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APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Enhancement of A82846B yield and proportion by overexpressing the halogenase gene in *Amycolatopsis orientalis* SIPI18099

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Abstract

The glycopeptide antibiotic A82846B (chloroeremomycin) produced by *Amycolatopsis orientalis* is the precursor of the semi-synthetic antibiotic oritavancin. However, during the industrial production of A82846B, two major impurities, A82846A (63.6%) and A82846C (12%) which are structurally similar to A82846B (24.4%), are also produced. In this study, to improve the ratio of A82846B to A and C, the genes encoding halogenase in A82846B and vancomycin synthesis were integrated into *A. orientalis* SIPI18099 to test their halogenation ability, respectively. The results indicated that *chal* from the A82846B biosynthesis pathway was more efficient in reducing A and C factors. Moreover, by increasing the *chal* copy number, the proportion of A and C were gradually reduced while the titer and proportion of A82846B were improved. In a scaled-up industrial process, the proportion of A and C were decreased to 11.6% and 0.2% in the recombinant strain *A.orientalis chal-3* with three gene copies of *chal* and the titers of A82846B (2.2 g/L) has increased by 2.8-folds compared to 780 mg/L produced by the parental strain, suggesting that the recombinant strain was suitable for the industrial production of A82846B with lower impurities.

Keywords A82846B · Vancomycin · *Amycolatopsis orientalis* · Halogenase gene · Overexpression

Introduction

Resistance to Gram-positive bacteria has been increasingly threatening public health, such as the appearance of methicillin-resistant *Staphylococcus aureus* (MRSA) (Edelsberg et al. 2014). Vancomycin was successfully used to treat MRSA in clinical practice for more than one decade; however, the emergence of vancomycin-resistant *S. aureus* (VRSA) and vancomycin-resistant enterococci (VRE) in recent years has presented an urgent challenge to human health, which requires the development of new antibiotics against these pathogens (Chang et al. 2003; Arthur and Courvalin 1993).

A82846B, a vancomycin-like lipoglycopeptide, is the precursor of the new semi-synthetic antibiotic, oritavancin (Fig. 1a), which was recently approved by the Food and Drug Administration (FDA) for treating acute bacterial skin and skin structure infections (ABSSSIs) caused by Gram-positive pathogens including MRSA (Brade et al. 2016; Corey et al. 2014). Oritavancin is synthesized by adding a 4-(4-chlorophenyl) benzyl group to chloroeremomycin via reductive alkylation (Leadbetter et al. 2010). Compared with previous glycopeptide antibiotics, oritavancin has a prolonged half-life of about 200–300 h, allowing for a single dose for a 7-day course of treatment, which makes it a promising drug for treating pathogen infections in both approved and unapproved treatments (Kaatz et al. 1998; Rupp et al. 2001).

A82846 is a complex of structurally related components including A, B, and C constituents (Fig. 1a) produced by *A. orientalis* (Hamill et al. 1998). This antibiotic is structurally similar to heptapeptide antibiotics like vancomycin (Fig. 1b). Although the chemical structures of A82846A, A82846B, and A82846C only differ in their chlorine atom number, the separation and purification of these compounds require very tedious procedures (Rafai et al. 2016) including successive resin

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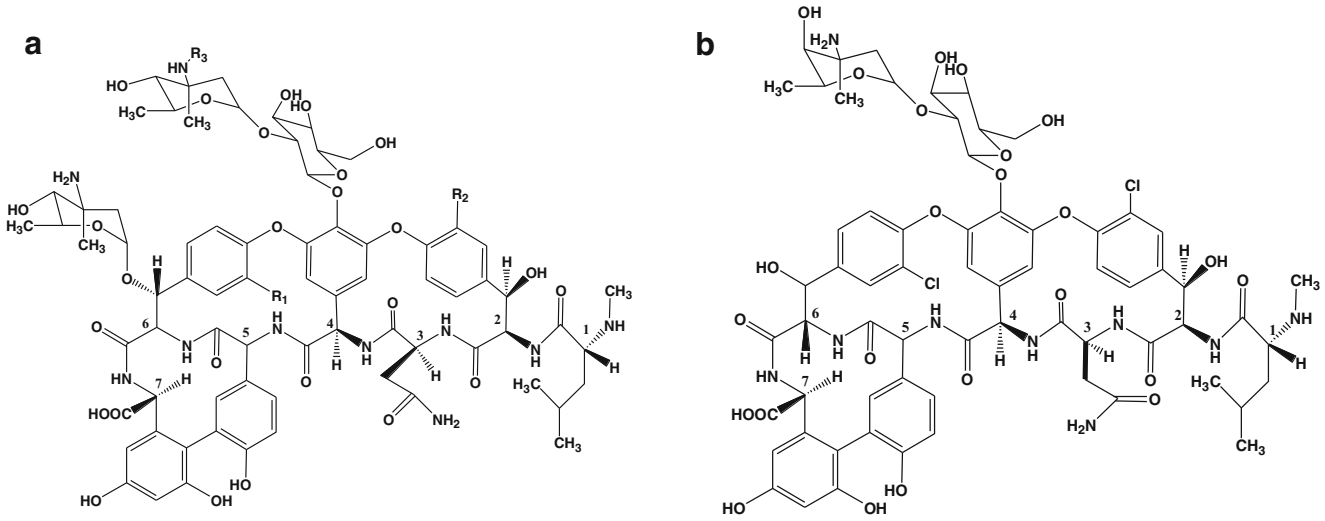

Fig. 1 **a** Structure of A82846 and oritavancin. **b** Structure of vancomycin

chromatography and high-pressure preparatory chromatography. The complex and costly isolation process of A82846B has stressed the commercialization of oritavancin. Furthermore, since the antimicrobial activity and safety of some impurities are not clearly understood, the existence of those impurities might cause side effects, so it is urgent to find ways to reduce the levels of impurities.

