTC) and 6-demethyltetracycline (6-DMTC) were also utilized as the polyketide substrates under the same condition.

### 2.7. Overexpression and transcriptional analysis of the two-component halogenase under PermE* in Streptomyces

To investigate whether the halogenase was inefficient or not *in vivo*, the associated genes were over-expressed in *S. aureofaciens*. The two-component genes, *ctcP* and *ctcQ* were integrated into chromosme with 1–5 extra copies under the control of the consecutive promoter *PermE**, respectively. The XbaI/SpeI fragment from pJTU4321 underwent repeats of self-cutting-insertion (Fig. 5A), resulting pJTU4321-25, all of which were introduced into *Streptomyces*. The fragments integrated from heterologous vector were investigated by PCR with general primers M13(−47)/(48). For details of the constructions of multiple-copy plasmids, see Fig. 5A.

Mycelia in fermentation media were collected during a beginning period of 96 h for transcriptional analysis. Total RNA extraction processed using the Redzol reagent according to standard protocol (SBS Genetech Co., Ltd.). The concentration of DNase I (MBI Fermentase) treated RNA was determined by the absorbance at 260/280 nm with Nanodrop (Thermo Co., Ltd.). Reverse transcription was done using RevertAid$^{TM}$ H Minus First Strand cDNA Synthisis Kit protocol (MBI Fermentase) and followed a second step amplification: 25 °C for 5 min, 42 °C for 60 min and 72 °C for 5 min. The specific primers chl3F/R were used for the transcriptional analysis of *ctcP* and another pair of primers RT-QF/R for *ctcQ*. The 16S rRNA gene of *Streptomyces* and the gene *ctcV* encoding the initial keto-synthase ($KS_\alpha$) in the *ctc* cluster were used as the internal controls.

## 3. Results

### 3.1. Analysis of the ctc gene cluster from industrial S. aureofaciens

Setting out with an industrial strain of *S. aureofaciens* F3, we constructed the complete genomic DNA library of *S. aureofaciens* F3 for the cloning of CTC biosynthesis cluster. One fosmid, 11D1, harboring the whole *ctc* gene cluster, was identified by a PCR screening with primers chl3F/R according to the halogenase gene sequence (Table S2). Shotgun sequencing resulted two contigs and a combined primer walking technique closed a 20 bp gap. The *ctc* gene cluster was analyzed using Frameplot 4.0 and the predicted roles of all proteins were assigned with BLAST (Table S3). A total 43.9 kb fragment contained 35 open reading frames (ORFs) and 28 of them were associated with TCs biosynthesis. Two of them, at the 3′-terminal encoding efflux pumps, were not included in previous sequence (Ryan et al., 1999). One of the ORFs, *ctcP,* was predicted to encode an $FADH_2$-dependent halogenase and adjacent to a falvin reductase gene, *ctcQ*. The complete DNA sequence and deduced protein function have been deposited in GenBank under the accession number HM627755. Details of the protein functions are listed in Table S3.

### 3.2. Inactivation of ctcP resulted in accumulation of high yield "clean" TC in S. aureofaciens

In order to eliminate the undesired side product, TC, we decided to find the role of the halogenase gene by knocking out *ctcP*. It was reported that the pIJ702 derivate plasmids could be introduced into *S. aureofaciens* NRRL3203 protoplast at a frequency less than $10^{-6}$ (Dairi et al., 1995). Based on the molecule mechanism of recombination between large DNA fragments, we overcame the obstacle of DNA engineering in *S. aureofaciens*, which produced CTC (15.0 g/l) with minor TC (1.4 g/l). The *ctcP* disruptive mutant, ZT04, abolished the production of CTC and accumulated "clean" TC with a yield of about 18.88 ± 2.68 g/l (Fig. 2C). This indicated that

