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Research Article

Engineered Production of Tryprostatins in *E. coli* through Reconstitution of a Partial *ftm* Biosynthetic Gene Cluster from *Aspergillus* sp.

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Abstract

Tryprostatin A and B are indole alkaloid-based fungal products that inhibit mammalian cell cycle at the G_2/M phase. They are biosynthetic intermediates of fumitremorgins produced by a complex pathway involving a nonribosomal peptide synthetase (FtmA), a prenyltransferase (FtmB), a cytochrome P450 hydroxylase (FtmC), an O-methyltransferase (FtmD), and several additional enzymes. A partial fumitremorgin biosynthetic gene cluster (*ftmABCD*) from *Aspergillus* sp. was reconstituted in *Escherichia coli* BL21(DE3) cells, with or without the co-expression of an Sfp-type phosphopantetheinyltransferase gene (*Cv_sfp*) from *Chromobacterium violaceum* No. 968. Several recombinant *E. coli* strains produced tryprostatin B up to 106 mg/l or tryprostatin A up to 76 mg/l in the fermentation broth under aerobic condition, providing an effective way to prepare those pharmaceutically important natural products biologically.

Keywords: *ftm* Biosynthetic Gene Cluster; Heterologous Production; Tryprostatins

Abbreviations:

A: Adenylation Domain;
BCRP: Breast Cancer Resistance Protein;
C: Condensation Domain;
IPTG: Isopropyl-β-D-Thiogalactopyranoside;
LC-MS: Liquid Chromatography-Mass Spectrometry;
MAP2: Microtubule Associated Protein 2;
MCS: Multiple Cloning Site;
MDR: Multidrug Resistance;
NRPS: Nonribosomal Peptide Synthetase;
PCP: Peptidyl Carrier Protein Domain;
PPTase: Phosphopantetheinyltransferase;
TPS-A: Tryprostatin A;
TPS-B: Tryprostatin B;
RT-PCR: Reverse Transcription-Polymerase Chain Reaction

Introduction

Tryprostatin A (TPS-A) and tryprostatin B (TPS-B) are anticancer natural products containing an isoprenylated diketopiperazine indole (Figure 1a), first isolated as mammalian cell cycle inhibitors from the fermentation broth of marine fungus *Aspergillus fumigatus* BM939 [1-3]. They are biosynthetic intermediates of fumitremorgins [3, 4]. Cui *et al*. showed that TPS-A, TPS-B and related demethoxyfumitremorgin C inhibit cell cycle progression of mouse tsFT210 cells at the G2/M phase with minimum inhibitory

concentrations in the low µM range [1]. Usui *et al*. demonstrated that TPS-A specifically blocks MAP2 (microtubule associated protein 2)-dependent assembly of microtubules [5]. Furthermore, both TPS-A and fumitremorgin C were reported to be potent inhibitors of breast cancer resistance protein (BCRP), a member of the ABC transporter family, which has been associated with multidrug resistance (MDR) of various cancers [6-8].

F is subsequently converted to TPS-B by a prenyltransferase, FtmB, as demonstrated by *in vitro* assays [10]. TPS-B undergoes hydroxylation at C-6 position of the indole ring catalyzed by a cytochrome P450 hydroxylase, FtmC, and followed by methylation catalyzed by an O-methyltransferase, FtmD, to produce TPS-A [4, 11]. Further biosynthesis leads to several fumitremorgins and verruculogen [4].