The current dilemma in industrial production of A82846B is to remove A82846A and A82846C. In our research, we proposed that the halogenation activity of *chal* (NCBI Accession No:AJ223998.1), which encodes halogenase, was not high enough in A82846B-producing strains, so we focused on reducing A82846A and A82846C content by overexpressing the halogenase genes. This is the first example of lowering impurity levels by genetic manipulation, which represents a cost-effective method for A82846B production.

Materials and methods

Strains, plasmids, and primers

Bacterial strains and plasmids used in this study are listed in Table 1; primers are listed in Table 2.

DNA manipulation

The manipulations of genomic DNA, plasmid DNA isolation, restriction endonuclease digestion, and DNA ligation were performed according to standard procedures (Sambrook and Russell 2001). The enzymes were purchased from Takara, Japan, and Thermo Fisher Scientific (Thermo, USA). All chemicals used were molecular biology grade and commercially available. The computer-based analysis and comparisons of nucleotide and protein sequences were performed with BLAST in NCBI databases.

Construction of plasmids

The integrative plasmid p*vhal*152 was obtained by inserting the synthetic vancomycin halogenase gene (*vhal*, NCBI Accession No: HQ679900.1) into the sites *Nde*I/*Bam*HI of p*eva*152. To construct p*chal*152, the A82846 halogenase gene (*chal*) was amplified by PCR with primer pairs *chal*-pF/pR from genomic DNA of *A. orientalis* SIPI18099, and cloned into the *Nde*I/*Asc*I sites of p*eva*152. Plasmids p*chal-2*-152 with two *chal* gene copies and p*chal-3*-152 with three *chal* gene copies were constructed based on p*chal*152. The 1.8-kb *Asc*I/*Bam*HI fragment containing *chal* and the *ermE** promoter was amplified from p*chal*152 by *chal-2*-pF/pR and inserted into p*chal*152 digested with *Asc*I/*Bam*HI to obtain p*chal-2*-152. In addition, p*chal-3*-152 was constructed by inserting another *ermE*-chal* amplified by *chal*-3-pF/R into *Bam*HI/*Not*I sites of p*chal-2*-152.

Construction of recombinant *A. orientalis*

The plasmids were introduced into *A. orientalis* SIPI18099 by the *Escherichia coli–Streptomyces* conjugation method

Table 1 Strains and plasmids used in this study

Strains or plasmids	Description	Source or reference
Plasmid vectors		
pSET152	*E. coli–Streptomyces* shuttle vector, Ap^r, *Streptomyces* φC31 integration site (*attP*)	Bierman et al. (1992)
P*eva*152	pSET152 derivative vector containing *ermE** promoter and *ter** terminator	This study
pMD18-T vector	*E. coli* cloning vector, Amp^r	Takara, Japan
P*chal*152	*chal* inserted into NdeI/AscI sites of P*eva*152	This study
p*vhal*152	*vhal* inserted into NdeI/BamHI sites of P*eva*152	This study
p*chal-2*-152	*chal* inserted into *Asc*I/*Bam*HI sites of P*chal*152	This study
p*chal-3*-152	*chal* inserted into *Bam*HI/*Not*I sites of P*chal-2*-152	This study
Bacterial strains		
A. orientalis SIPI18099	A82846B-producing mutant strain derived from the parental strain *A. orientalis* NRRL18099	Our laboratory
A. orientalis chal	SIPI18099 integrated with p*chal*152, Ap^r	This study
A. orientalis vhal	SIPI18099 integrated with p*vhal*152, Ap^r	This study
A. orientalis chal-2	SIPI18099 integrated with p*chal-2*-152, Ap^r	This study
A. orientalis chal-3	SIPI18099 integrated with p*chal-3*-152, Ap^r	This study
A. orientalis SET152	SIPI18099 integrated with pSET152, Ap^r	This study
E. coli		
DH5α	Host for general cloning	CWBIO, China
ET12567/pUZ8002	Donor strain for intergenetic conjugation between *E. coli* and Streptomyces, Ap^r, Kan^r, Chl^r	YouBio, China

described previously (Kieser et al. 2000). The *E. coli* ET12567/pUZ8002 containing the plasmid was grown in the presence of antibiotics (50 mg/L apramycin, 25 mg/L chloramphenicol, and 50 mg/L kanamycin) to an OD_{600} of 0.4–0.6. The cells were washed twice with an equal volume of LB medium and resuspended in 2 mL LB medium. The *A. orientalis* SIPI18099 was incubated in 25 mL TSB medium (15 g tryptone, 5 g soy peptone, 5 g NaCl, 15 g agar in 1 L tap water, pH 6.8–7.0) at 28°C for 48 h, then 10% of the culture was transferred into 25 mL TSB medium for 36-h growth. Mycelium was collected by centrifugation and resuspended in 2 mL LB. Mix 2-mL ET12567/pUZ8002 cells and the resuspended *A. orientalis* SIPI18099 and spread the mixture on mannitol-soya (MS) plates. The plates were incubated for 16–20 h at 28°C and then overlaid with 1 mL water containing 1 μg nalidixic acid and 200 μg apramycin. The plates were incubated again for 7–10 days at 28°C until the exconjugants were obtained. The exconjugants were then transferred onto slant medium containing 50 mg/L apramycin. The recombinant strains were designated as *A. orientalis vhal*, *A. orientalis chal*, *A. orientalis chal-2*, *A. orientalis chal-3*, and *A. orientalis* SET152, respectively.