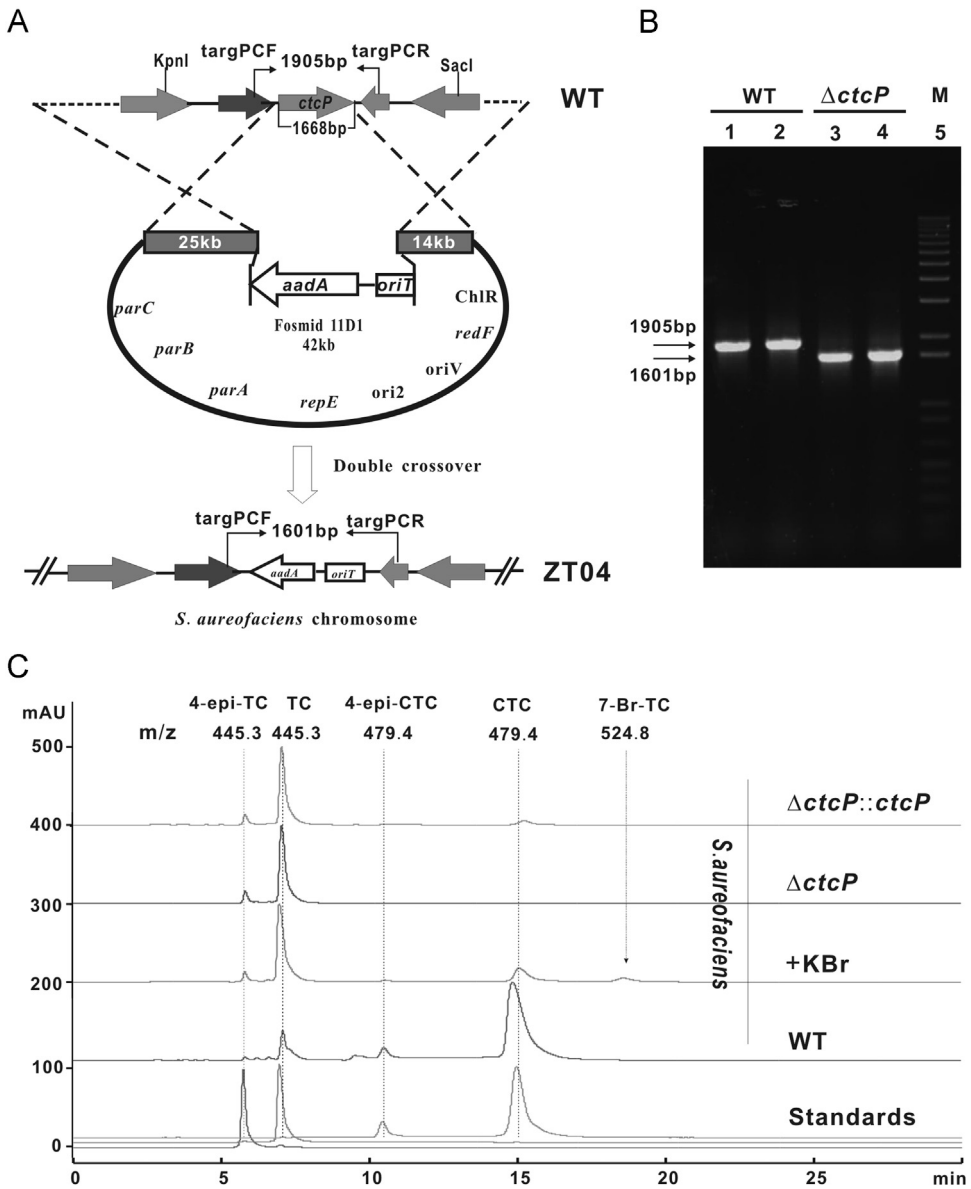



**Fig. 2.** *In vivo* features indicated that the halogenase was responsible for the last step during CTC biosynthesis in *S. aureofaciens*. (A) The complete CDS of the halogenase gene, *ctcP*, was replaced with an *oriT+aadA* cassette using fosmid-based gene replacement in *S. aureofaciens*. (B) The gene replaced was confirmed by PCR. (C) HPLC UV (360 nm) of the product profiles during *Streptomyces* fermentation. In the wild type, CTC was predominant during the fermentation. However, TC became the main product when 0.25% KBr was added to the fermentation broth. Deletion of *ctcP* resulted in an accumulation of high yield "clean" TC and CTC restored after complemented with *ctcP*.

halogenation was possibly the last step during CTC biosynthesis, which was supported by the consequent complementation of the halogenase gene. The complete CDS of *ctcP* was amplified and inserted into pIB139 under the control of the consecutive promoter *PermE** and then integrated into the *ctcP* deficient mutant. All of the complementary strains harboring *ctcP* restored the production of CTC (Fig. 2C and Table S1).

