Li et al. Bioresour. Bioprocess. (2016) 3:46
DOI 10.1186/s40643-016-0123-7

Bioresources and Bioprocessing

RESEARCH

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Protein expression analysis of a high-demeclocycline producing strain of *Streptomyces aureofaciens* and the roles of CtcH and CtcJ in demeclocycline biosynthesis

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Abstract

Background: *Streptomyces aureofaciens* strain A6-9, obtained with traditional mutagenesis, produces elevated levels of 6-DCT. The increased formation of 6-DCT may be attributable to the changes in the expression of some proteins in the 6-DCT biosynthetic pathway. For this reason, we explored the differences in protein expression between A6-9 and wild-type (WT) strains of *Streptomyces aureofaciens*, and based on the differences (CtcH and CtcJ were overexpressed in A6-9), investigated the roles of CtcH and CtcJ in biosynthesis.

Results: Two-dimensional gel electrophoresis and a Mascot search indicated that some enzymes (including CtcH and CtcJ) involved in the primary and secondary metabolism were more strongly expressed in the high-6-DCT-yielding strain A6-9 than in the WT strain DT1. To examine the roles of CtcH and CtcJ in 6-DCT biosynthesis, ctcH-deleted, ctcJ-deleted, ctcH-overexpressing, and ctcJ-overexpressing mutants and a mutant overexpressing both ctcH and ctcJ were constructed. Compared with WT, 6-DCT production was 50 and 37 % higher in the ctcH-overexpressing and ctcJ-overexpressing strains, respectively, and increased by 60 % in the ctcH–ctcJ-overexpressing strain. The ctcH-deleted and ctcJ-deleted strains produced almost no 6-DCT. Analysis of the metabolic flux distribution indicated that ctcH encodes a hydroxyacyl-CoA dehydrogenase and ctcJ encodes a monooxygenase that are essential for 6-DCT biosynthesis.

Conclusion: Protein expression differs between high-6-DCT-yielding and WT strains, and the enzymes increased in the high-6-DCT-yielding strain explain the increased 6-DCT production. ctcH encodes a hydroxyacyl-CoA dehydrogenase and ctcJ encodes a monooxygenase that are essential for 6-DCT biosynthesis.

Keywords: Biosynthesis, Demeclocycline, *Streptomyces aureofaciens*, Two-dimensional gel electrophoresis

Background

Four tetracycline antibiotics, chlortetracycline, tetracycline, demeclocycline (6-DCT), and 6-demethyltetracycline, are secondary metabolites of *Streptomyces aureofaciens* (Zhu et al. 2001). 6-DCT is produced when the 6-methylation step is blocked in chlortetracycline biosynthesis pathway, and is used for the industrial production of semisynthetic tetracyclines (Nakano et al. 2000). There has been a little research into 6-DCT production, whereas many papers are available concerning the gene clusters and ribosomes involved in the biosynthesis of chlortetracycline and other tetracyclines. Chlortetracycline production depends on the expression of proteins involved in its biosynthetic pathway, so identifying and understanding the corresponding proteins are required for the industrial production of 6-DCT biosynthesis (Mikulik et al. 1983; Li et al. 2001). *Streptomyces aureofaciens* strain A6-9, obtained with the traditional mutagenesis, produces the elevated levels of 6-DCT. This increased formation of 6-DCT may be attributable to changes in the expression of some proteins in the 6-DCT

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biosynthetic pathway. Modulating the expression of these proteins may significantly affect 6-DCT biosynthesis.

A type II polyketide synthase (PKS) constructs the skeleton of tetracycline antibiotics, and a series of subsequent reactions modify the skeleton to produce four final products (Nakano et al. 2000). The modification reactions (e.g., chlorination, methylation, and oxidation) are important for the diversity of tetracyclines, and 6-DCT is formed when the 6-methylation step in the pathway is lacking. Above all, oxidation reactions catalyzed by monooxygenases are necessary for the formation of 6-DCT (Fig. 1) (Taguchi et al. 2011; Bentley et al. 2002).

Although certain features of the secondary metabolism of *Streptomyces* have been studied in detail, including the synthesis of antibiotics and its differentiation processes, its primary metabolism has not been (Al-Shamma et al. 1983; Hensel et al. 1991). Hydroxyacyl-CoA dehydrogenase is involved in the β-oxidation of fatty acids, which is essential for the whole metabolism of the microorganism (Fujita et al. 2007; Rock and Cronan 1996; DiRusso and Nystrom 1998; Schujman et al. 2003; Lu and Rock 2006). Hydroxyacyl-CoA is oxidized by hydroxyacyl-CoA dehydrogenase to generate acetyl-CoA, NADH, and H^+, which are essential for the biosynthesis of 6-DCT (Fig. 2).