Culture of *A. orientalis*

The parental and recombinant *A. orientalis* strains were grown on solid medium containing 10 g/L soy peptone, 10 g/L

Table 2 Oligonucleotide primers used in this study

Oligonucleotide	Sequence (5′-3′)	Purpose
chal-pF/pR	CATATGATGTCGGTCGAAGACTTCGATGTGG GGCGCGCC TATTTCGTAGATCCTCGA	Amplification of *chal*
chal-2-pF/pR	GGCGCGCCCTAGAGGTACCAGCCCGA GGATCCTCATGCCG GATGGTGCGGCAGC	Amplification of *ermE*- chal* for construction of p*chal-2*-152
chal-3-pF/R	GGATCCCTAGAGGTACCAGCCCGACCCGAGC GGATCCCT AGAGGTACCAGCCCGACCCGAGC	Amplification of *ermE*- chal* for construction of p*chal-3*-152
Apra-F/R	GTGCAATACGAATGGCGAAAAGCC TCAGCCAATCGACT GGCGAGC	Amplification of apramycin resistance gene
RT*chal*-pF/R	ATGTCGGTCGAAGACTTCGATGTGGTTGTT ACGCGCAC CCCAGCGGAAAGT	Amplification of 252-bp fragment in *chal* sequence for RT-PCR
RT16s-pF/R	GGGCGACATCCACGTTGTCC CGACAGCCATGCACCACCTG	Amplification of 407-bp fragment in 16s rRNA sequence in *A. orientalis* SIPI18099 genome

starch, 15 g/L agar, and pH 7.0–7.2 at 28°C for 5–6 days. For fermentation culture, a 1-cm^2 agar piece was transferred into a 250-mL flask with 20 mL seed medium containing 3 g/L yeast extract, 11 g/L polypeptone, 3 g/L malt extract, and 11 g/L glucose pH 6.8–7.0, and incubated at 28°C, 200 rpm for 72 h. Then 10% seed culture was inoculated into a 250-mL flask with 30 mL production medium containing 8 g/L glucose, 0.6 g/L yeast extract, 5 g/L maltodextrin, 0.54 g/L KCl, 0.5 g/L $CaCO_3$, 0.017 g/L KH_2PO_4, and pH 6.5–6.7, and incubated at 28°C, 200 rpm for 6 days.

Selection procedures of *A. orientalis* SIPI18099

Strain *A. orientalis* SIPI18099 was selected by the following procedures: NRRL18099 was the original strain and it was naturally separated for three times on solid medium described above. The obtained strain was named SIPI-S-32 in this procedure, then suspension of this strain was induced with 1 mg/mL NTG for 40 min and strain SIPI-N-101 selected from this procedure was used for next UV mutant for 90 s. Each selection course had improved the yield of A82846B to a certain extent, and the *A. orientalis* SIPI18099 was finally screened out as the original strain in our study.

Production of A82846B in 5-L fermenter

To investigate A82846 production, 2- to 3-cm^2 agar pieces of the *A. orientalis* SIPI18099 or *A. orientalis chal-3* from slant medium were cultured in 750-mL flasks with 100 mL seed medium at 33°C, 200 rpm, for 48 h and then 10% seed culture was inoculated into 2 L production medium in a 5-L fermenter. The fermentation temperature was kept at 34°C. The pH was adjusted to 6.7 with NaOH at the beginning, and during the fermentation process, this value was automatically kept at about 6.5–7.0 with $NH_3{\cdot}2H_2O$. Fifty percent glucose solution was added when the content of glucose dropped below 1.0%, and its concentration was kept at about 3.0–5.0% during the fermentation period.

Analytic method

One milliliter of culture broth was centrifuged at 12000 rpm for 20 min to remove the cells, then the supernatant was diluted tenfold and determined using HPLC with a gradient elution program (0→20 min, A:B = 95:5→85:15; 20→25 min, A:B = 85:15→95:5) in an Agilent C18 chromatographic column (4.6 × 150, 3.5 μm, Agilent, USA) with mobile phases of 0.1% trifluoroacetic acid (A), acetonitrile (B) at 1.0 mL/min, and detection at 235 nm. To measure biomass (PMV%), 10 mL fermentation broth was centrifuged for 20 min, 12000 rpm. Measure the volume of supernatant (v), the value of (10-v) 100%/10 was the biomass which could reflect the growth of the strains.

Assay of transcript levels by QRT-PCR analysis

Total RNAs from *A. orientalis* SIPI18099 and *A. orientalis chal*, *A. orientalis chal-2*, and *A. orientalis chal-3* were isolated from 5- and 6-day fermentation cultures of each strain. The mycelia were suspended in TRIzon Reagent (CWBIO, China) after being thoroughly ground. Ultrapure RNA Kit (CWBIO, China) was used for RNA isolation. To obtain cDNAs, DNA in the sample was eliminated using RNase-free DNase (Promega, USA). Then, reverse transcription was performed using M-MLV Reverse Transcriptase (Promega, USA). The transcript levels of the *chal* gene were assayed in a Bio-Rad iQ5 Real-Time PCR System; RT-PCR was performed with a QuantiTect SYBR Green RT-PCR Kit (Qiagen) with the following program: 95 °C for 2 min, followed by 40 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s. To assess the transcript level of the 16s rRNA gene (as a control), RT16s-F/R was used to amplify a 407-bp fragment in the 16s rRNA gene sequence in *A. orientalis* SIPI18099. For assessing the transcript level of the *chal* gene, RT*chal*-pF/R was used as the primer to amplify *chal*.