### 3.3. Heterologous expression and anaerobic purification of recombinant CtcP/CtcQ

In order to investigate the role of CtcP, both the halogenase and the flavin reductase CtcQ were expressed in *E. coli* for *in vitro* reconstitution. BLAST and Pfam analysis showed that CtcP was a member of the flavin-dependent halogenase family and shared 72% (377/522) identity to DacE (Wang et al., 2012), 47% (252/532) to PrnA (Dong et al., 2005) and 41% (195/478) to PltM (Zhang and Parry, 2007). CtcP has previously been reported to be a protein containing 452 amino acids, but here showed as a 555 amino acid-long protein. In fact, the N-terminal (residues 22–27) contained the FAD/FMN binding motif G(G/S)GXXG, which was necessary for attachment of $FADH_2$ during the reaction (Bellamacina, 1996) and the shorter enzyme was insoluble during our initial attempt to express it in *E. coli*. The purified 66.3 kDa N-terminal His-tagged CtcP revealed an unusual dimmer style (Fig. 3A).

At the same time, we set out to purify the flavin reductase. Initial attempts to produce N-terminal His-tagged CtcQ yielded soluble, but inactive enzyme. In the contrast, the N-terminal His-tagged flavin reductase, *fre*, from *E. coli* worked well (Fig. S2). We were able to over express the C-terminal His-tagged CtcQ, which catalyzed the reduction of FAD quite efficiently when NADH provided (Fig. S2).

### 3.4. In vitro reconstitution of the halogenation step catalyzed by CtcP/Q

The flavin-dependent halogenation requires reduced $FADH_2$, chlorine ion and oxygen as cosubstrates, as the formation of a FAD (C4a)-OOH intermediate was detected by stopped-flow spectroscopy. It has been proposed that this intermediate generated HOCl first, followed the formation of an enzyme Lys-$\varepsilon$NH-Cl, lysine chloramine, as the proximal chlorinating agent which will chlorate the substrate. The content of oxygen in the reaction buffer is the critical element (Dong et al., 2005; Yeh et al., 2006, 2005). The halogenase system must be mixed under anaerobic glove box first and then exposed to air. As a matter of fact, the reaction could be finished occasionally when we first set to manipulate the protein purification of CtcP/Q under the aerobic condition and the enzyme activity was quite low if the solution prepared under aerobic condition.

To identify the enzymatic activity, the final samples were applied to HPLC. The product profiles illustrated two new compounds, which were identical to the authentic CTC (4*S*) and 4-epi-CTC (4*R*), respectively, with the same retention time and UV spectra. The exactly m/z of these compounds were verified by the LC–MS ($[M+H]^+ = 479.1$ corresponding to 4-epi-CTC or CTC, Fig. 3C). As shown in