In this study, we examined the differences in protein expression between A6-9 and the parental wild-type (WT) strain to identify the enzymes that are important for 6-DCT biosynthesis, using two-dimensional (2D) gel electrophoresis and mass spectrometry. Based on the results, the roles of CtcH (hydroxyacyl-CoA

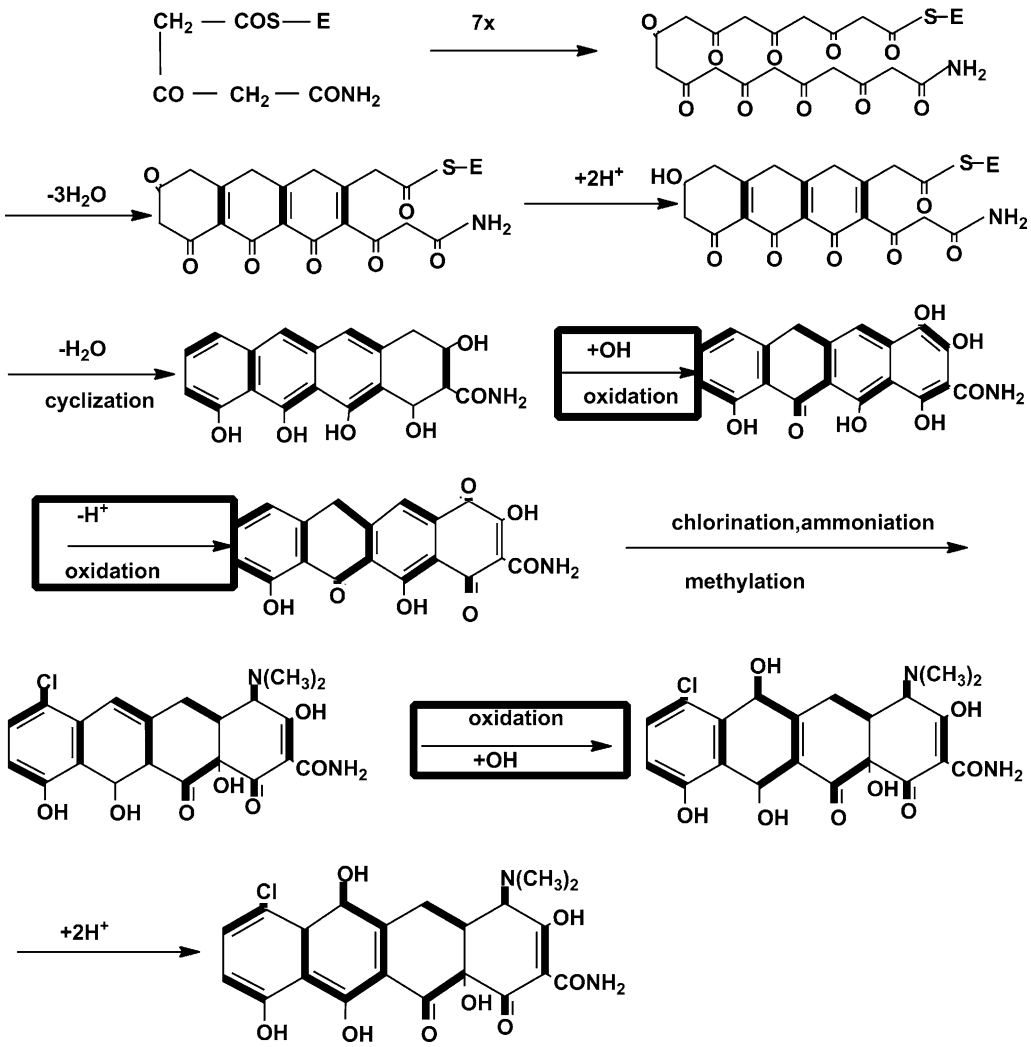


Fig. 1 Detailed pathway of the precursors involved in the synthesis of 6-DCT in *S. aureofaciens*. *Black boxes* show the oxidation steps (CtcJ) in the 6-DCT-forming pathway