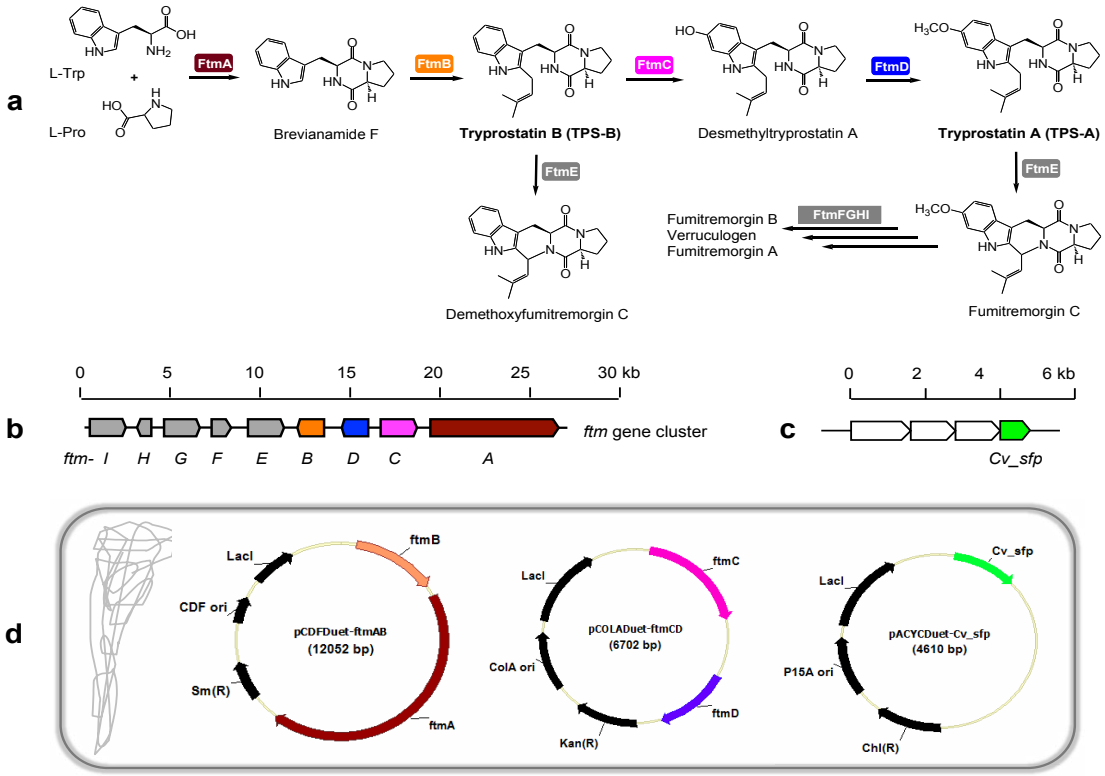

Figure 1. Schematic depiction of heterologous production of tryprostatins in recombinant *E. coli* strains. (a) The biosynthetic pathway of tryprostatins and fumitremorgins. (b) The *ftm* gene cluster in the genome of *A. fumigatus* BM939. (c) Source of a Sfp-type phosphopantetheinyltransferase gene from *Chromobacterium violaceum* No. 968 [15]. (d) Scheme of a representative recombinant *E. coli* BL21(DE3) cell (GS07Plus strain) that harbors all three gene expression constructs.

The tryprostatin and fumitremorgin biosynthetic pathway (Figure 1a) is encoded by the fumitremorgin biosynthetic cluster (*ftm*) which contains nine genes (Figure 1b). The same orthologous gene cluster has been identified in three *A. fumigatus* isolates (Af293, A1163 and BM939) and in *Neosartorya fischeri* NRRL 181 [4]. The biosynthesis of tryprostatins and fumitremorgins was proposed to begin with the condensation of a tryptophan (L-Trp) and a proline (L-Pro) to form brevianamide F. This reaction is catalyzed by FtmA, a dimodular nonribosomal peptide synthetase (NRPS) with a domain organization of A-PCP-C-A-PCP-A, where A stands for adenylation domain, PCP for peptidyl carrier protein domain, and C for condensation domain. This reaction was proven by heterologous expression of *ftmA* in *A. nidulans* and identification of brevianamide F as the biosynthetic product [9]. Brevianamide

Although the *ftm* gene cluster was first identified in the genome of Af293 [10], it was thought to be not expressed in Af293 because no fumitremorgins could be detected in this strain [9]. However, a recent study showed by RT-PCR that all *ftm* genes are expressed almost equally well in both Af293 and BM939 strains [12]. Furthermore, a point mutation was found in *ftmD* in the genome of Af293 to cause an arginine to leucine substitution at position 202 of FtmD, resulting in a dramatic decrease of the catalytic efficiency of FtmD. This mutated form of FtmD appeared not functioning under physiological conditions in Af293 to produce any detectable levels of TPS-A or any downstream metabolites [12].