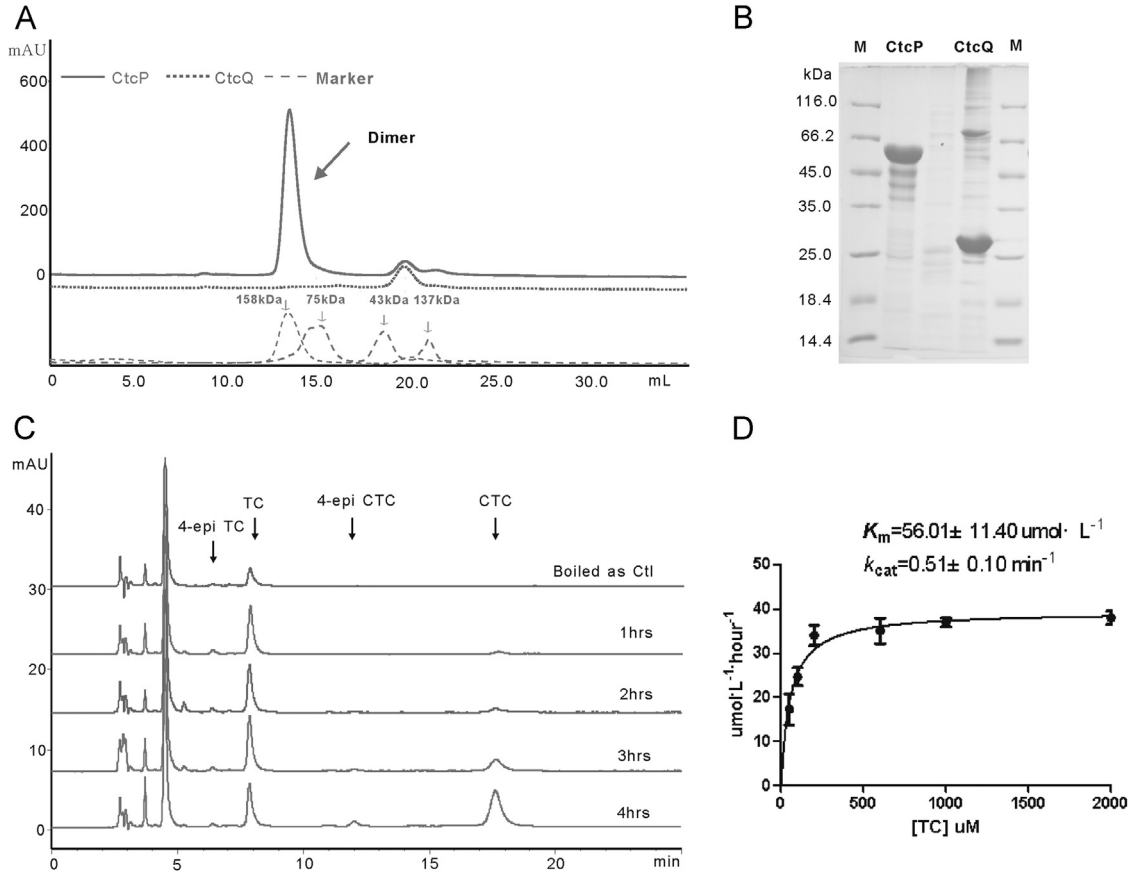



**Fig. 3.** Heterologous expression and *in vitro* enzymatic assay of the two-component halogenase CtcP/Q. (A) Superdex GL200 Gel filtration and (B) SDS-PAGE analysis of the purified CtcP and CtcQ. The $His_6$-CtcP ($M_r$=66.3 kDa) from *E. coli* behaved as a dimer. (C) Time course of the addition of chlorine to TC by the two-component halogenase, CtcP/Q. Reactions