Results

Production of A82846B by *A. orientalis* SIPI18099

The *A. orientalis* SIPI18099 used in this study is an industrial strain used to produce A82846B, which has high yield of A82846B due to a series of selections from *A. orientalis* NRRL18099. A fed-batch culture was carried out in a 5-L fermenter. As can be seen in Fig. 2, during the whole fermentation process, *A. orientalis* SIPI18099 produced a high titer of A82846A and A82846C, and the maximum yield of A82846B, A, and C was around 780,2000, and 370 mg/L, respectively, at about 140 h. A82846B was only about 24.2% of the total A82846 components. Traditional methods such as optimizing the culture medium and fermentation techniques as well as

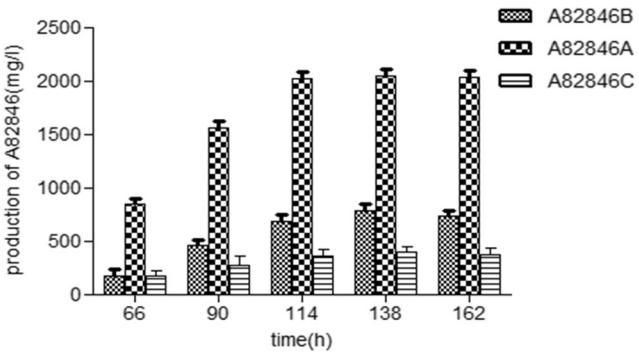

Fig. 2 The A82846 produced by *A. orientalis* SIPI18099 in a 5-L fermentation process at different culture times. Levels of A82846A, B, and C were analyzed by HPLC. The values shown are the average of three determinations

Fig. 3 Alignment of proteins encoded by *chal*, *vhal*, *bhaA* and *thal* in the align program, *chal* has 93.5% sequence identity with *vhal*, 95.5% with *bhaA*, and 85.1% with *thal.*

optimizing the strain by mutation have much randomness and might be difficult to obtain satisfied strain with low impurities.

Overexpression of *vhal* and *chal* in *A. orientalis* SIPI18099

In the biosynthesis of some antibiotics, halogenase is responsible for FADH2-dependent chlorination reactions (Puk et al. 2002; Dorrestein et al. 2005). We speculate that the halogenase activity in *A. orientalis* is not high enough to completely chlorinate the B component. To enhance halogenation activity, we overexpressed the halogenase gene. The protein encoded by ORF18 which named *chal* here in chloroeremomycin biosynthesis pathway (Van Wageningen et al. 1998) was aligned with the protein encoded by *vhal* encoding vancomycin halogenase (Xu et al. 2014), *bhaA* encoding balhimycin halogenase (Puk et al. 2002) and the gene named *thal* here encoding teicoplanin halogenase (Li et al. 2004), respectively, and they showed high identity (Fig. 3), suggesting that *chal* might be responsible for the halogenation of A82846.

Two plasmids, p*vhal*152 and p*chal*152, containing *vhal* and *chal* expression cassettes, respectively, controlled by a strong promoter *ermE** were constructed. Then, these plasmids were introduced into *A. orientalis* SIPI18099 by conjugal transformation and selected on apramycin-containing media to obtain recombinant strains *A. orientalis vhal* and *A. orientalis chal*, respectively. The plasmids p*vhal*152 and p*chal*152 were integrated into the chromosome of *A. orientalis* SIPI18099 via the φC31 integration site (*attP*). The recombinant strains were cultured in a shake flask to determine the fermentation products. As shown in Fig. 4a, *A. orientalis chal* produced slightly more A82846B compared to production by the parental strain, and the ratios of factors A, B, and C were 43, 54, and 3%, respectively, which displayed an obvious change from ratios of the parental strains (66% A, 24% B, 10% C). Expression of *vhal* also lowered the A82846C production, but the ratio of A to B was much higher than that of *A. orientalis chal*. The result indicated that *chal* from