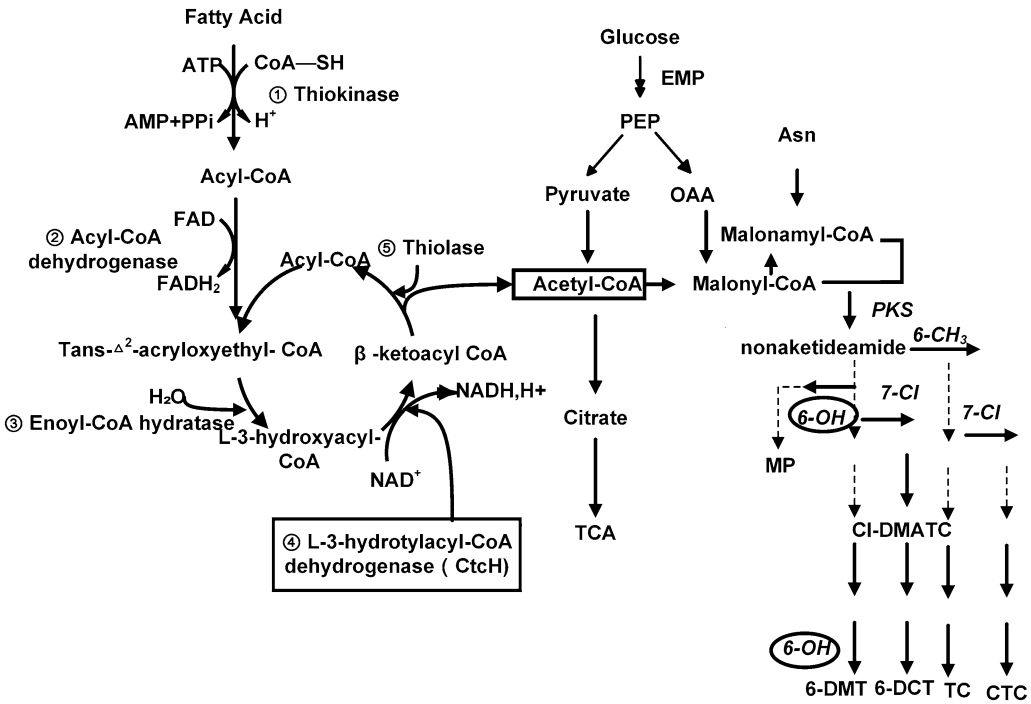


Fig. 2 Pathway of the β-oxidation of fatty acids, the biosynthetic pathway of demeclocycline (6-DCT) in *S. aureofaciens*. *Black rectangle* shows the function of hydroxyacyl-CoA dehydrogenase (CtcH) and the acetyl-CoA generated by the β-oxidation of fatty acids used for the biosynthesis of 6-DCT. *Black ellipse* shows the function of 6-hydroxylase in the 6-DCT biosynthetic pathway. Abbreviations: *PKS*, polyketide synthase reaction; *6-CH$_3$*, 6-methylation reaction; *6-OH*, 6-hydroxylation reaction; *7-Cl*, 7-chlorination reaction; CoA, coenzyme A; CTC, chlortetracycline; TC, tetracycline; 6-DMT, oxytetracycline

dehydrogenase) and CtcJ (monooxygenase) in 6-DCT biosynthesis were examined by inactivating or overexpressing them individually and together in DT1.

Methods

Microbial strains and plasmids

The strains and plasmids used in this study are listed in Table 1.

Media and culture conditions

Escherichia coli DH5α and its derivatives were routinely grown aerobically in LB medium (37 °C, 180 rpm) (Jian et al. 2010; Zhang et al. 2006). ISP4-containing 80 mmol l^{-1} $MgCl_2$ was used for *E.coli* ET12567/pUZ8002-*Streptomyces* conjugative medium (Liu et al. 2015).

The mycelia of *S. aureofaciens* were stored in 20 % glycerol solution at −80 °C. A 250-ml shaker flask containing 30 ml of seed medium was inoculated with 3 ml of mycelial solution. After incubation for 48 h at 27 °C, a 250-ml fermentation flask containing 30 ml of fermentation medium was inoculated with 3 ml of seed medium, and shaken at 27 °C and 240 rpm for 148 h. All experiments were performed in triplicate. The seed medium and fermentation medium were adjusted to pH 6.5.

Generation of gene-inactivated and gene-overexpressing strains

Genes *ctcH* and *ctcJ* were deleted with the non-replicating plasmid pOJ260-neo, which produced deletion mutants substituted with a kanamycin-resistance gene cassette. The transformants (Δ*ctcH* and Δ*ctcJ*) were grown on ISP4 plates-containing kanamycin and nalidixic acid, selected on MS plates-containing kanamycin and apramycin, and verified with DNA sequencing and PCR. All primers used in this study are shown in Additional file 1: Online Resource 1 and the plasmid used for the gene-overexpressing constructs is shown in Additional file 2: Online Resource 2.