TPS-A and TPS-B are produced at merely 0.4 mg/l by the native *A. fumigatus* BM939 strain in shaker flasks under

laboratory conditions [1]. Maiya *et al*. heterologously co-expressed *ftmA* and *ftmB*, under the control of a strong promoter, P_{alcA}, in a naïve host *A. nidulans*, and obtained TPS-B at an impressive titer of 250 mg/l [13]. However, to date no study has been reported for the overproduction of TPS-A. Here we report that, through reconstitution of a partial *ftm* gene cluster (four genes, *ftmABCD*, on two compatible plasmids) from *Aspergillus* sp., we obtained recombinant *E. coli* strains that produce TPS-B up to 106 mg/l and TPS-A up to 76 mg/l in shaker flask fermentation, providing an effective way to prepare those pharmaceutically important natural products.

Materials and Methods

General microbiology and molecular biological manipulations

Bacterial and fungal strains, and plasmids used in this study are listed in Table 1. Bacterial culture conditions and general molecular biological manipulations were performed according to standard protocols [14], or according to manufacturer's manuals. Chemicals and biochemicals were purchased from Fisher Scientific Inc. (Pittsburgh, OH), and enzymes from New England BioLabs (Ipswich, MA), unless otherwise indicated. YPD medium (1% yeast extract, 2% peptone, 2% dextrose) was used to grow *Aspergillus* sp. cultures, from which all fungal genomic DNA sample were prepared with a Fungi/Yeast Genomic DNA Isolation Kit (Norgen Bioteck Co., Ontario, Canada) and all fungal total RNA samples were prepared with an RNeasy Mini Kit (Qiagen, Valencia, CA) after mycelia having been frozen in nitrogen and ground into fine powder.

Construction of gene expression constructs

The Duet vectors (Novagen, Madison, WI) were used for the partial *ftm* gene cluster reconstitution to allow for multiple vector combinations and expression of one or two genes on each vector when necessary. All cloned genes are arranged under the control of the same inducible T7 promoter. PCR primers used for gene amplification are listed in Table 2. The 1.4-kb intron-less *ftmB* gene was amplified by PCR using the high fidelity Phusion DNA polymerase and the primer set AscI-*ftmB*-F/NotI-*ftmB*-R from the genomic DNA of *A. nidulans* PSM An(355)-8, which was engineered to overproduce TPS-B by Maiya *et al*. [13], and was cloned into the MCS1 of pCDFDuet-1 vector at the AscI/NotI sites to create pCDFDuet-*ftmB*. The 6.6-kb intron-less *ftmA* gene was amplified similarly by PCR using the primer set KpnI-*ftmA*-F/PspXI-*ftmA*-R, and cloned into the MCS2 of pCDFDute-*ftmB* at the KpnI/XhoI sites to create pCDFDuet-*ftmAB*. The 1.7-kb cDNA of *ftmC* gene was amplified by reverse transcription (RT)-PCR using an OneStep RT-PCR Kit (Qiagen) and the primer set EcoRI-*ftmC*-F/HindIII-*ftmC*-R from the purified RNA sample of *A. fumigatus* BM939, and was cloned into the MCS1 of pCOLADuet-1 vector at the EcoRI/HindIII sites to create pCOLADuet-*ftmC*. The 1.0-kb cDNA of *ftmD* gene was amplified similarly by RT-PCR using the primer set NdeI-*ftmD*-F/KpnI-*ftmD*-R, and cloned into the MCS2 of pCOLADuet-*ftmC* at the NdeI/KpnI sites to create pCOLADuet-*ftmCD*. To convert the apo-form of PCP domains on FtmA to its holo-form, the 0.7-kb *Cv_sfp* gene encoding an Sfp-type phosphopantetheinyltransferase (PPTase) from *Chromobacterium violaceum* No. 968 (Figure 1c) was excised from pCDFDuet-*sfp* [15] by NcoI/HindIII digestion and subcloned into the same sites on pACYCDuet-1 vector to create pACYCDuet-*Cv_sfp*. Every cloned gene was re-sequenced to ensure DNA sequence fidelity.

Table 1. Strains and plasmids used in the study.