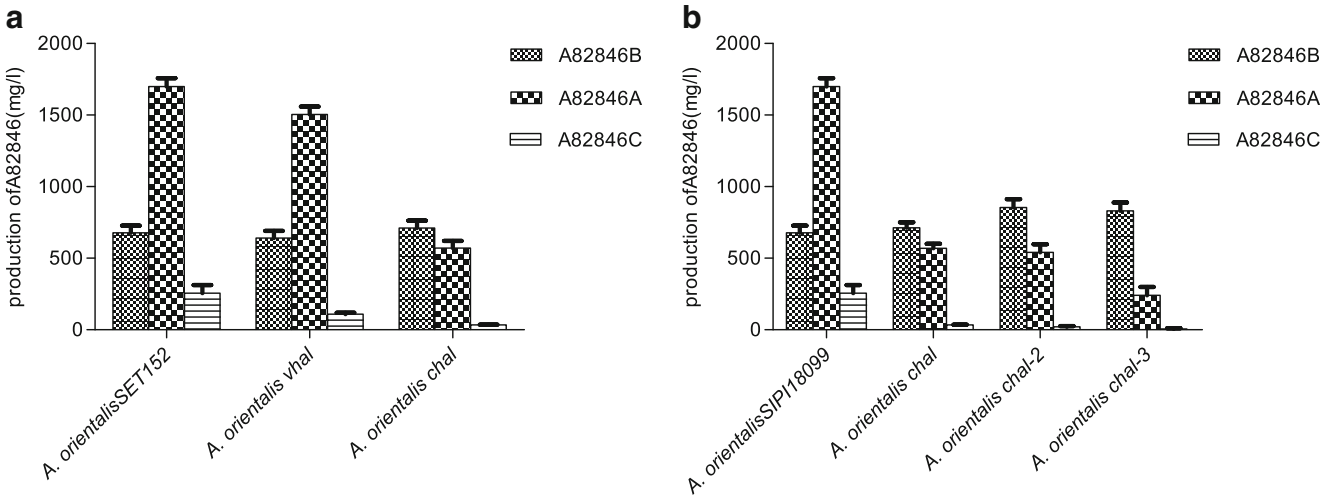

Fig. 4 **a** Comparison of yield of A82846 analogs in control strain, *A. orientalis vhal*, and *A. orientalis chal.* Each strain was cultured in shake-flask fermentation for 6 days. A82846 production values shown are the average of three determinations. **b** Effects of *chal* copy number on yield of A82846 analogs. Each strain was cultured in a shake flask for 6 days. A82846 production values shown are the average of three determinations

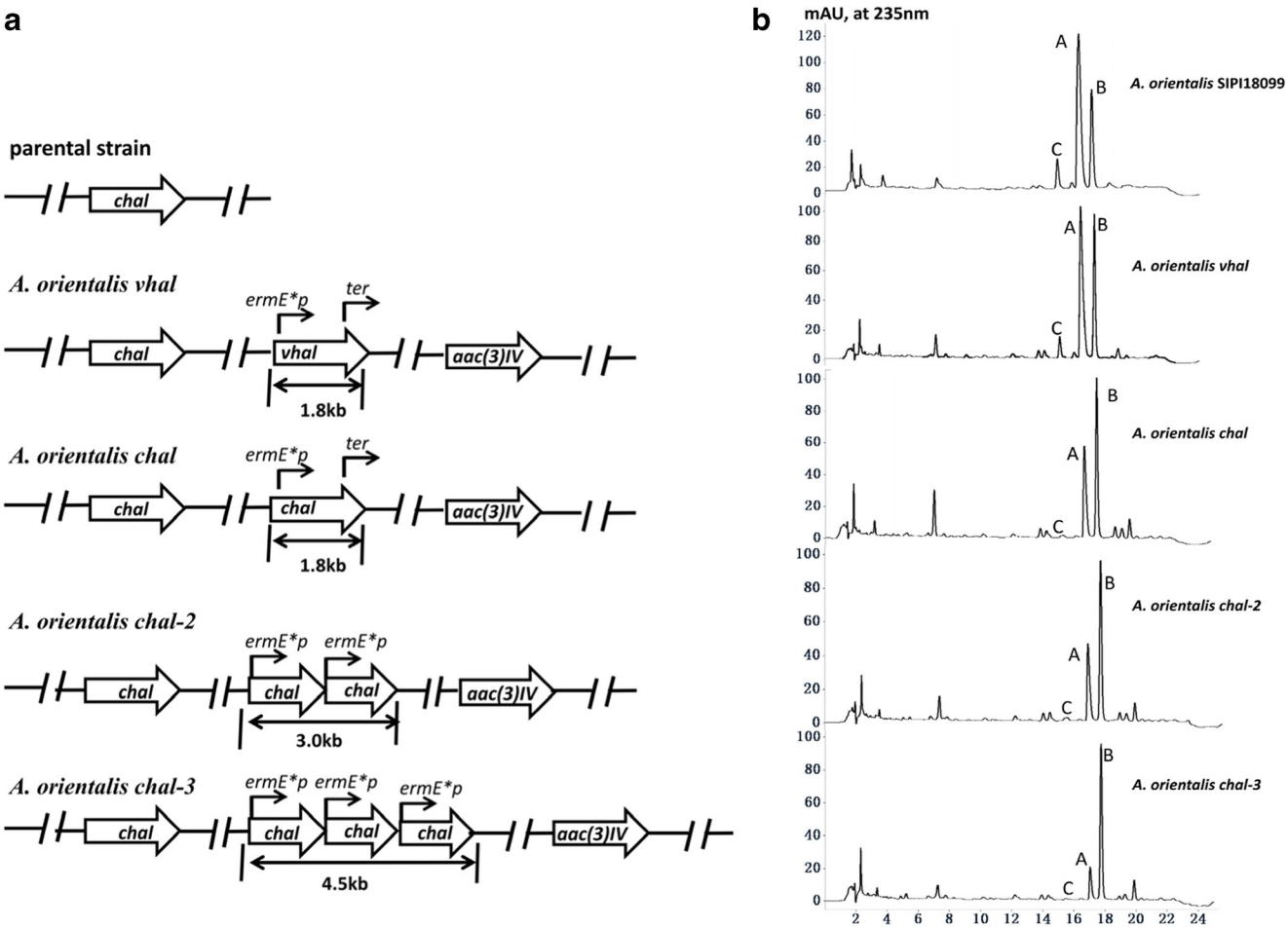

Fig. 5 **a** Genotypes of *A. orientalis* SIPI18099 and its recombinant strains. **b** HPLC analysis of the A82846 components production in fermentation broths; each strain was cultured in shake-flask fermentation for 6 days. The values shown are the average of three determinations