Genes *ctcH* and *ctcJ* were overexpressed individually or both genes were overexpressed simultaneously with an integrative plasmid, pIB139, containing a strong promoter from the erythromycin-resistance gene and the ϕC31 *attp* site, which can integrate into the *attB* site of *Streptomyces*. A 719-bp DNA fragment containing *ctcH* was cloned and inserted into pIB139 to generate pIB139-H, and a 763-bp DNA fragment containing *ctcJ* was inserted into pIB139 to generate pIB139-J. The two fragments were inserted into pIB139 to generate pIB139-H-J. The genotypes of WT and the overexpressing mutants are shown in Fig. 5a. The transformants (*ctcH*+, *ctcJ*+, and

Table 1 Strains and plasmids used in this study

Strains or plasmids	Genotype and/or relevant characteristics	Source or reference
Strains		
Escherichia coli		
DH5α	Host for general	
ET12567/ pUZ8002	Donor strain for conjugation between *E. coli* and *Streptomyces*	Invitrogen Lab. collection
S. aureofaciens		
DT1	producer of *S. aureofaciens* Industrial 6-DCT-producing strain	Lab. collection
A6-9	6-DCT high-yield strains mutated from DT1	Lab. collection
Δ*ctcH*	The deletion mutant of *ctcH* derived from DT1	This study
Δ*ctcJ*	The deletion mutant of *ctcJ* derived from DT1	This study
ctcH+	*ctcH* gene-duplicated derivative of DT1, with genotype *PermE*-ctcH* gene	This study
ctcJ+	ctcJ gene-duplicated derivative of DT1, with genotype *PermE*-ctcJ* gene	This study
ctcH-ctcJ+	*ctcH* and *ctcJ* gene-duplicated derivative of DT1, with genotype *PermE*-ctcH-ctcJ* gene	This study
Plasmids		
pOJ260	Non-replicating vector, Apms	Lab. collection
pIB139	attφC31, oriT, Apms, PermE*	Lab. collection
pOJ260-neo	pOJ260 derivative carrying a 1145 bp DNA fragment containing the *neo* gene, Kans, Apms	This study
pOJ260-neo-H	pOJ260-neo containing a 959 bp upstream fragment and a 889 bp downstream fragment of *ctcH*, Kans, Apms	This study
pOJ260-neo-J	pOJ260-neo containing a 911 bp upstream fragment and a 1021 bp downstream fragment of *ctcJ*, Kans, Apms	This study
pIB139-H	pIB139 derivative carrying a 719 bp DNA fragment containing the *ctcH* gene	This study
pIB139-J	pIB139 derivative carrying a 763 bp DNA fragment containing the *ctcJ* gene	This study
pIB139-H-J	pIB139 derivative carrying a 763 bp and a 719 bp DNA fragments containing the *ctcJ* and *ctcH* gene	This study

ctcH–ctcJ+) were isolated on ISP4 plates under apramycin and nalidixic acid selection, cloned on MS plates, and verified with PCR and a transcription analysis.

Determination of 6-DCT

The production of 6-DCT was determined with high-performance liquid chromatography (HPLC), using the linear regression equation: $U = 3 \times 10^{-4}A - 10.98$, $r^2 = 0.9956$, where U and A represent the concentration of 6-DCT and the peak area, respectively. Samples (1, 2, 3, 4, or 5 μl) of a standard 6-DCT solution (4000 μg/ml) were injected and eluted after 15 min, and the peak areas were calculated. The linear regression equation was obtained and plotted, with the concentration of 6-DCT on the horizontal axis and the peak area on the vertical axis. As a control, WT DT1 was processed under the same fermentation conditions.

Analytical methods

After fermentation for 7 days, the pH of the culture broth was adjusted to 1.8–2.0 with oxalic acid. The filtration supernatant was assayed with HPLC using an Extend-C18 reverse phase column (4.6 mm × 150 mm, 5 μm; Agilent, USA) at 350 nm with 0.01-M oxalic acid–0.08-M ammonium oxalate solution and 40 % methanol/acetonitrile/water as the mobile phase at a flow rate of 0.4 ml/min. The column temperature was at 25 °C.

2D gel electrophoresis and Mascot search

The mycoproteins were extracted with ultrasonication and their concentrations determined with Coomassie Brilliant Blue. The mycelia were washed in low-salt buffer (2.5 mM KH_2PO_4, 5 mM NaH_2PO_4) precooled to 4 °C, and then resuspended in 1 ml of lysis buffer (8 M carbamide, 1 % IPG buffer, 2 % dithiothreitol). Protein sample preparation and 2D gel electrophoresis were performed as previously described (Helmel et al. 2014; Jan et al. 2014). (All chemicals were from Sigma-Aldrich, St. Louis, MO, USA). A Mascot search was performed by Shanghai Bo-Yuan Biological Technology Co., Ltd.

RNA isolation and quantitative real-time reverse transcription-PCR (RT-qPCR)

The transcription levels of *ctcH* and *ctcJ* were assayed with RT-qPCR (Bio-Rad CFX96). Sample preparation, RNA isolation, and the PCR were all conducted according to a previous study (Zhu et al. 2013). RT-qPCR was performed

with the SYBR Green Two-Step RT-qPCR SuperMix (Takara). The transcription levels were normalized to the expression of 16S rRNA. RT-qPCR was performed in three independent experiments, each in triplicate.