Strain or plasmid	Description	Reference or source
Aspergillus sp.		
A. nidulans PSM An(355)-8 (FGSC A1209)	Engineered strain with *ftmA* and *ftmB* from *A. fumigatus* Af293 (under *alcA* promoter) inserted into the chromosome	[13], FGSC
A. fumigatus BM939 (FERM P-15067)	Wild-type producer of tryprostatins and fumitremorgins	[2], IPOD
Escherichia coli		
DH5α	General cloning host strain	Lab stock
BL21(DE3)	Host strain for gene expression	Novagen
GS01	BL21(DE3) harboring pCDFDuet-1 vector	This work
GS02	BL21(DE3) harboring pCDFDuet-*ftmB*	This work
GS03	BL21(DE3) harboring pCDFDuet-*ftmAB*	This work
GS03Plus	GS03 harboring pACYCDuet-*Cv_sfp*	This work
GS04	BL21(DE3) harboring pCOLADuet-1 vector	This work
GS05	BL21(DE3) harboring pCOLADuet-*ftmC*	This work
GS06	BL21(DE3) harboring pCOLADuet-*ftmCD*	This work
GS07	BL21(DE3) harboring pCDFDuet-*ftmAB* and pCOLADuet-*ftmCD*	This work
GS07Plus	GS07 harboring pACYCDuet-*Cv_sfp*	This work
GS08	BL21(DE3) harboring pACYCDuet-*Cv_sfp*	This work
Plasmids		
pCDFDuet-1	Sm^r, CDF *ori*, gene expression vector	Novagen
pCOLADuet-1	Kan^r, ColA *ori*, gene expression vector	Novagen
pACYCDuet-1	Chl^r, P15A *ori*, gene expression vector	Novagen
pCDFDuet-*ftmB*	*ftmB* cloned into the MCS1 of pCDFDuet-1	This work
pCDFDuet-*ftmAB*	*ftmA* cloned into the MCS2 of pCDFDuet-*ftmB*	This work
pCOLADuet-*ftmC*	*ftmC* cloned into the MCS1 of pCOLADuet-1	This work
pCOLADuet-*ftmCD*	*ftmD* cloned into MCS2 of pCOLADuet-*ftmC*	This work
pCDFDuet-*sfp*	*Cv_sfp* cloned into MCS1 of pCDFDuet-1	[15]
pACYCDuet-*Cv_sfp*	*Cv_sfp* cloned into MCS1 of pACYCDuet-1	This work

FGSC, Fungal Genetic Stock Center, Kansas City, MO, USA; IPOD, In-

ternational Patent Organism Depositary, Tsukuba, Japan; Smr, streptomycin resistant; Kanr, kanamycin resistant; Chlr, chloramphenicol resistant.

Table 2. Primers used for gene amplification and detection of gene expression by RT-PCR.

Experiment	Primer name	Sequence (5' > 3')	Amplicon size
ftmA cloning	KpnI-*ftmA*-F	TCGGTACCATGGCGATGGCTCTTGCGGTAGGC	6.6 kb
	PspXI-*ftmA*-R	CTAGGATCTCGAGTGGTAGCGAAGGTATTTCCTATC	
ftmB cloning	AscI-*ftmB*-F	ATAGGCGCGCCAAATGCCGCCAGCACCACCGG	1.4 kb
	NotI-*ftmB*-R	ACTGCGGCCGCTCAATTGGGGAACGACACATCC	
ftmC cloning	EcoRI-*ftmC*-F	CGGAATTCGATGAAACCGAGTCACTCTG	1.7 kb
	HindIII-*ftmC*-R	CAGAAGCTTATGGCGTCTGGTCAAAACG	
ftmD cloning	NdeI-*ftmD*-F	AGACGTCATATGATGACACAGGCAGTCGAC	1.0 kb
	KpnI-*ftmD*-R	ATAGGTACCCTCGCAGATCCAACTGAGC	
16S rDNA detection	16S-RT-PCR-F	GACTCCTACGGGAGGCAGC	199 bp
	16S-RT-PCR-R	GTATTACCGCGGCTGCTGGC	
ftmA detection	*ftmA*-RT-PCR-F	ATTTCCTGGGATGGCTCATG	230 bp
	ftmA-RT-PCR-R	GCGAGATATCCTTCAGATCGC	
ftmB detection	*ftmB*-RT-PCR-F	GACCGTCCTGGTCAAGGCG	332 bp
	ftmB-RT-PCR-R	CATCTGCAGATTCACATGTCGC	
ftmC detection	*ftmC*-RT-PCR-F	ATTAGCCCGCCATCTTGATGAGG	288 bp
	ftmC-RT-PCR-R	GCGGCGTACATTGATCCTGAGAT	
ftmD detection	*ftmD*-RT-PCR-F	TCCTCACATCCAAATCACCGTCC	251 bp
	ftmD-RT-PCR-R	GCAAGACGGCATCCATAATGAGC	