were prepared separately in 1.5 ml tubes and incubated at 30 °C. Antibiotics were isolated during 4 h with an interval of 1 h. CtcP denatured at 100 °C for 10 min was added as a control. (D) Enzymatic kinetics assay of TC to CTC conversion by CtcP/Q. Samples were incubated at 30 °C for 6 h using 0–2000 μM TC.

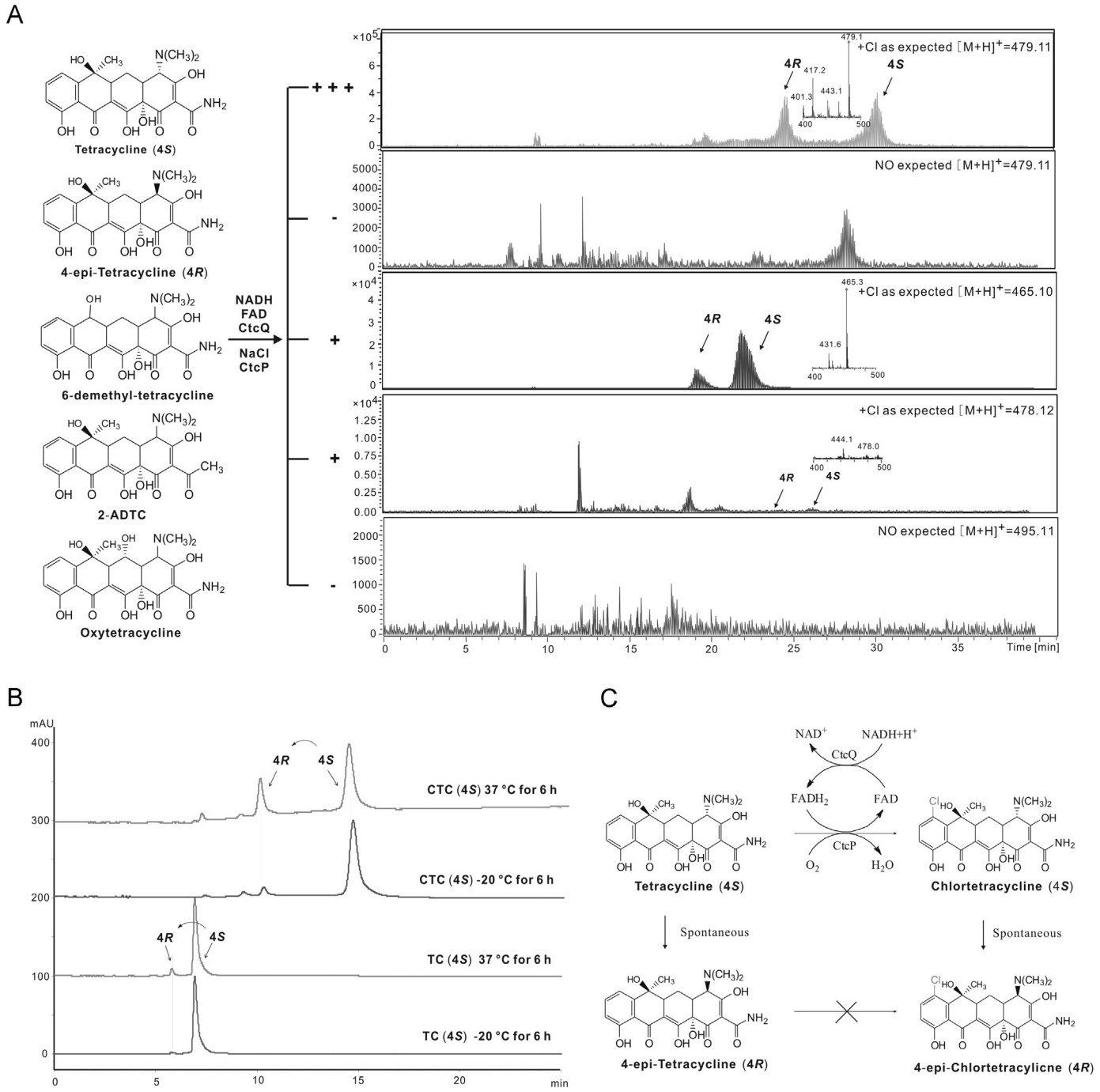
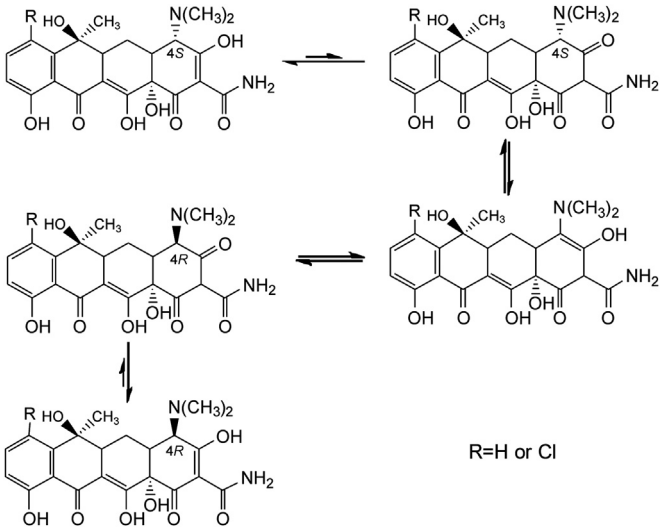