Results

Differences in protein expression by *S. aureofaciens* strains A6-9 and DT1

A high-6-DCT-yield *S. aureofaciens* strain A6-9 was generated with the traditional mutagenesis. Compared with the parental strain under the same flask fermentation conditions, A6-9 showed a 20 % increase in 6-DCT production, which might be attributable to changes in protein expression. For this reason, 2D gel electrophoresis and a Mascot search were used to explore the differences in protein expression between the mutant A6-9 and the parental strain DT1.

Seven differentially expressed proteins were identified with mass spectrometry using the PDquest software (Fig. 3; Table 2). Malate dehydrogenase, glutamine synthetase, 6-hydroxylation enzyme, CtcH, and CtcJ were expressed more strongly and ATP synthetase less strongly in A6-9 than in DT1.

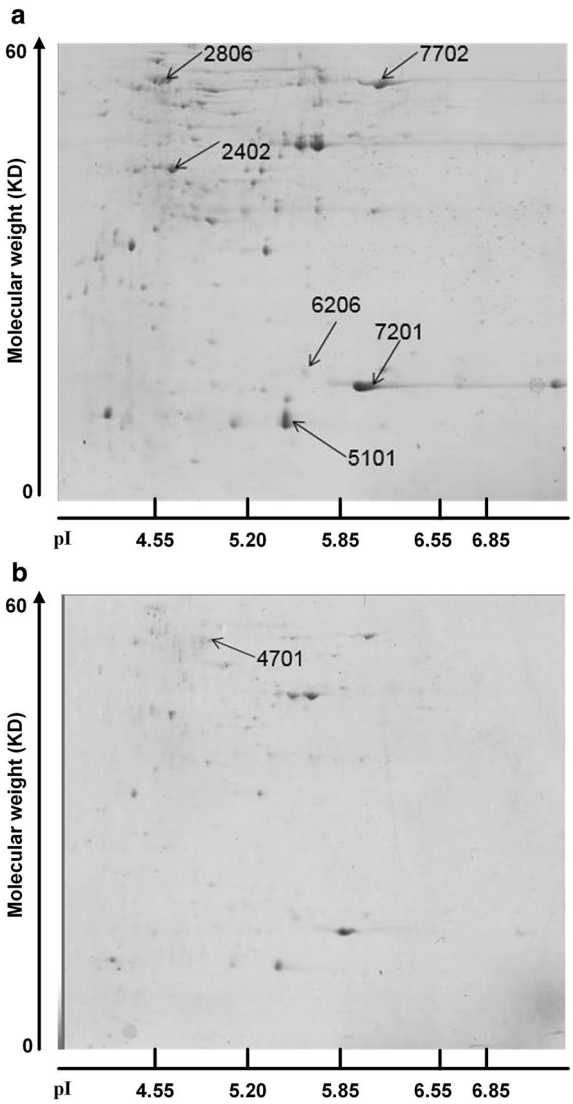


Fig. 3 Two-dimensional electrophoresis patterns of *S. aureofaciens* strains A6-9 and DT1. According to the PDquest software, seven protein spots were differentially expressed. **a** Two-dimensional electrophoresis pattern of strain A6-9. **b** Two-dimensional electrophoresis pattern of DT1

6-DCT biosynthesis requires CtcH and CtcJ

The proteins expressed more strongly in A6-9, according to 2D gel electrophoresis and the Mascot search, might explain, at least in part, the increase in 6-DCT production. CtcH and CtcJ are encoded by *ctcH* and *ctcJ*, respectively, which belong to the chlortetracycline biosynthesis gene cluster, and occur near one another on the *S. aureofaciens* chromosome, but have not yet been investigated. Therefore, their roles in 6-DCT biosynthesis were examined first, and the other enzymes upregulated in A6-9 will be explored in a future study. *ctcH* and *ctcJ* were knocked out individually to study their effects on 6-DCT biosynthesis.

The *ctcH* and *ctcJ* deletion mutants of *S. aureofaciens* DT1 were generated by in-frame deletion (Fig. 4a) and confirmed with PCR (Fig. 4b). The mutants and WT were grown in fermentation culture for 7 days and the 6-DCT concentrations detected with HPLC. Less 6-DCT was detected in Δ*ctcH*, and no 6-DCT was detected in Δ*ctcJ* (Fig. 4c). The HPLC analyses of the mutants and WT are shown in Additional file 3: Online Resource 3.