A. orientalis SIPI18099 itself is more efficient than *vhal* in reducing impurities during A82846 fermentation.

Effects of *chal* copy number on A82846 production

Since introducing one copy of *chal* was effective in reducing impurities, we increased the copy number of the *chal* gene. Expression cassettes containing two *chal* genes and three *chal* genes were separately constructed and integrated into *A. orientalis* SIPI18099 to generate the recombinant strains *A. orientalis chal-2* and *chal-3* (Fig. 5a). The genetically engineered strains were cultured in shake flasks for 6 days, and the fermentation broth samples were analyzed by HPLC (Fig. 5b). As shown in Fig. 4b, with increasing *chal* copy number, the contents of A82846A and C were gradually lowered, and factor B became the major component in the fermentation broth. For *A. orientalis chal-2*, the proportions of A and C in total A82846 were 38 and 1.7%, while in *A. orientalis chal-3*, the proportions of A and C dropped to 22.5% and below 1.0%, respectively. Besides reducing the impurities, the titer of A82846B was also improved by increasing the *chal* copy number; the production of A82846B improved by 23% (831 mg/L) in *A. orientalis chal-3* in comparison with parental strain (678 mg/L). The results suggested that overexpressing *chal* in the parental strain is a highly effective way to reduce structural analogs.

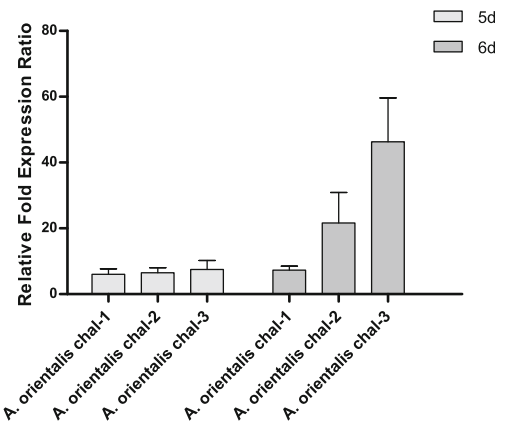

Fig. 6 Relative transcript levels of *chal* gene in recombinant strains compared with that in the parental strain, the total RNAs of each strain were extracted after 5 and 6 days of flask culture

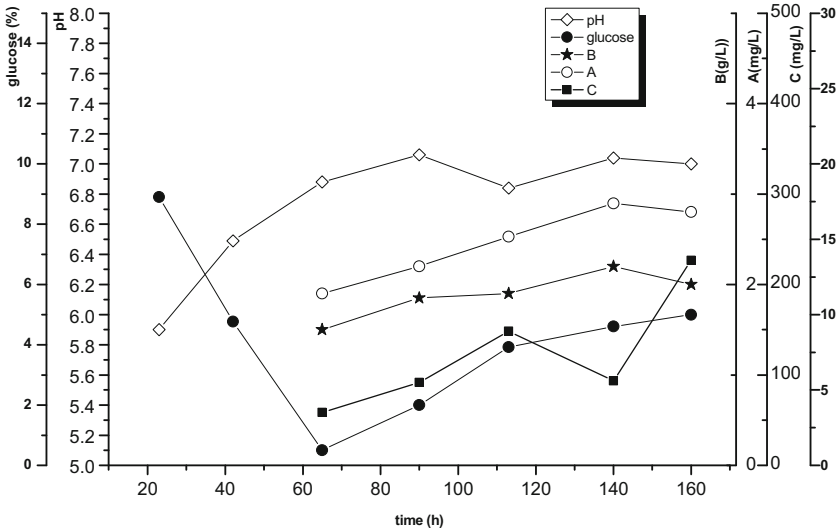

Fig. 7 Time-course of engineered strain *A. orientalis chal-3* cultured in a 5-L fermenter; culture conditions and process control were similar to the *A. orientalis* SIPI 18099

In addition, the biomass was around 25 ± 5% with no obvious difference among engineered and wild-type strains, indicating that increasing the copy number of this gene did not affect strain growth.

Assay of the transcript levels of *chal* by QRT-PCR analysis

RT-PCR was carried out to assay *chal* gene transcript levels in parental and recombinant strains. The transcript level of the 16s rRNA gene fragment served as a control gene in this test. As the data shows in Fig. 6, the transcript levels of *chal* in strains *A. orientalis chal*, *A. orientalis chal-2*, and *A. orientalis chal-3* were about 6-fold, 6.5-fold and 7.5-fold of that in strain SIPI18099 (set as 1) in the 5th day, while in the 6th day, the transcript level were about 7-fold, 23-fold, and 46-fold, indicating that the transcript levels of *chal* genes were obviously increased in the overexpressing strains, and increasing *chal* copy number significantly affected the transcriptional level of this gene. This result is consistent with the reduced production of A and C impurities and enhanced production of A82846B in recombinant strains.

