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Overexpression of the ABC transporter AvtAB increases avermectin production in *Streptomyces avermitilis*

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Abstract Avermectins are 16-membered macrocyclic polyketides with potent antiparasitic activities, produced by *Streptomyces avermitilis*. Upstream of the avermectin biosynthetic gene cluster, there is the *avtAB* operon encoding the ABC transporter AvtAB, which is highly homologous to the mammalian multidrug efflux pump P-glycoprotein (Pgp). Inactivation of *avtAB* had no effect, but increasing the concentration of *avtAB* mRNA 30–500-fold, using a multi-copy plasmid in *S. avermitilis*, enhanced avermectin production about two-fold both in the wild-type and in a high-yield producer strain on agar plates. In liquid industrial fermentation medium, the overall productivity of avermectin B1a in the engineered high-yield producer was improved for about 50%, from 3.3 to 4.8 g/l. In liquid YMG medium, moreover, the ratio of intracellular to extracellular accumulation of avermectin B1a was dropped from 6:1 to 4.5:1 in response to multiple copies of *avtAB*. Additionally, the overexpression of *avtAB* did not cause any increased expression of the avermectin biosynthetic genes through RT-PCR analysis. We propose that the AvtAB transporter exports avermectin, and thus reduces the feedback inhibition on avermectin production inside the cell. This strategy may be useful for enhancing the production of other antibiotics.

Keywords Antibiotic overproduction · Avermectin · Drug efflux pump · ABC transporter

Jingfan Qiu and Ying Zhuo contributed equally to this work.

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Introduction

Avermectins (Fig. 1a), produced by *Streptomyces avermitilis*, are important antiparasitic (nematode and insect) drugs for veterinary and human medicine (Ikeda and Omura 1997). *S. avermitilis* also produces large amounts of the usually unwanted, toxic macrolide oligomycin. The genome sequence of *S. avermitilis* is known (Ikeda et al. 2003), and there have been several studies aimed at increasing avermectin production (Yoon et al. 2004; Zhuo et al. 2010).

S. avermitilis has 428 putative ORFs related to ABC transporters, but the transported substrates are mostly unknown. The overexpression of maltose importer-type ABC transporter could enhance the production of avermectin and ivermectin (Li et al. 2010), but few studies have reported on exporters in *S. avermitilis*, and little is known about drug efflux pumps for avermectins. Upstream of the avermectin biosynthetic gene cluster is the *avtAB* operon (*sav933–934*) which encodes a putative ABC multidrug efflux transporter (Fig. 1b). There is an untranslated 478-bp sequence between the end of the *avtAB* and the global LAL-family regulator gene, *aveR*, which is the pathway-specific transcriptional activator for avermectin biosynthesis (Kitani et al. 2009).

ABC transporters share a common domain organization: two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs, also called ABC ATP-binding cassette) (Hollenstein et al. 2007). Most prokaryotic ABC