Overexpression of *ctcH*- and/or *ctcJ*-enhanced 6-DCT production

The results of *ctcH* or *ctcJ* deletion suggested that the production of 6-DCT may increase when the genes are overexpressed. To improve 6-DCT production and explore the effect of each gene on it, *ctcH* and *ctcJ* were overexpressed individually and together in DT1 (Fig. 5b). As predicted, the *ctcH*+, *ctcJ*+, and *ctcH*–*ctcJ*+ mutants expressed considerably more 6-DCT than did DT1, with increases of ca. 50, 37, and 60 %, respectively. Compared with the 3164-μg/ml 6-DCT produced by DT1, *ctcH*+ produced 4715 μg/ml, *ctcJ*+ produced 4332 μg/ml, and *ctcH*–*ctcJ*+ produced 5075 μg/ml (Fig. 5c). The transcription levels of *ctcH* and *ctcJ* were also high in the

Table 2 Appraisal results of protein points by mass spectrometry (MS)

Sample ID	Protein ratio grayscale of A6-9 and DT1	The name of the protein	Protein ID	Molecular weight/Da
2402	2.00	Malic dehydrogenase (MDH)	GI:357389982	34778
2806	0.39	ATP synthase containing α-subunit	GI:357391910	56264
4701	11665.80	Glutamine synthetase	GI:357389171	52225
5101	4.92	CtcJ	GI:338776751	10625
6206	3.22	CtcH	GI:338776749	17213
7201	6.69	CtcH	GI:338776750	17213
7702	10.42	6-Hydroxylation enzyme	GI:1100766	50588

mutants (Fig. 5d), indicating that the overexpression of these genes caused the increase in 6-DCT production. The HPLC analysis of the mutants and WT is shown in Additional file 3: Online Resource 3.

Discussion

In recent years, engineering the primary metabolism has been used to supply more precursors for the production of the secondary metabolites of *Streptomyces* and rationally engineering the secondary metabolism has shown potential utility in strain improvement, allowing the production of high levels of antibiotics and to reduce the byproducts (Pang et al. 2015; Zhuo et al. 2010; Zhang et al. 2008). Gene overexpression has been an effective way to enhance antibiotic production (Liu et al. 2015; Du et al. 2013; Yuan et al. 2011; Malla et al. 2010). In this study, the overexpression of some key enzymes involved in the 6-DCT biosynthetic pathway in the high-yield strain A6-9 explained the enhanced production of 6-DCT.

ctcH is an endogenous gene that encodes a 3-hydroxylacyl-CoA dehydrogenase, an NADH-dependent enzyme that also has Δ^3-*cis*, Δ^2-*trans*-enoyl-CoA isomerase and (S)/(R)-epimerase activities. It is responsible for the oxidation of 3-hydroxyacyl-CoA, generating NADH, H^+, and β-ketoacyl-CoA, which can also generate acetyl-CoA when it is catalyzed by thiolase during the β-oxidation of fatty acids (Fig. 2) (Volodina and Steinbuchel 2014; Fujita et al. 2007; Kunau et al. 1995; Black and DiRusso 1994; Pramanik et al. 1979). Here, the results of modulating *ctcH* can be explained by the requirement for NADH, H^+, and acetyl-CoA in 6-DCT biosynthesis. First, the regulation of *ctcH* expression has a direct effect on the generation of NADH and H^+, which are the major reductants in the 6-DCT biosynthetic pathway, and second, it affects the amount of acetyl-CoA, a major source of the precursors of 6-DCT.

In Δ*ctcH*, 3-hydroxyacyl-CoA could not be oxidized to a keto group, so the mutation of *ctcH* reduced the generation of NADH, H^+, and acetyl-CoA, further reducing 6-DCT production. One cycle of β-oxidation produces one molecule each of NADH, H^+, and acetyl-CoA, which are necessary for the 6-DCT biosynthesis (Fig. 2). However, in *ctcH*+ and *ctcH–ctcJ*+, the reaction catalyzed by 3-hydroxylacyl-CoA dehydrogenase was enhanced, increasing the amounts of NADH, H^+, and acetyl-CoA, and thus increasing 6-DCT production.

ctcJ encodes a monooxygenase involved in antibiotic biosynthesis, and is also involved in the monooxygenation of intermediates in the macrolide biosynthetic pathways (Gaisser et al. 2002). These oxygenases belong to the so-called post-PKS-modifying (tailoring) enzymes, which play crucial roles in the formation of interesting and unique molecular structures. The monooxygenases are representative, tailoring enzymes that are especially important in providing the structural elements essential for the special biological activity of these structures (Taguchi et al. 2011; Faust et al. 2000).