Scaled-up culture of *A. orientalis chal-3* in 5-L fermenter

The production of A82846B by *A. orientalis chal-3* was carried out in a 5-L fermenter. The fermentation process is shown in Fig. 7. During the culture process, the pH value gradually rose from 0 to 80 h and thereafter was maintained at 6.8 ± 0.2 by adding 3 mol/L ammonium solution. The glucose was rapidly consumed before 60 h and then was controlled at about 3.0–5.0% by feeding 50% glucose solution. As can be seen, the proportion of impurities in scaled-up fermentation of the recombinant strain remained at a low level, while higher A82846 yields were obtained compared with that in flask culture. The maximum production of A82846B, A, and C was 2200, 290, and 56 mg/L, respectively, at about 140 h. The results suggested that A82846C was nearly eliminated in *A. orientalis chal-3* and the A82846A proportion decreased to 11.6%; meanwhile, the yield of A82846B was nearly threefold of that in the wild-type strain *A. orientalis* SIPI18099 in the same fermentation conditions (Table 3). During fermentation, few differences in growth pattern were found between the engineered and parental strains, indicating that the hereditary characters of *A. orientalis chal-3* were quite stable. The fermentation broth could be purified by normal-pressure chromatography to obtain high-purity A82846B for semi-synthesizing oritavancin, thereby avoiding high-pressure preparatory chromatography used in industrial production. Therefore, the *A. orientalis chal-3* allows for a simple and cost-effective fermentation process to produce A82846B.

Discussion

Recombinant DNA technology has provided new tools for improving yields of product of interest through genetic engineering

Table 3 Comparison of yield and proportion of A82846 in scaled-up fermentation

Strains	B (%)	A (%)	C (%)	Yield of A82846B (mg/L)
A. orientalis chal-3	88.2%	11.6	0.2	2200
A. orientalis SIPI18099	24.2%	63.6	12	780

of biosynthetic pathways. Heterogeneous expression and overexpression of some regulatory genes and biosynthetic structural genes have been used to alter the production of secondary metabolites (Lee et al. 2016; Olano et al. 2008; Shao et al. 2010). In 1998, van Wageningen et al. sequenced and analyzed the genes involved in chloroeremomycin biosynthesis. Through sequence comparisons, they annotated that the enzymes encoded by ORF18 and ORF10 were non-heme haloperoxidases and haloperoxidase that were responsible for chlorinating residues 2 and 6 of chloroeremomycin. However, ORF10 and 18 showed relatively low homologies in the report due to lack of enough microorganisms' genomic data in early study (24–29% identity to haloperoxidases found, 20% identity to haloperoxidase). Secondary metabolites with similar structures often share similar biosynthetic pathways. The chemical structures of vancomycin-type antibiotics such as balhimycin and chloroeremomycin have the similar heptapeptide core and have similarities in their biosynthetic pattern (Hubbard and Walsh 2003; Puk et al. 2004). By sequence re-analysis, we found the ORF18 showed 93.5% amino-acid sequence identity with the *vhal* gene in the vancomycin biosynthetic pathway, and 95.3% identity with the *bhaA* (NCBI Accession No: Y16952.3) gene in balhimycin biosynthesis. Deleting *bhaA* yields dechloro-balhimycin (Puk et al. 2002), and deleting *vhal* also abolishes halogenation of vancomycin, indicating that a single halogenase is required for chlorinating balhimycin and vancomycin, respectively. We also found that ORF10 showed similarities to perhydrolases of balhimycin and vancomycin (83.2 and 85.6% identity), suggesting that ORF10 is a perhydrolase rather than a haloperoxidase. Therefore, only ORF18 is responsible for chlorinating residues 2 and 6. Also, the fact that overexpressing ORF18 has significantly enhanced the content of A82846B suggests that this gene encodes a halogenase catalyzing both aromatic residues of A82846B.

Many glycopeptide antibiotics are characterized by the presence of chlorine atoms, which are strikingly important for antibiotic activity (Gerhard et al. 1993; Harris et al. 1985). However, the biosynthetic step during which halogenation takes place is still unclear, and the actual substrates for halogenation also have not been identified (Puk et al. 2004; Xu et al. 2014). Given the fact that vancomycin has two chlorine substitutes without any mono- or non-chlorine analogs in current industrial production, introducing the halogenase gene for vancomycin could theoretically help reduce the generation of A82846A and C as well as enhance the main product A82846B. But as the results showed, *vhal* was less effective than *chal* in reducing impurities in spite of its high homology with *chal*, indicating that halogenases from different sources have different substrate specificity. By overexpressing the endogenous A82846B halogenase gene, the halogenation capability significantly improved and the halogenase activity increased with increased *chal* copy number. The enhanced halogenase level can catalyze more substrates to synthesize a product with two chlorine atoms. On the other hand, fed-batch fermentation in the 5-L fermenter showed increased antibiotic production compared to that in flask culture, indicating that sufficient carbon source by feeding glucose and sufficient oxygen through aeration and agitation are beneficial for mycelium growth and A82846B production.