**Fig. 4.** Substrate scope of CtcP and thermal stability of TCs. (A) Extracted ion chromatograms of the products from different substrates by LC–MS. CtcP specifically catalyzed the addition of chlorine to TC (4*S*). On the contrary, TC (4*R*) and OTC could not be halogenated at all. 6-DMTC and 2-DMTC gave no UV–visible chlorinated product, but trace amounts of 7-Cl product as expected were detected by LC–MS. (B) 4*S* configuration could transformed to 4*R* spontaneously around room temperature. (C) Halogenation is proved to be the final step during CTC biosynthesis. CtcP chlorinated TC specifically with absolute 4*S* configuration. Both of the 4*S* products isomerized to the 4*R* spontaneously.

Fig. 3D, the $K_m$ and $k_{cat}$ values for TC were determined. CtcP resulted a lower turnover number ($k_{cat}=0.51\pm0.01$ min$^{-1}$) when catalyzing the chlorination of TC, which might result in the accumulation of TC in the wild producer.

### 3.5. *TC as the stereo-specificity substrate of the two-component halogenase CtcP/Q*

The substrate scope of CtcP was specific to authentic TC, while different analogs isolated from *S. aureofaciens* mutants were tested. First, commercial TC was used as the substrate. The product profiles were identical to both CTC 4-epimers, intricately indicating their origins, since TC commercially provided contained both 4*S* configuration and a small amount of 4*R*. Later, both TC (4*S*) and 4-epi-TC (4*R*) were separated as the substrate. Surprisingly, no trace of 4-epi-CTC could be detected even by LC–MS when 4-epi-TC added as substrate (Fig. 4A). As a matter of fact, we found that 4*S* TCs transformed to their 4*R* counterparts at room temperature spontaneously (Fig. 4B). A mount of CTC (4*R*) product came out during fermentation, especially after the stationary phase (Fig. S3).




**Scheme 1.** Keto-Enol tautomerism during the conversion from the 4*S* to the 4*R* isomers.

It concluded that CtcP could only recognize the absolute 4*S* configuration as substrate, while the 4*R* product originated from 4*S* spontaneously due to the thermal effect in favor (Scheme 1).