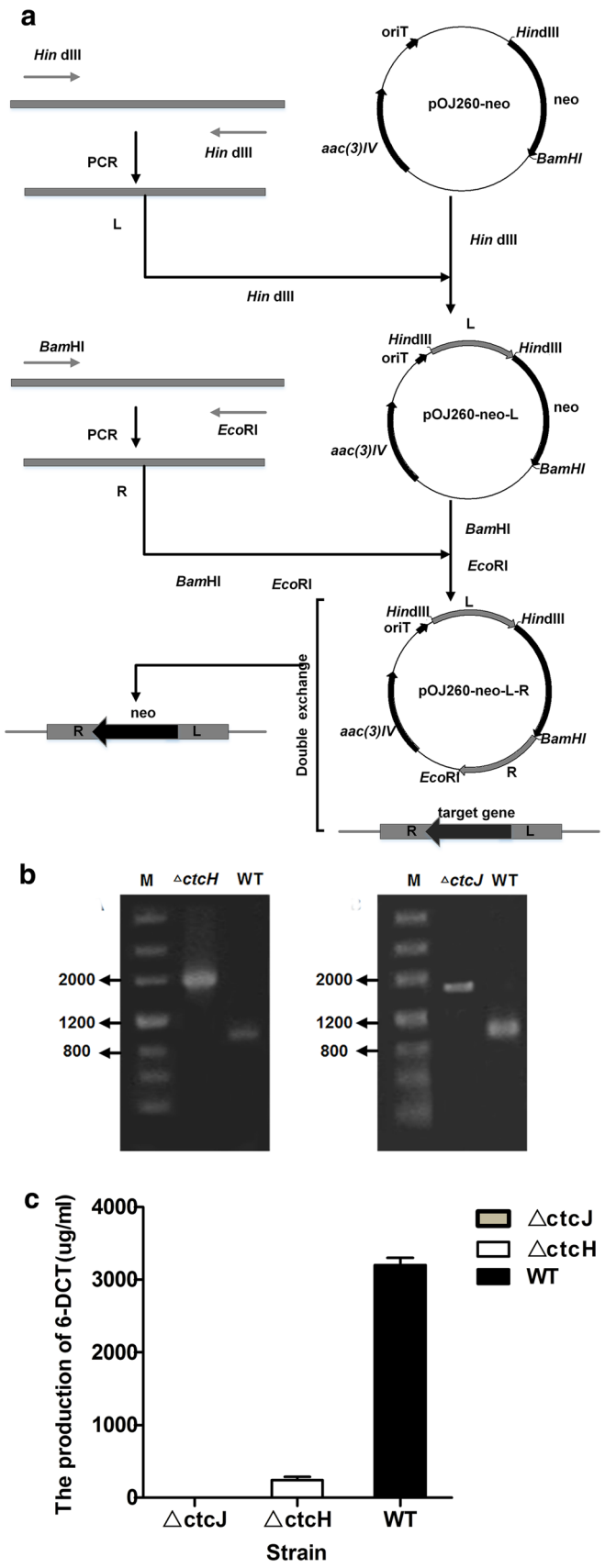
In Δ*ctcJ*, no 6-DCT was formed, and instead, an unknown chemical was produced that was also produced in DT1. These results indicate that Δ*ctcJ* is unable to catalyze the oxidation steps (Fig. 1) required in the 6-DCT biosynthetic pathway. In *ctcJ*+ and *ctcH–ctcJ*+, the oxidation steps involving CtcJ in the 6-DCT biosynthesis pathway were enhanced.

Conclusion

This study mainly focused on the differences in protein expression between *S. aureofaciens* strains A6-9 and DT1 and the effects of CtcH and CtcJ on the production of

(See figure on next page.)