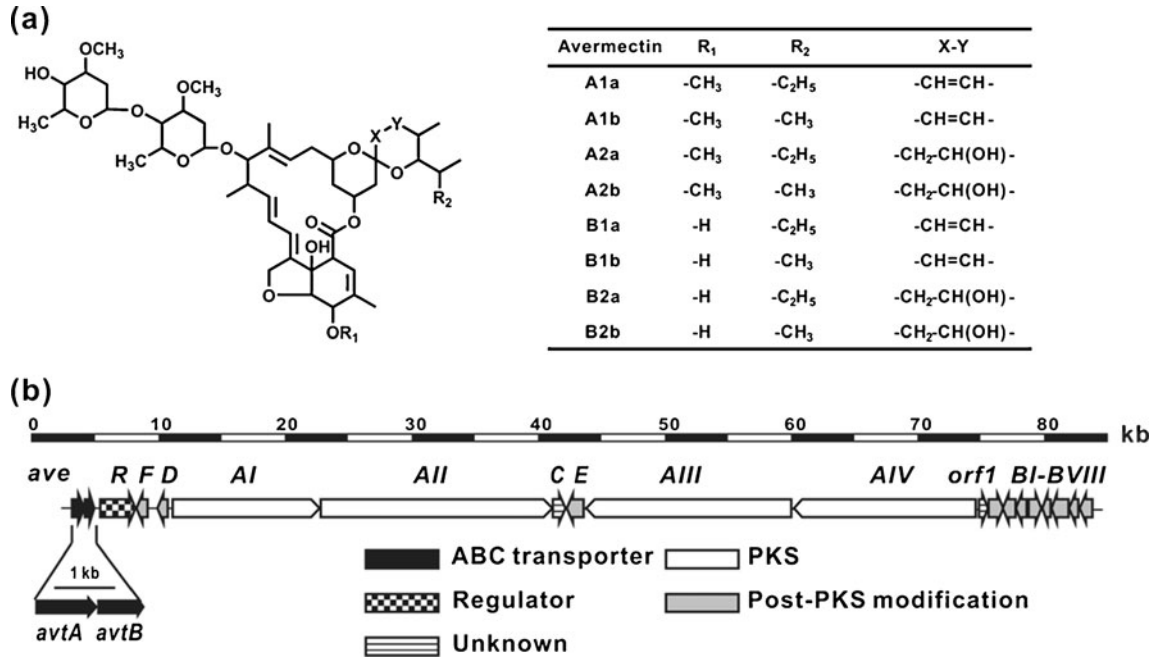


Fig. 1 Avermectins and *S. avermitilis* avemectin biosynthetic gene cluster. **a** Structures of eight avermectins. Avermectin B1a is the medically most effective compound. **b** *S. avermitilis* 80-kb avermectin (*ave*) biosynthetic gene cluster, and the *avtAB* operon encoding a putative ABC transporter. *aveR* positive regulator, *aveF* C5-ketoreductase, *aveD* C5-*O*-methyltransferase, *aveAI*, *aveAII*, *aveAIII* and *aveAIV* type I PKS genes, *aveE* cytochrome P450 hydroxylase

transporters consist of TMD and NBD polypeptides, which form a half transporter that then dimerizes to become a functional transporter (Eckford and Sharom 2009; Kerr et al. 2010). AvtA features the NBD domain with the conserved P-loop NTPase motif. AvtB features the typical transmembrane helices of ABC transporter permease proteins. The amino acid sequences of AvtA and AvtB are 24% identical and 45% similar to the mammalian P-glycoprotein (Pgp), an ABC drug efflux pump conferring resistance to the medically used semisynthetic ivermectin (Aziz et al. 1982). The HMMTOP program (Tusnady and Simon 2001) predicted that AvtA was located in the cytoplasm, and AvtB had six transmembrane helices like Pgp (Eckford and Sharom 2009).

When highly expressed, ABC efflux pumps confer multidrug resistance (MDR) in all organisms. In prokaryotes, ABC drug efflux pumps can also export endogenously produced secondary metabolites and thus prevent self-poisoning (Ikeno et al. 2000), reduce the feedback inhibition and increase metabolite production (Martin et al. 2005). Improving the export efficiency of avermectins could potentially enhance their production. However, the transport mechanisms for avermectins were poorly understood. Because of the proximity of the *avtAB* operon to the avermectin biosynthesis gene cluster and the sequence homology analysis, we speculated that the AvtAB transporter may export avermectins and possibly increase their production by reducing feedback inhibition.

Materials and methods

Bacterial strains and plasmids

Strains and plasmids are listed in Table 1.

General techniques

Spores of *S. avermitilis* NRRL 8165 and 3-115, a high-yield producer of avermectins were stored, activated and cultivated as described (Zhuo et al. 2010). Unless indicated otherwise, *S. avermitilis* was cultivated at 30°C. *E. coli* was cultivated at 37°C in LB (Luria-Bertani) broth agar. Total DNA was isolated from *Streptomyces* as described (Kieser et al. 2000). In vivo PCR-targeted gene replacement was achieved using REDIRECT® Technology (Gust et al. 2003). *S. avermitilis* was propagated on SFM agar (2% agar, 2% mannitol, 2% soybean powder, pH 7.2, *w*/*v*) with apramycin (30 μg/ml) or thiostrepton (25 μg/ml) when appropriate. The antibiotic concentrations were halved for liquid cultures.

Fermentation and HPLC antibiotic assay

Avermectins produced by wild-type and mutant strains were detected as follows: for plate cultures, a seed culture was prepared in 10.3% TSBY (3% TSB, 1% yeast extract, 10.3% sucrose, pH 7.2, *w*/*v*). After 36 h, three 9-cm

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant phenotype and/or characteristics	Reference or source
E. coli		
DH10B	*recA*	GibcoBRL
ET12567/pUZ8002	*recE*, *dam*, *dcm*, *hsdS*, Cm^r, Str^r, Tet^r, Km^r	(Paget et al. 1999)
BW25113	K12 derivative, *ΔaraBAD*, *ΔrhaBAD*	(Gust et al. 2003)
S. avermitilis		
NRRL 8165	Wild-type producer of avermectins	(Burg et al. 1979)
3-115	CGMCC no. 3229, industrial avermectin producer	(Zhuo et al. 2010)
QJF-8	NRRL 8165 derivative, 129-bp internal sequence of *avtA* replaced by the *aac(3)IV-oriT* cassette	This study Fig. 2
QJF-9	NRRL 8165 derivative, 138-bp internal sequence of *avtB* replaced by the *aac(3)IV-oriT* cassette	This study Fig. 2
Plasmids		
Fosmid 20B4	pCC1FOS™ containing part of the avermectin biosynthetic gene cluster (genes *sav927* - *aveAI*); source of *avtAB*	This study
pBluescript II SK(+)	*bla*, *lacZ*, *ori*(f1)	Stratagene
pJTU1278	*bla*, *tsr*, *lacZ*, *oriT*, ori^{pIJ101}, ori^{ColE1}	(He et al. 2010)
pJTU3468	pBluescript II SK(+) containing the 6.2-kb BglII/SacI fragment (*sav932*, *avtAB*) from Fosmid 20B4	This study
pJTU3469	3.7-kb BclI/EcoRI fragment (*avtAB*) from pJTU3468 in pJTU1278	This study
pJTU3470	pJTU1278 containing the 4.5-kb KpnI fragment (*avtAB*) from Fosmid 20B4	This study Fig. 3
pJTU3471	1.4-kb *aac(3)IV-oriT* cassette from pIJ773 (Gust et al. 2003) cloned into pJTU3469	This study Fig. 2
pJTU3472	1.4-kb *aac(3)IV-oriT* cassette from pIJ773(Gust et al. 2003) cloned into pJTU3470	This study Fig. 2