In addition to TC and its 4-epimer analog, the isolated 2-ADTC, 6-DMTC and OTC also were utilized as the substrate. All of these TC analogs yielded no enzymatic products could be visible by HPLC under the same reconstitution condition. However, trace amounts of 7-Cl derivatives of both 2-ADTC and 6-DMTC could be detected by LC–MS. To the contrary, no product could be detected when OTC was treated under the same condition (Fig. 4A).

### *3.6. In vivo overexpression showed enhancement of two-component halogenase in S. aureofaciens*

The disability of total conversion from TC to CTC in *Streptomyces* indicated that the substrate may not be accepted by halogenase with sufficient catalytic efficiency. It was further evidenced by the low $k_{cat}$ of CtcP when catalyze the halogenation of TC under the *in vitro* reconstitution condition, compared to halogenases originated from other research (Dorrestein et al., 2005; Lin et al., 2007; Yeh et al., 2005). On the other hand, the flavin reductase, CtcQ, exhibited efficiently in the reduction of FAD *in vitro* (Fig. S2B). To verify that the two-component halogenase was not efficient in *S. aureofaciens*, the gene of *ctcP* or *ctcQ* was introduced with 1–5 extra copies under the control of the consecutive promoter *PermE**, respectively (Fig. 5A).

Consequently, most of mutants showed CTC product improvement, especially the strain harboring three extra copies of the halogenase gene (Table 1). The amount of CTC from the strain integrated with an extra copy of *ctcP* presented a slight 10–18% increase. The percentages of CTC production increased along with the going up extra gene copy number of *ctcP*, reaching the highest 1.73 folds at three in copy number. The control, harboring the empty plasmid pZT139, decreased CTC production dramatically (Fig. 5B). The *ctcQ* overexpressed mutants, corresponding with the efficiency of CtcQ activity supposed from *in vitro* enzymatic construction, did not engage in the CTC yield improvement, comparing to the wild type (Fig. S2C).

Further RT-PCR verified the overexpression of *ctcP* during a beginning 96 h, compared to the wild type and the strains harboring the plasmid pZT139 as control. The control gene associated with the halogenation, *ctcQ*, also showed no overexpression. At the same time, the mini-PKS gene *ctcV* ($KS_{\alpha}$) in the gene cluster and 16S rRNA gene showed the same level of transcription (Fig. 5).

## 4. Discussion

Improvement and engineering of the productivity of commercially viable microorganisms is very important (Li et al., 2009; Xiang et al., 2009; Zhou et al., 2011). Although a number of high-throughput methods have been developed for the analysis of whole genome, with respect to fundamental information content, none can replace the genetic analysis of organisms that have had each gene mutated, which is very important for functional studies of genes in all aspects. The oriented DNA manipulation will provide a platform for the evolution of structural diversity in nature products (Kosec et al., 2012; Nic Lochlainn and Caffrey, 2009). In this work, we reported the genetic manipulation in *S. aureofaciens*, an industrial producer of CTC. Gene knockout and *in vitro* enzymatic reconstitution demonstrated that the halogenation was the final step during CTC biosynthesis. Taken together the improved CTC yield through the overexpression of the halogenase gene, it is suggested that the inefficient halogenase played an essential role in the accumulation of TC during the predominant CTC fermentation.

Halogenase are involved into a broad range of secondary metabolites biosynthesis, improving pharmacological activity, especially halogenation on small extender unit of NRPS. The flavin-dependent halogenase catalyzes the chlorination of electron-rich aromatic or heteroaromatic rings, for example $C_2$, $C_5$, $C_6$, $C_7$ of tryptophan, $C_4$, $C_5$ of proline and $C_3$, $C_5$ of tyrosine (Freitag et al., 2006; Fujimori and Walsh, 2007; Lin et al., 2007; Neumann et al., 2008; Vaillancourt et al., 2006). Unlike other halogeases of this class, CtcP represents a subgroup of bacterial aromatic polyketide tailoring enzymes (Wang et al., 2012; Zhang et al., 2012) that are responsible for C–Cl bond formation which are quite different from NRPS halogenase. Although the C7 chlorine in CTC is not responsible for antibiotic activity, it is crucial for the modification of C7 position during the semisynthesis of TCs toward higher potency, better solubility profile and favorable pharmacological activity derivatives (Doan et al., 2006; McCormick et al., 1960b). As far as we know, it was the first report of *in vitro* reconstruction of halogenating type II PKS core structure.