Bacterial fermentation and quantification of tryprostatin production

To measure tryprostatin production, recombinant *E. coli* strains were first fermented aerobically in shaker flasks for 4 d at 37°C under constant agitation (200 rpm) in LB medium supplemented with appropriate antibiotics individually or in combination (streptomycin was added at 50 µg/ml to select for pCDFDuet-1 based plasmid, kanamycin at 50 µg/ml to select for pCOLADuet-1 based plasmid, and chloramphenicol at 34 µg/ml to select for pACYCDuet-1 based plasmid). Gene expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG; from Sigma-Aldrich, St. Louis, MO) to a final concentration of 0.5 mM when bacterial culture reached an optical density of 0.4 at 600 nm (OD_{600}). For production of tryprostatins under anaerobic condition, bacterial culture was grown still in tightly capped glass bottles with occasional shaking in a Coy anaerobic chamber (Grass Lake, MI) for 5 d at room temperature. Sampling, extraction, detection and quantification of TPS-B and TPS-A by liquid chromatography-mass spectrometry (LC-MS) on an Agilent 1100 Series LC-MSD Trap SL equipped with an Eclipse XDB-C18 column (Agilent, 5 µm particle size, 2.1 x 50 mm), was performed according to previously reported methods with minor modifications [13]. The solvent systems consist of buffer A (water with 0.1% formic acid) and buffer B (acetonitrile with 0.1% formic acid). The LC program included a linear gradient from 40% buffer B to 100% buffer B in 10 min, an isocratic elution in 100% buffer B for 2 min, followed by a linear return to 40% buffer B in 1 min. Flow rate was set at 0.5 ml/min and UV spectrum was monitored at 226 nm. The inert nitrogen gas flow rate, nebulizer pressure and drying temperature for the MS system were 10 l/min, 30 psi and 350°C, respectively. MS was scanned in positive mode in a range of 100 to 1,000 *m/z*; the target compounds, TPS-A (381.1 ± 0.1 *m/z* for $[M + H]^+$) and TPS-B (351.7 ± 0.2 *m/z* for $[M + H]^+$), were eluted at 3.7-4.6 min or 5.1-5.4 min, respectively, under those conditions. Standard curves were generated by plotting the concentrations (ranging from 1 to 10 mg/l) of authentic compounds (TPS-A purchased from Santa Cruz Biotechnology, Santa Cruz, CA; TPS-B was a gift from James Cook, University of Wisconsin-Milwaukee) as a function of ion signal peak areas detected by LC-MS in triplicates (Figure 2). The standard curves showed a good linear correlation between the varying concentrations of injected analytes and peak areas of the extracted ion signal of tryprostatins.

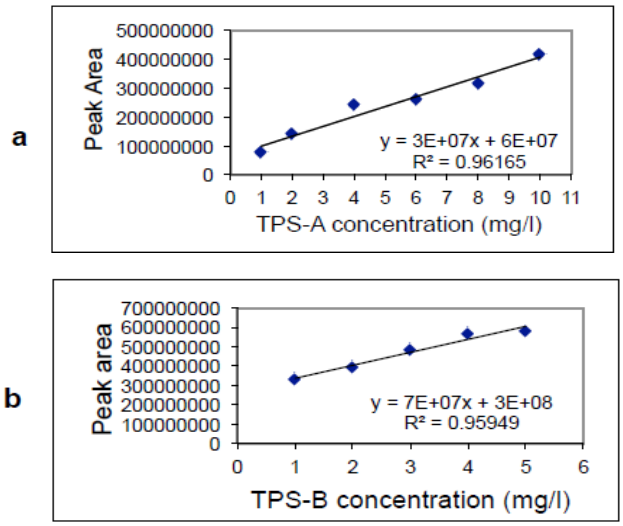

Figure 2. Standard curves for the quantification of TPS-A (a) and TPS-B (b) accumulation in the fermentation broths by LC-MS.