diameter plates each with 20 ml SFM were inoculated using 1 ml seed culture. Production of avermectin B1a (the most effective and commercially available avermectin component) was measured after 14 days. The agar and mycelia were extracted overnight with methanol, and the filtered methanol phase was evaporated. The residue was redissolved in 1 ml methanol, filtered through a 0.22-μm membrane filter and assayed using HPLC (Agilent SB C18 column, 2.1×150 mm, 3.5 μm; absorbance was monitored at 246 nm; the column was developed using 85% methanol). For liquid cultures in industrial medium, the seed medium was corn starch 3.0%, soya flour 0.8%, peanut meal 1.0%, yeast extract 4.0%, $CoCl_2$ 0.003%, α-amylase 0.004%, pH 7.0, *w*/*v*. The seed culture was incubated for 40–48 h at 28°C and 220 rpm, and used to inoculate (2%, *v*/*v*) 250 ml flasks with steel springs, containing 30 ml fermentation broth (Gao et al. 2009). After 10-day fermentation, the culture broths were analyzed. Strains containing vectors without inserts (8165/pJTU1278 or 3-115/pJTU1278) were used as controls. Data were collected from at least three independent experiments.

Detection of extracellular and intracellular avermectin B1a

A seed culture was prepared in 10.3% TSBY at 30°C and 220 rpm. After 72 h, it was used to inoculate (2%, *v*/*v*) three 250 ml flasks with steel springs, containing 30 ml YMG (0.4% yeast extract, 1% malt extract, 0.4% glucose, pH 7.2, *w*/*v*). After 10-day fermentation, the culture broths were filtered using gauze. Then mycelia adhere to the gauze were washed with 30-ml YMG medium. The liquid (about 60 ml) and the mycelia were extracted overnight with methanol separately. The methanol phase was evaporated, redissolved in 1 ml methanol and analyzed using HPLC.

Inactivation of *avtA*

A 6.2-kb SacI/BglII fragment from Fosmid 20B4 (*sav932–934*) was inserted into *Sac*I/*Bam*HI-digested pBluescript II SK(+) to give pJTU3468. This plasmid was digested with *Eco*RI/*Bcl*I, and a 3.7-kb fragment carrying *avtA* was inserted into *Eco*RI/*Bam*HI-digested pJTU1278 to give pJTU3469. Subsequently, we amplified the gene disruption cassette *aac(3)IV-oriT* from pIJ773 using primers SAV933-C1 and SAV933-C2. We then used REDIRECT® Technology to replace the 129 bp of *avtA* with1.37-kb *aac(3)IV-oriT* cassette to give pJTU3471 (Gust et al. 2003). pJTU3471 was then used for the targeted replacement in the wild-type *S. avermitilis* NRRL 8165 by conjugation. Total DNAs from the mutant strains and from the wild-type were used as templates for PCR analysis with primers SAV933-C5 and SAV933-C6. The PCR products of the wild-type and mutant strain were sequenced.

Inactivation of *avtB*

A 4.5-kb *Kpn*I fragment from Fosmid 20B4 carrying *avtA* and *avtB* was inserted into *Kpn*I site of pJTU1278 to give

pJTU3470. The gene disruption *aac(3)IV-oriT* cassette was amplified as previously described using the two oligonucleotide primers SAV934-C1 and SAV934-C2. We use REDIRECT® Technology to replace 138 bp of *avtB* with the *aac(3)IV-oriT* cassette, generating pJTU3472. The resulting plasmid pJTU3472 was used for the targeted replacement in *S. avermitilis* NRRL 8165 of a 138-bp DNA fragment internal to *avtB* with the 1.37-kb *aac(3)IV-oriT* cassette. The inactivation of the chromosomal *avtB* was confirmed through PCR using primers SAV934-C3 and SAV934-C4, and the PCR products were sequenced.

Overexpression of *avtA* and *avtB* in *S. avermitilis*

The gene dosage of the *avtAB* operon was increased by introducing the pIJ101-derived multi-copy plasmid pJTU3470 (Fig. 3) into *S. avermitilis* by RP4-mediated intergeneric conjugation.