Above all, this study demonstrates for the first time that overexpressing halogenase can modify the chlorination pattern of glycopeptide antibiotics. Then, we developed an engineered strain which could be used in the industrial production of A82846B with much lower levels of components A82846A and A82846C.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Arthur M, Courvalin P (1993) Genetics and mechanisms of glycopeptide resistance in enterococci. Antimicrob Agents Chemother 37:1563–1571

Bierman M, Logan R, O'Brien K, Seno ET, Rao RN, Schoner BE (1992) Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces spp*. Gene 116(1):43–49

Brade KD, Rybak JM, Rybak MJ (2016) Oritavancin: a new lipoglycopeptide antibiotic in the treatment of gram-positive infections. Infect Dis Ther 5(1):1–15

Chang S, Sievert DM, Hageman JC, Boulton ML, Tenover FC, Downes FP, Shah S, Rudrik JT, Pupp GR, Brown WJ, Cardo D, Fridkin SK (2003) Infection with vancomycin-resistant *staphylococcus aureus* containing the *vanA* resistance gene. N Engl J Med 348(14):1342–1347

Corey GR, Kabler H, Mehra P, Gupta S, Overcash JS, Porwal A, Giordano P, Lucasti C, Perez A, Good S, Jiang H, Moeck G, O'Riordan W (2014) Single-dose oritavancin in the treatment of acute bacterial skin infections. N Engl J Med 370(23):2180–2190

Dorrestein PC, Yeh E, Garneau-Tsodikova S, Kelleher NL, Walsh CT (2005) Dichlorination of a pyrrolyl-S-carrier protein by FADH2-dependent halogenase PltA during pyoluteorin biosynthesis. Proc Natl Acad Sci U S A 102(39):13843–13848

Edelsberg J, Weycker D, Barron R, Li X, Wu H, Oster G, Badre S, Langeberg WJ, Weber DJ (2014) Prevalence of antibiotic resistance in US hospitals. Diagn Microbiol Infect Dis 78(3):255–262

Gerhard U, Mackay JP, Maplestone RA, Williams DH (1993) The role of the sugar and chlorine substitutes in the dimerization of vancomycin antibiotics. J Am Chem Soc 115(1):232–237

Hamill RL, Mabe JA, Mahoney DF, Nakatsukasa WM, Yao RC (1998) A82846 antibiotics. US Pat NO.005843437A

Harris CM, Kannan R, Kopecka H, Harris TM (1985) The role of the chlorine substituents in the antibiotic vancomycin: preparation and characterization of mono-and didechlorovancomycin. J Am Chem Soc 107(23):6652–6658

Hubbard BK, Walsh CT (2003) Vancomycin assembly: nature's way. Angew Chem 42(7):730–765

Kaatz GW, Seo SM, Aeschlimann JR, Houlihan HH, Mercier RC, Rybak MJ (1998) Efficacy of LY333328 against experimental methicillin-resistant *staphylococcus aureus* endocarditis. Antimicrob Agent Chemother 42(4):981–983

Kieser T, Bibb MJ, Buttner MJ, Chater K, Hopwood DA (2000) Practical Streptomyces genetics: a laboratory manual. The John Innes Foundation, Norwich

Leadbetter MR, Linsell MS, Lee J, Liu J (2010) Process for preparing glycopeptide phosphonate derivatives. US Pat NO.7728104B2

Lee KS, Lee BM, Ryu JH, Kim DH, Kim YH, Lim S-K (2016) Increased vancomycin production by overexpression of MbtH-like protein in *Amycolatopsis orientalis* KFCC10990P. Lett Appl Microbiol 63(3): 222–228

Li TL, Huang F, Haydock SF, Mironenko T, Leadlay PF, Spencer JB (2004) Biosynthetic gene cluster of the glycopeptide antibiotic teicoplanin: characterization of two glycosyltransferases and the key acyltransferase. Chem Biol 11(1):107–119

Olano C, Lombó F, Méndez C, Salas JA (2008) Improving production of bioactive secondary metabolites in *Actinomycetes* by metabolic engineering. Metab Eng 10(5):281–292

Puk O, Huber P, Bischoff D, Recktenwald J, Jung G, Süssmuth RD, Pée KH, Wohlleben W, Pelzer S (2002) Glycopeptide biosynthesis in *Amycolatopsis mediterranei* DSM5908: function of a halogenase and a haloperoxidase/perhydrolase. Chem Biol 9(2):225

Puk O, Bischoff D, Kittel C, Pelzer S, Weist S, Stegmann ED, Süssmuth R, Wohlleben W (2004) Biosynthesis of chloro-beta-hydroxytyrosine, a nonproteinogenic amino acid of the peptidic backbone of glycopeptide antibiotics. J Bacteriol 186(18):6093–6100

Rafai FA, Krishna G, Ding M, Chemburkar SR, Knable CM, Petzel JJ, Pruyne JJ, Reamer DM (2016) High purity Oritavancin and method of producing same. WO Pat No.2016011245 A1

Rupp ME, Fey PD, Longo GM (2001) Effect of LY333328 against vancomycin-resistant enterococcus faecium in a rat central venous catheter-associated infection model. J Antimicrob Chemother 47(5): 705–707

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Habor Laboratory Press, Cold Spring Habor

Shao L, Huang J, Jing L, Chen J, Kan S, Wang M, Li J, Chen D (2010) Overexpression of *aveBIV* leading to the improvement of 4′-epidaunorubicin production in *Streptomyces coeruleorubidus* strain SIPI-A0707. Appl Microbiol Biotechnol 87(3):1057–1064

Van Wageningen AM, Kirkpatrick PN, Williams DH, Harris BR, Kershaw JK, Lennard NJ, Jones M, Jones SJ, Solenberg PJ (1998) Sequencing and analysis of genes involved in the biosynthesis of a vancomycin group antibiotic. Chem Biol 5(3):155–162

Xu L, Huang H, Wei W, Zhong Y, Tang B, Yuan H, Zhu L, Huang W, Ge M, Yang S, Zheng H, Jiang W, Chen D, Zhao G, Zhao W (2014) Complete genome sequence and comparative genomic analyses of the vancomycin-producing *Amycolatopsis orientalis*. BMC Genomics 15(1):363