Fig. 4 Deletion of *ctcH* and *ctcJ* genes. **a** Schematic representation of the in-frame deletion of *ctcH* and *ctcJ*. A 410-bp fragment of *ctcH* and a 254-bp fragment of *ctcJ* were replaced with the 1145-bp *neo* gene by double crossover. **b** Confirmation of the *ctcH* deletion and *ctcJ* deletion mutants with PCR amplification. **c** 6-DCT production during the fermentation of the parental strain DT1 and the *ctcH*-deleted, *ctcJ*-deleted strains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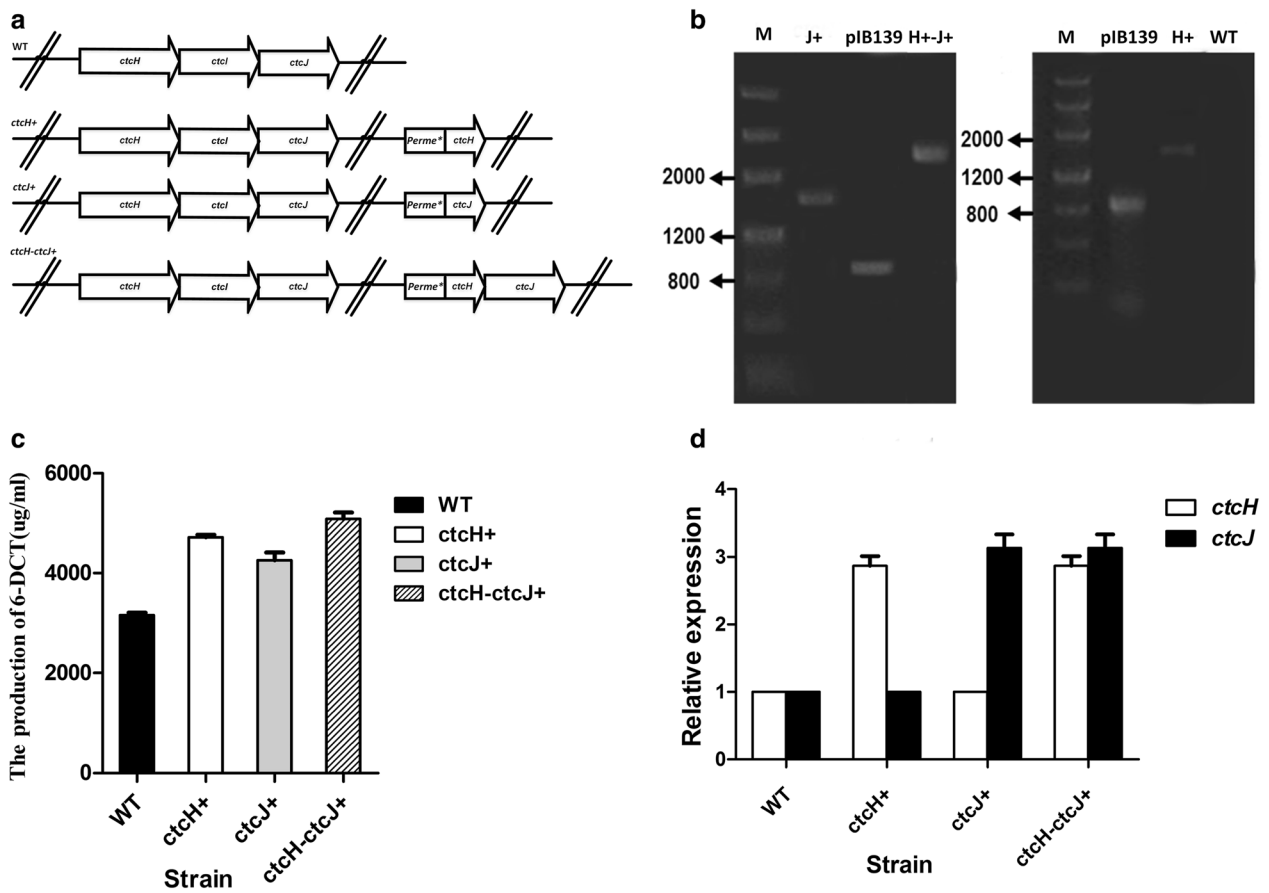

Fig. 5 Overexpression of the individual *ctcH* and *ctcJ* genes and the simultaneous overexpression of *ctcH* and *ctcJ*. **a** Genotypes of WT and overexpressing mutants. **b** Confirmation of the *ctcH*-overexpressing, *ctcJ*-overexpressing, and *ctcH–ctcJ*-overexpressing mutants with PCR. The PCR products were 900 bp for pIB139 (empty vector), 1663 bp for *ctcJ*+, 1619 bp for *ctcH*+, and 2382 bp for *ctcH–ctcJ*+. All the PCR products were sequenced to confirm each mutation. **c** 6-DCT production during the fermentation of the parental strain DT1, and the *ctcH*-overexpressing, *ctcJ*-overexpressing, and *ctcH–ctcJ*-overexpressing mutants. **d** RT-qPCR analysis of *ctcH* and *ctcJ* expression in the three overexpressing mutants and WT. Relative levels of *ctcH* and *ctcJ* transcripts were determined after normalization to the internal reference, 16S rRNA. RT-qPCR was performed in three independent experiments, each in triplicate

6-DCT in *S. aureofaciens*. Our experiments demonstrate that CtcH and CtcJ play important roles in the formation of 6-DCT. Further exploration of all the enzymes overexpressed in A6-9 will allow us to clarify the 6-DCT biosynthetic pathway. Our findings should be useful in controlling this metabolic node and modifying the metabolic pathway to increase the production of 6-DCT in an industrial strain.

Additional files

Additional file 1: Online Resource 1. Primers used in this study.

Additional file 2: Online Resource 2. The plasmids construction of gene overexpression.

Additional file 3: Online Resource 3. The HPLC analysis of demeclocycline in mutants.

Authors' contributions
All authors have participated in the interpretation of results during preparation of the manuscript. All authors read and approved the final manuscript.

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Acknowledgements
This work was financially supported by Topfond Pharmaceutical Co., Ltd., Henan, China.

Competing interests
The authors declare that they have no competing interests.

Received: 3 June 2016 Accepted: 15 September 2016
Published online: 22 September 2016

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