RT-PCR analysis

The mRNA levels of *avtAB*, *aveR* and *aveAIII* were determined by quantitative RT-PCR. RNA was extracted from the *S. avermitilis* using the Total RNA Isolation Kit (SBS). Genomic DNA was removed using DNase I (MBI) according to the manufacturer's procedure. First-strand cDNA was synthesized from 4 μg total RNA in a 20-μl reaction volume using the First Strand cDNA Synthesis Kit (MBI). RT-PCR was performed using the Maxima™ SYBR Green qPCR Master Mix (MBI) and the Applied Biosystems 7500 Fast Real-time PCR System. Gene-specific primers were designed using Primer Premier 5.0, and the sequences of the primer pairs are listed in Table 2. The fluorescence change of SYBR Green I was monitored by the system software for every cycle, and the threshold cycle (CT), the melting curves, and the relative gene expression levels ($2^{-\Delta\Delta CT}$) were measured automatically. *hrdB* was used as the internal control.

Results

The genes *avtA* and *avtB* are expressed in *S. avermitilis* and form an operon

The sequence analysis suggested that *avtA* and *avtB* specify an ABC drug efflux pump, and the proximity of these genes to the avermectin biosynthetic gene cluster suggested that the pump might be for secreting avermectins, or transporting effector molecules for avermectin biosynthesis. The *avtA* stop codon overlaps the *avtB* start codon in ATGA, making it likely that the genes were co-transcribed in an operon. This was confirmed by RT-PCR using the primers SAV933–SAV934-boundary-C1 and SAV933–SAV934-boundary-C2, which match the end of *avtA* and the start of *avtB*. The expected 545 bp fragment was produced (data not shown), showing that the *avtAB* operon was co-transcribed.

Mutation of *avtA* or *avtB* had no effect on avermectin production

Both ORFs of the *avtAB* operon were inactivated in separate *S. avermitilis* strains by replacing an internal sequence (129 or 138 bp) by the *aac(3)IV-oriT* cassette with *aac(3)IV* in the same orientation as the replaced genes (Fig. 2a, b).

Four thiostrepton-sensitive, apramycin-resistant (Thio-^{S}Apr^{R}) *avtA* recombinant mutants (QJF-8-A-D) were obtained. Using primers SAV933-C5 and SAV933-C6, the wild-type gave a 0.36-kb PCR product, whereas the mutants gave a 1.60-kb PCR product (Fig. 2c). This confirmed that a 129-bp DNA fragment internal to *avtA* had been replaced by the 1371-bp disruption cassette.

Similarly, from *avtB* replacement, four independent ex-conjugants (QJF-9-A, QJF-9-B, QJF-9-C and QJF-9-D) were obtained and tested using primers SAV934-C3 and SAV934-C4. A new 2.10-kb band appeared in QJF-9, and the original 0.87-kb band disappeared (Fig. 2d).

HPLC quantification of the fermentation extracts of three independent mutants of each *avtA* and *avtB* clearly demonstrated that the amount of avermectin B1a produced was similar to the amount produced by the wild-type *S. avermitilis* NRRL 8165 (Fig. 2e). These results suggested that *avtAB* was not required for avermectin production in *S. avermitilis* NRRL 8165 under our conditions.

Overexpression of AvtA and AvtB in wild-type *S. avermitilis* increased avermectin production

We noticed that in liquid cultures only a small amount of the avermectin was found outside, suggesting that most of the avermectin was intracellular or remained cell bound. It seemed possible that increasing the expression of AvtAB would increase the proportion of exported avermectins.

The expression of AvtA and AvtB was enhanced by increasing the copy number of *avtAB* in *S. avermitilis*. For this, the pIJ101-derived multi-copy plasmid pJTU3470 (thiostrepton selection, Fig. 3a) was introduced into wild-type NRRL 8165. The yield of all eight avermectins (Fig. 1) increased almost uniformly (data not shown). Only avermectin B1a, for which there was a commercially available standard, and oligomycin A were quantitated in further experiments.

Figure 3b shows the relative antibiotic yields of three independent *S. avermitilis* NRRL 8165/pJTU3470 deriva-