Analysis of gene expression by semi-quantitative RT-PCR

Cultivation of recombinant *E. coli* strains in LB growth medium supplemented with appropriate antibiotics. Extraction and purification of RNA samples, and RT-PCR detection of the expression level of each gene were performed according to previously described protocols [16]. Primers used in RT-PCR are listed in Table 2. RNA samples that had not been subjected to reverse transcription were used as negative controls; a positive control was set to amplify the 16S rRNA gene.

Results and Discussion

Generation of a serial of recombinant *E. coli* strains

Aimed at developing a bacterium-based fermentation platform for large-scale production of TPS-A and TPS-B, two empty vectors (pCDFDuet-1 and pCOLADuet-1, as controls) and five expression constructs (pCDFDuet-*ftmB*, pCDFDuet-*ftmAB*, pCOLADuet-*ftmC*, pCOLADuet-*ftmCD*, and pACYCDuet-*Cv_sfp*) were transformed individually or in combination into *E. coli* BL21(DE3) cells to generate a series of 10 recombinant strains listed in Table 1. Those strains were designed to not only test the production of TPS-B or TPS-A under both aerobic and anaerobic fermentation conditions, but also to confirm the assigned function of FtmABCD enzymes (Figure 1a).

Aerobic production of TPS-A and TPS-B

Under aerobic fermentation conditions, the control strains GS01 (harboring pCDFDuet-1 vector) and GS04 (harboring pCOLADuet-1 vector), and the intermediate strains GS02 (harboring pCDFDuet-*ftmB*), GS05 (harboring pCOLADuet-*ftmC*), GS06 (harboring pCOLADuet-*ftmCD*) or GS08 (harboring pACYCDuet-*Cv_sfp*) did not produce any TPS-B or TPS-A, as expected. The target strains GS03 (harboring pCDFDuet-*ftmAB*) and GS03Plus (GS03 with pACYCDuet-*Cv_sfp*) accumulated 98.1-106.0 mg/l of TPS-B in the bacterial broth after 4 d of fermentation, and the target strains GS7 (harboring both pCDFDuet-*ftmAB* and pCOLADuet-*ftmCD*) and GS07Plus (GS07 with pACYCDuet-*Cv_sfp*) accumulated 8.2-9.0 mg/l of TPS-B and 70.1-76.0 mg/l of TPS-A in the bacterial broth after 4 d of fermentation (Table 3). Those observations confirmed that *ftmAB* genes are necessary and sufficient to make TPS-B, and likewise, *ftmABCD* genes to make TPS-A. The quantitative results demonstrated that reconstruction of a partial *ftm* gene cluster in *E. coli* could produce impressive amounts of target products TPS-B and TPS-A. Production of secondary metabolites in a heterologous host such as *E. coli* drastically reduces the cost, time and effort in downstream natural product purification process [17]. Furthermore, the observed insignificant difference of compound accumulation levels between strains without and with pYCACDuet-*Cv_sfp* (GC03 vs. GC03Plus, GC07 vs. GS07Plus) prompted us to postulate that the PCP domains on FtmA could be fully phosphopantetheinylated in *E. coli* BL21(DE3) by one of its three endogenous PPTases, namely AcpS involved in the biosynthesis of fatty acids, EntD involved in the biosynthesis of enterobactin siderophore, and AcpT involved in the biosynthesis of O-antigens [18]. EntD is the most likely candidate for the PCP phosphopantetheinylation because it is known to be able to modify the carrier proteins of heterologous NRPS proteins [19].

Anaerobic production of TPS-A and TPS-B

We were also interested in assessing the production of TPS-A and TPS-B under anaerobic fermentation conditions. Recombinant strains (GS03, GS03Plus, GS07, and GS07Plus) were fermented in LB medium supplemented with appropriate antibiotics in strictly anaerobic conditions in a Coy anaerobic chamber for 5 d at room temperature with occasional manual agitation. All four strains were found to accumulate 18.1-20.0 mg/l of TPS-B in the fermentation broth; but neither GS07 nor GS07Plus strain produced detectable level of TPS-A (Table 3).