Classically, enzymes hold their preciseness to act as highly stereo- or region-specific catalysts. The substrate flexibility of enzymes is attenuated due to the few amount products for detection from progenitor microorganisms, compared to the high yield industrial ones. This work showed the promiscuity of enzymes to recognize their substrates during natural products biosynthesis. Early studies investigating the incorporation of bromine instead of chlorine CTC resulted the formation of the corresponding bromide analog (Lein et al., 1959), giving the first indication that the involved enzyme was not exclusively specific for chlorine (Fig. 2C). The C-terminal sequence in CtcP variable from other NRPS halogenase suggested an additional biochemical characterization of the type II PKS substrate. Therefore, we proposed that CtcP probably encompassed much broader substrate scope beyond amino acids and their derivatives, reported before. This reminds us the long reach of halogenation biology and maybe the elucidation of the protein crystal structure in future will provide many detail insights into the chemical mechanism.

It was apparent that CtcP had a stereo selectivity of substrate, specific to absolute 4*S* TC along with the capability of catalyzing 2-ADTC and 6-DMTC into their 7-Cl derivatives, while 4*R*-epimers and OTC could not be recognized (Figs. 3C and 4A). The natural 4*S* isomer is required for optimal antibacterial activity, while

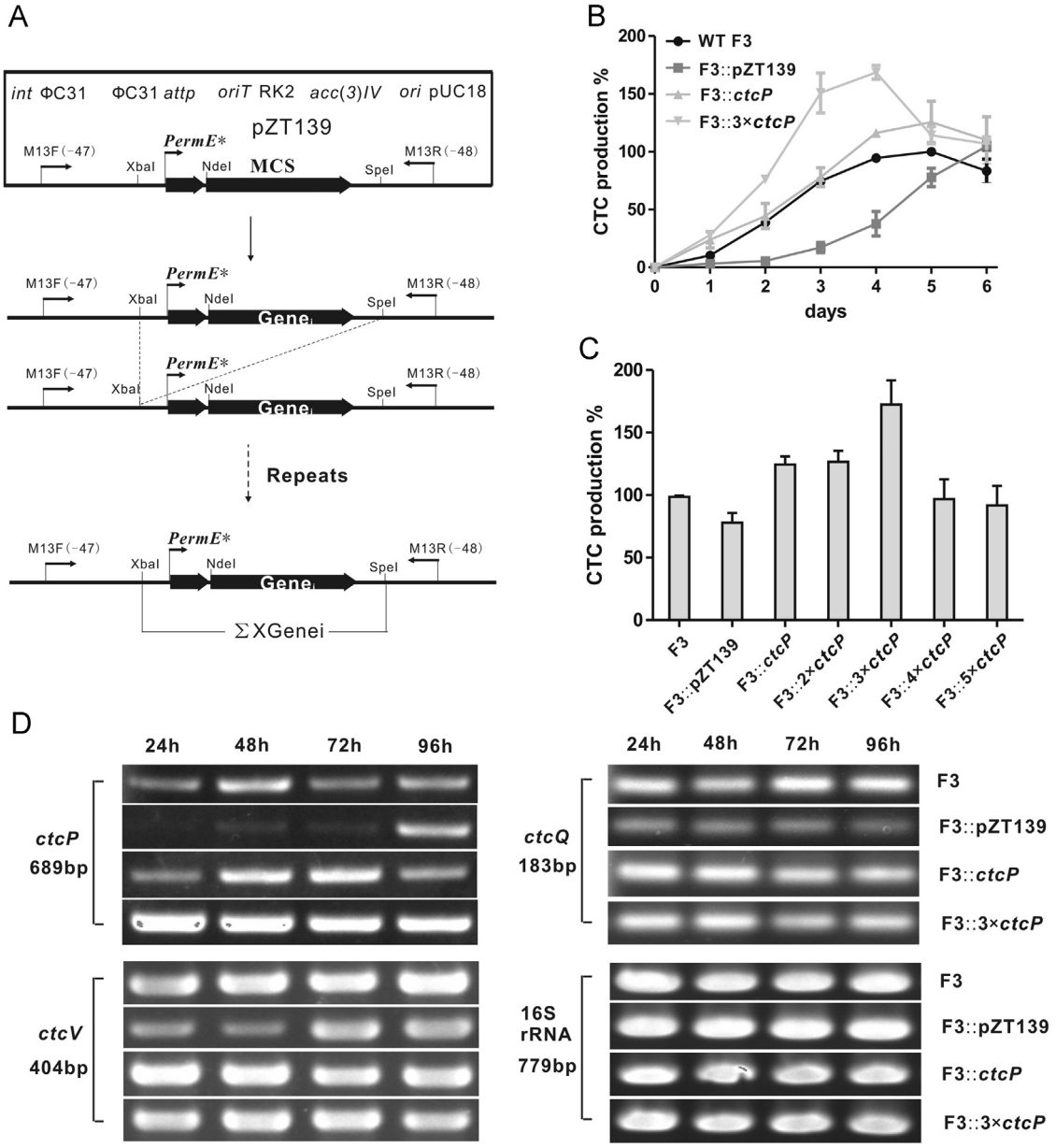



**Fig. 5.** *ctcP* overexpression improved CTC production and transcriptional analysis of the strains with extra multiple copies of *ctcP*. (A) Strategy for gene overexpression. The *Streptomyces* integrative vector pZT139 containing the consecutive *PermE** promoter was used. Note, XbaI and SpeI produce identical 5′-overhangs. (B) and (C) Production profiles of *S. aureofaciens* WT and *ctcP* overexpressing mutants. (D) RT-PCR analysis of gene transcription in *S. aureofaciens* WT and *ctcP* overexpressing derivatives (indicated to the right). The halogenation associated genes (halogenase *ctcP*, FAD reductase *ctcQ*), the mini-PKS gene $KS_{\alpha}$ (*ctcV*) and the control 16S rRNA were checked during a beginning 96 h with an interval of 24 h.

epimerization to 4*R* isomer decreases Gram negative activity (Doerschuk et al., 1955). This is quite important for the storage and transportation of commercial product. It was reported that 5-hydroxy-7-chlortetracyclin could be obtained by incubation of 5a, 11a-dehydrochlortetracycline with cell free preparation of *S. rimosus* (Mitscher et al., 1966). Since the 5-hydroxyl group added already in OTC, we supposed that the existence of the C5-OH blocked the action of halogenase. It was noteworthy that 6-DMTC could be also catalyzed by CtcP at a quite low efficiency, as chlorine was necessary to obtain the important demeclocycline. The addition of chlorine at C7 position of demeclocycline provided its property to be modified for the diversity of new semisynthetic TCs (Church et al., 1971; Doan et al., 2006). The commercial strains used for demeclocycline product yield much less product than the CTC producer. This is probably due to the unfavorable 6-DMTC substrate recognized by the halogenase. As shown in the work of PrnA (Lang et al., 2011), we believe that direct protein engineering according to the crystal structure will provide CtcP improved probability of halogenation favor with 6-DMTC, to meet the commercial antibiotic production improvement.

Notably, the twentieth century marked the introduction and widespread use of antibiotics, followed clinical resistance with the introduction of each new antibiotic. It is an urgent for identification of new antimicrobial targets and development of new antibiotics with few side effects (Thaker et al., 2010). The TC analogs are specifically designed to overcome common mechanisms of TC resistance, namely resistance mediated by acquired efflux pumps, ribosomal protection and enzymatic inactivation (Alekshun and Levy, 2007; Donhofer et al., 2012; Petkovic et al., 2006). Although, the elegant total synthesis of 6-deoxytetracyclines by Myers's group has highlighted the success of TC derivatives through chemical approach (Charest et al., 2005; Sun et al., 2008), current

**Table 1**
Product profiles in *Streptomyces aureofaciens* WT and halogenase (*ctcP*) engineered strains with industrial potential.

| Strains | TC (g/l or %) | CTC (g/l) |
|---|---|---|
| *S. aureofaciens* WT | 7.8% | $14.80 \pm 0.13$ |
| *S.aureofaciens*Δ*ctcP* | $18.88 \pm 2.68$ | 0 |
| *S. aureofaciens*+*ctcP* | 6.5% | $18.66 \pm 0.99$ |
| *S. aureofaciens*+2 × *ctcP* | 7.1% | $18.98 \pm 1.34$ |
| *S. aureofaciens*+3 × *ctcP* | 5.5% | $25.86 \pm 2.90$ |

TCs commercially used totally originate from the fermentation products of microorganisms. Biosynthetic engineering of such a structural complexity is an attractive route of generating pharmaceutically important analogs. Our combined genetic manipulation and molecular identification of enzymes during TCs biosynthesis will underpin ongoing efforts aimed at those microbial pharmaceutical purposes.

## Accessional numbers

The complete DNA and deduced protein sequences of the *ctc* gene cluster reported in this paper have been deposited in GenBank under accession number HM627755.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2013.06.003.

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