Table 2 Primers used in this study

Name	Sequence (lower case indicates 5′ non-homologous tails)	Use
SAV933-C1	agccggtcaacgggctcgacccggaaggcgtcctgtggc ATTCCGGGGATCCGTCGACC	Replacement of *avtA* by REDIRECT® Technology
SAV933-C2	ccacgtccgtgtacctgtcgatgaactcgctcatgctgg TGTAGGCTGGAGCTGCTTC	
SAV933-C5	TTCTCGCTGGGCATGAGTC	PCR analysis of QJF-8 (*ΔavtA*)
SAV933-C6	GCAGTCGGTGACCGTGATT	
SAV934-C1	gcgccattgtctacctgacgctgatctgcaccttgtcca ATTCCGGGGATCCGTCGACC	Replacement of *avtB* by REDIRECT® Technology
SAV934-C2	tccgtcgccgggaggtccggcaatacgcatcagttccag TGTAGGCTGGAGCTGCTTC	
SAV934-C3	CAACTTCAGTCCGCTCTTCGC	PCR analysis of QJF-9 (*ΔavtB*)
SAV934-C4	TCCTGGAGACATCCGTGCC	
DQ18F	GCAGCCTCAACCAGATCCTC	RT-PCR amplification of *hrdB*
DQ18R	TTGGCAGTCACCGTCTTCG	
aveR-R1	CGGCTGGCCCTTACCGAATG	RT-PCR amplification of *aveR*
aveR-R2	AGTGCCTCGCCCAGGAACGT	
AveA3-R3	ATCCGCAGCAGCGGTTGT	RT-PCR amplification of *aveAIII*
AveA3-R4	CGTTGCCGATGTAGCCCTC	
SAV933-R3	GCGGGTGGCGGTCATTCT	RT-PCR amplification of *avtA*
SAV933-R4	GCTTGGTCAGATCCGTGACTTCG	
SAV934-R3	CTGCTGCTGGCCTTCGTGC	RT-PCR amplification of *avtB*
SAV934-R4	GAAGAGCGGACTGAAGTTGACG	
SAV933-SAV934-boundary-C1	GCCATCTGATGAGCGAGGCG	Co-transcription analysis of *avtA* and *avtB*
SAV933-SAV934-boundary-C2	CGAAGAGCGGACTGAAGTTGACG	

tives grown on agar and in liquid industrial production medium. Avermectin B1a production was increased between 1.6 and 4.5-fold compared to the strain transformed with the control vector (7.5 mg/l on agar plates and 17.1 mg/l in liquid culture). Oligomycin A production was unaffected or slightly reduced. This indicated that overexpression of AvtAB enhanced avermectins production, presumably by increasing avermectin export and thus reducing feedback inhibition. The AvtAB transporter seemed to be specific for avermectins because oligomycin A production was not increased.

Effect of increased expression of *avtAB* on the transcription of avermectin biosynthetic genes

If the function of AvtAB is only to export avermectin from the cells, then the transcription of the avermectin biosynthetic genes was expected to remain largely constant. The transcription of four genes, *aveR* (LAL-family transcriptional regulator), *aveAIII* (PKS), *avtA* and *avtB* was measured using RT-PCR. Again three independent isolates of *S. avermitilis* NRRL 8165/pJTU3470 were tested, and each sample was measured three times using the primers listed in Table 2.

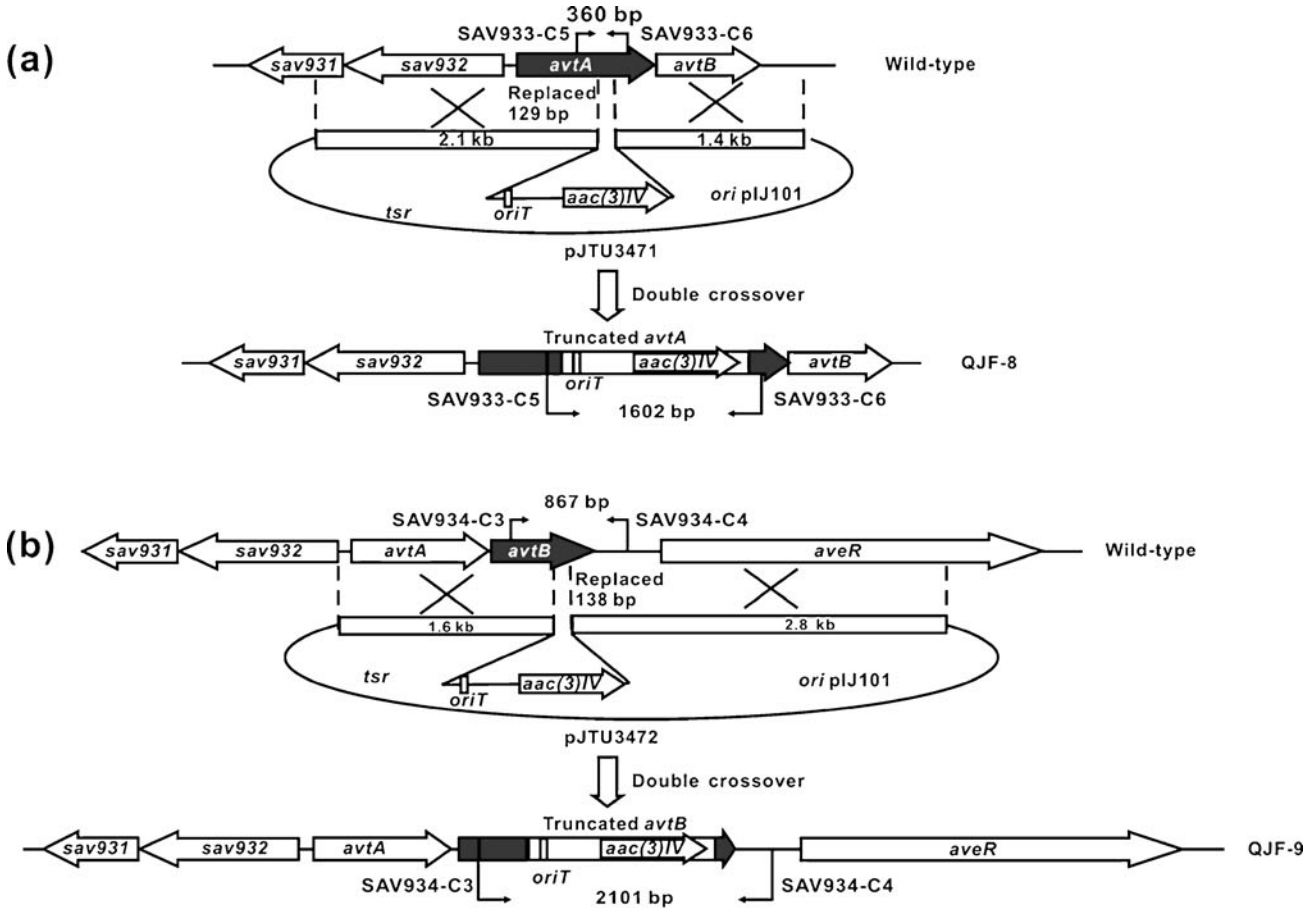
Figure 4 shows that transcription of *aveAIII* was unchanged compared to the control (8165/pJTU1278), and *aveR* was slightly (2^2-fold) induced. The mRNA abundance of *avtA* and *avtB* confirmed that the copy number of the *avtAB* operon was about 100 (2^7)-fold increased, in line with the reported copy number of pIJ101, the ancestor of pJTU3470 (Kieser et al. 1982).