Table 3. Levels of TPS-B and TPS-A accumulation in the fermentation broths of recombinant *E. coli* strains. Data are the mean values of results from triplicate experiments, with calculated standard deviation of the mean provided.

Fermentation condition	Strain	TPS-B titer (mg/l; μmol/l)	TPS-A titer (mg/l; μmol/l)
Aerobic	GS03	106.0 ± 14.2; 302.3 ± 40.5	N/A
	GS03Plus	98.1 ± 8.1; 279.7 ± 23.1	N/A
	GS07	8.2 ± 2.4; 23.4 ± 6.8	70.1 ± 11.2; 184.4 ± 29.5
	GS07Plus	9.0 ± 1.7; 25.7 ± 4.8	76.0 ± 13.1; 199.9 ± 34.5
Anaerobic	GS03	20.0 ± 5.7; 57.0 ± 16.3	N/A
	GS03Plus	18.5 ± 4.1; 52.8 ± 11.7	N/A
	GS07	18.1 ± 6.4; 51.6 ± 18.2	N/D
	GS07Plus	18.9 ± 4.7; 53.9 ± 13.4	N/D

N/A, not anticipated/not applied; N/D, not detected.

RT-PCR detection of gene expression

To examine whether *ftmABCD* genes were adequately expressed in a representative GS07 strain under either aerobic or anaerobic conditions, aliquots of the culture were collect-

ed at 0 h (pre-IPTG induction) and 24 h post-IPTG induction. Total bacterial RNA was extracted, cleaned and subjected to semi-quantitative RT-PCR. It was found that, prior to IPTG induction, none of the four genes was expressed under either aerobic or anaerobic fermentation conditions, whereas all four genes were expressed at 24 h post-IPTG induction under both aerobic and anaerobic conditions (Figure 3). Adequately induced gene expression under aerobic conditions is consistent with the observed accumulation of both TPS-A and TPS-B in the fermentation broth (Table 3). However, under anaerobic conditions the situations are not so straightforward. The induced gene expression is consistent with the observed TPS-B accumulation in the anaerobically fermented broth, whereas it is incongruent with the lack of TPS-A accumulation in the anaerobically fermented broth (Table 3). This incongruity is likely due to the fact that conversion from TPS-B to desmethyltryprostatin A, a TPS-A precursor, requires an oxygen-dependent P450 hydroxylase (FtmC) (Figure 1a). Although *ftmC* was overexpressed, the resultant P450 enzyme could not catalyze the biochemical reaction in the absence of oxygen.

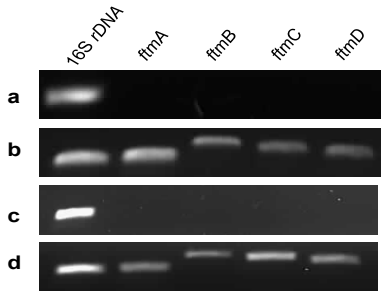

Figure 3. Analysis of the *ftm* gene expression in GS07 strain by RT-PCR. (a) Pre-induction (0 h) under aerobic conditions. (b) 24 h post-IPTG induction under aerobic conditions. (c) Pre-induction (0 h) under anaerobic conditions. (d) 24 h post-IPTG induction under anaerobic conditions. 16S rRNA gene amplification was included as a positive control.

Summary

Since the regulation of tryprostatins or fumitremorgins biosynthesis in *Aspergillus* sp. is totally unknown, yield improvement could be achieved either through classical iterative strain improvement processes, or through increasing the copy number of biosynthetic gene cluster, or through heterologous biosynthesis as demonstrated in this work. In fact, heterologous biosynthesis has emerged as effective route to mass production of natural products and intermediates [20, 21]. Although the production of TPS-B up to 106 mg/l by a recombinant *E. coli* strain (GS03) demonstrated in this work is less than the previously reported 250 mg/l of TPS-B production in a recombinant *A. nidulans* strain [13], it provides an alternative source for cost effective, large-scale production of TPS-B, because *E. coli* strain is more amenable for large-scale fermentation. Second, we engineered the first recombinant bacterial strains (GS07 and GS07Plus) to produce TPS-A up to 76.0 mg/l. Third, this work sets up an excellent platform for combinatorial biosynthesis and structural derivatization of tryprostatin analogues with potentially enhanced anticancer activities.

Acknowledgements

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