Effect of increased copy number of *avtAB* on the industrial producer *S. avermitilis* 3-115

To check the generality of the above results, we increased the copy number of *avtAB* in the industrial strain *S. avermitilis* 3-115 by introducing pJTU3470. In SFM agar plate culture, the yield of avermectin B1a was increased almost two-fold to about 170 mg/l compared to the control (Fig. 5). In shake flask cultures containing the industrial production medium, even the strain transformed with the control vector produced large amounts (3.3 g/l) of avermectin B1a. Adding extra copies of *avtAB* increased this production by 30–50% to 4.8 g/l.

Overexpression of AvtAB enhanced extracellular avermectin B1a accumulation in the industrial producer *S. avermitilis* 3-115

The extracellular and intracellular avermectins from the agar cultures cannot be accurately distinguished, because the mycelia and agar tightly blend with each other. Compared with agar, it is much easier to separate the extracellular and intracellular avermectins from liquid


(a)
360 bp
SAV933-C5
SAV933-C6
sav931
sav932
avtA
avtB
Wild-type
Replaced
129 bp
2.1 kb
1.4 kb
tsr
oriT
aac(3)IV
ori pIJ101
pJTU3471
Double crossover
Truncated avtA
QJF-8
SAV933-C5
1602 bp
SAV933-C6
(b)
867 bp
SAV934-C3
SAV934-C4
aveR
Replaced
138 bp
1.6 kb
2.8 kb
pJTU3472
Truncated avtB
QJF-9
SAV934-C3
2101 bp
SAV934-C4
(c)
1 2 3 4 5 6
1.60 kb
0.36 kb
1.65 kb
1.00 kb
0.40 kb
0.30 kb
(d)
2.10 kb
0.87 kb
3.00 kb
2.00 kb
1.00 kb
0.85 kb
(e)
Avermectin B1a production (mg/l)
11
10
9
8
7
6
NRRL 8165
QJF-8-A
QJF-8-B
QJF-8-C
QJF-9-A
QJF-9-B
QJF-9-C

◀ **Fig. 2** Inactivation of *avtA* and *avtB*, encoding putative ABC transporter. **a** Double crossover gene replacement to inactivate *avtA* (*black arrow*). The *top line* shows the genes in the wild-type strain. The *angled arrows* indicate the PCR primers (SAV933-C5 and SAV933-C6) that were used to amplify the DNA fragments shown in **c**. *Large crosses* indicate crossovers between the 2.1 and 1.4-kb identical sequences on pJTU3471 and the *S. avermitilis* genome. The *bottom line* shows the mutant QJF-8, in which a 129-bp fragment of *avtA* was replaced by the 1371-bp *aac(3)IV-oriT* cassette. **b** Construction of the *avtB* mutant strain QJF-9. The homologous arms were 1.6 and 2.8 kb. A 138-bp fragment of *avtB* was replaced by the *aac(3)IV-oriT* cassette. **c** and **d** Ethidium bromide-stained agarose gels showing the PCR analysis of the mutants QJF-8 (*ΔavtA*) and QJF-9 (*ΔavtB*). *Lanes 1–4* independent mutants, *lane 5* wild-type, *lane 6* 1-kb ladder (see Table 2 for primers). **e** Production of avermectin B1a by *S. avermitilis* NRRL 8165 and three independent ex-conjugants of QJF-8 (*ΔavtA*) and QJF-9 (*ΔavtB*) on SFM agar

medium. However, in the liquid industrial production medium, extracellular avermectins adhere to the medium particles, and thus would be trapped together with the medium particles during filtration. Therefore we use YMG, a simple and clear liquid medium, to separate extracellular and intracellular avermectins.

In shake flask cultures containing the YMG medium, the wild-type strain transformed with pJTU3470 only produced an extremely small amount of avermectin B1a (about 1.6 mg/l), which makes it difficult to quantitate avermectins by HPLC. However, the industrial strain 3-115 transformed with pJTU3470 produced relatively large and detectable amounts of avermectin B1a (about 25 mg/l). Therefore 3–115/pJTU3470 was used to quantitate and compare the extracellular and intracellular avermectins.

Compared with the control of 3-115/ pJTU1278, the yield of extracellular avermectin B1a for 3-115/pJTU3470 was increased almost three-fold from 2.0 mg/l to about 5.5 mg/l in

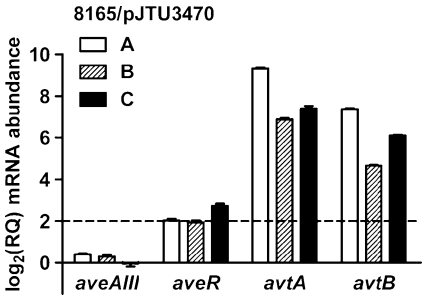


Fig. 4 Transcriptional activity in wild-type *S. avermitilis* NRRL 8165 in response to multiple copies of *avtAB*. Relative transcription levels of *aveAIII* (PKS), *aveR* (regulator), *avtA* and *avtB* (ABC transporter) in *S. avermitilis* NRRL 8165/pJTU3470 (containing *avtAB*) were compared to the vector control (8165/pJTU1278). Samples were collected from agar plates after 72 h at 30°C. *hrdB* was used as an internal control. Three independent ex-conjugants, *A*, *B* and *C*, of NRRL 8165/pJTU3470 were examined. Each ex-conjugant was tested in triplicate by quantitative RT-PCR, using gene-specific primers (Table 2). 0=no change; values higher than two (*dash line*) were considered significant increases

YMG. However, the production of intracellular avermectin B1a for 3-115/pJTU3470 was increased only by one-fold to 25 mg/l (Fig. 6). Moreover, the ratio of intracellular to extracellular accumulation of avermectin B1a was dropped from 6:1 to 4.5:1 in response to multiple copies of *avtAB*, suggesting that AvtAB enhanced the export of avermectins. Meanwhile, neither extracellular nor intracellular oligomycin A was found increased in YMG medium (data not shown).

Discussion

We identified a putative ABC efflux pump, AvtAB, consisting of two proteins encoded by the *avtAB* operon

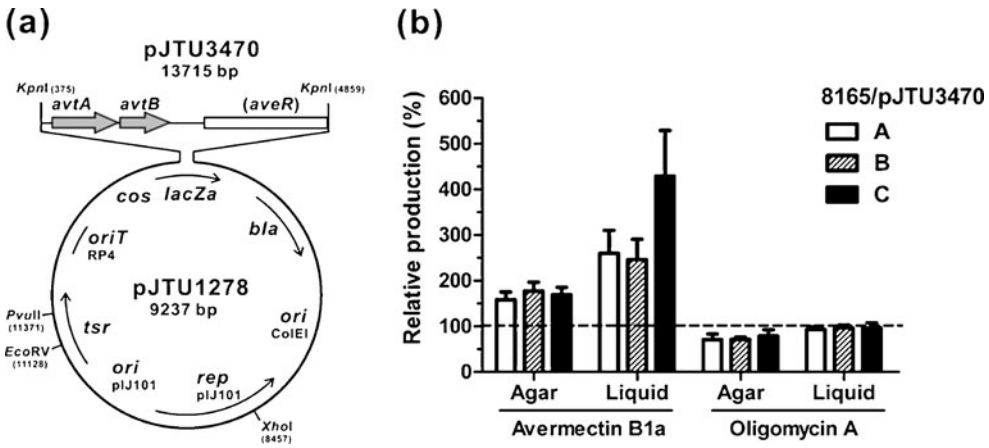


Fig. 3 Antibiotic production by wild-type *S. avermitilis* NRRL 8165 in response to multiple copies of *avtAB*. **a** pJTU3470 was derived from the pIJ101-derived multi-copy vector pJTU1278 by inserting a 4.5-kb *Kpn*I fragment from Fosmid 20B4 containing *avtAB*. *tsr* thiostrepton resistance gene. **b** Production of avermectin B1a and oligomycin A by *S. avermitilis* NRRL 8165/pJTU3470 was compared to the strain transformed with the control vector, both on SFM agar and in shake cultures containing liquid industrial medium. (100%=no change in production). *A*, *B* and *C* indicate three independent ex-conjugants, each of which was cultured and examined for three times

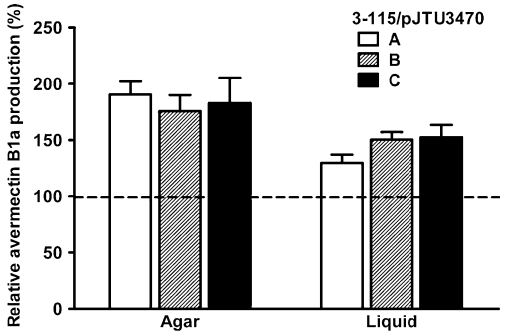


Fig. 5 Avermectin B1a production by the high-yield industrial strain *S. avermitilis* 3-115 in response to multiple copies of *avtAB*. *S. avermitilis* 3-115 containing pJTU3470 was compared to the strain transformed with the control vector, both on SFM agar and in liquid industrial medium

next to the avermectin biosynthetic gene cluster. This transporter was not required for avermectin production in *S. avermitilis* NRRL 8165, but adding multiple copies of *avtAB* increased avermectin production by wild-type *S. avermitilis* and also by an industrial avermectin production strain.

No obvious decrease was detected after inactivation of *avtAB*. Similarly, inactivation of an ABC transporter, LndW, conferring landomycin E resistance, did not affect Landomycin E production in *S. globisporus* (Ostash et al. 2008). Similar results were obtained for the multidrug efflux pump CefT. The gene replacement showed it was not essential for cephalosporin production in *Acremonium chrysogenum*. However, amplification of *cefT* increased the yield of cephalosporin in the transformants (Ullan et al. 2002). Although little is known about avermectin export, we proposed that AvtAB may not be the only avermectin exporter. In addition, avermectin is non-toxic for bacteria (Ikeda and Omura 1997), and therefore, avermectin resistance could not be detected in *Streptomyces*.

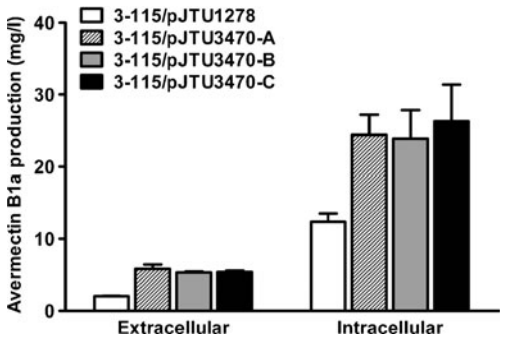


Fig. 6 Extracellular and intracellular avermectin B1a production by the industrial strain *S. avermitilis* 3-115 in response to multiple copies of *avtAB*. *S. avermitilis* 3-115 containing pJTU3470 was compared to the strain transformed with the control vector. Extracellular and intracellular avermectin B1a produced in YMG liquid medium were quantitated separately. 3-115/pJTU3470-A, -B and -C indicate three independent ex-conjugants. The data shown are the mean values obtained from at least three independent experiments

Quantitative RT-PCR confirmed that increasing the copy number of *avtAB* gave the expected increase of *avtAB* mRNA (Fig. 4). However, the relative level of *aveR* mRNA increased only slightly, just reaching the level of significance (Fig. 4). Considering the bioinformatic predictions that AvtAB is a drug efflux pump, AvtAB was believed unlikely to act as a transcriptional inducer of *aveR*. We speculated that the extra copies of *avtAB* increased the efflux of avermectin. This in turn may have reduced a hypothetical feedback regulation on avermectin production. Besides, transcription of the type I PKS gene *aveAIII* did not change in response to the increased copy number and expression of *avtAB*, excluding the possibility that AvtAB might uptake signal molecules that modulate the biosynthesis of avermectin.

We initially expected the additional ABC efflux transporter genes to have an even bigger effect in a high-yielding strain, but this strain may have lost feedback inhibition, and gained improved ability to export avermectins during strain development. Or avermectin production may be limited by the supply of precursor molecules. With these considerations, it seems remarkable that increasing the copy number of *avtAB* in *S. avermitilis* 3-115 did increase the already high avermectin production by a further 30–50%.

Overexpression of AvtAB increased the yield of extracellular avermectin B1a about three-fold and changed the ratio of intracellular to extracellular accumulation of avermectin B1a from 6:1 to 4.5:1 in YMG medium. This data supports the hypothesis that AvtAB exports avermectin and reduces the feedback inhibition of avermectin biosynthesis inside the cell.

Like other ABC transporters, AvtAB might export multiple drugs including oligomycin, another macrolide produced by *S. avermitilis*. Oligomycin production was, however, not affected (Fig. 3). The slight decrease in oligomycin production on agar may have been caused by the competition for methylmalonate which is used for polyketide biosynthesis. This would be consistent with AvtAB being specific for avermectin transport. It will also be interesting to find out whether AvtAB can increase production of antibiotics other than avermectins.

Even if the secondary metabolite is not toxic for the producer, even an inert substance could fill up the cells and reduce their fitness. So increasing the copy number of genes for drug efflux found in the vicinity of antibiotic biosynthetic clusters may be a generally useful strategy for increasing the production of secondary metabolites even in high-yield production strains.

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