Enhancement of the Diversity of Polyoxins by a Thymine-7-Hydroxylase Homolog outside the Polyoxin Biosynthesis Gene Cluster[∇]†

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Polyoxins consist of 14 structurally variable components which differentiate at three branch sites of the carbon skeleton. Open reading frame (ORF) SAV_4805 of *Streptomyces avermitilis*, showing similarity to thymine-7-hydroxylase, was proved to enhance the diversity of polyoxins at the C-5 site of the 1-(5′-amino-5′-deoxy-β-D-allofuranuronosyl) pyrimidine moiety.
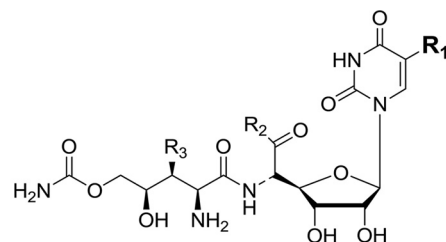
The antifungal nucleoside antibiotic polyoxins synthesized naturally by *Streptomyces cacaoi* subsp. *asoensis* (*S. cacaoi* here) consist of a mixture of at least 14 different compounds called polyoxins A to N (Fig. 1) (6, 13, 25). Most of the polyoxins have a common nucleoside skeleton that is attached with variable side groups at three different places (polyoxins C, I, and N have a modified skeleton). Polyoxins are grouped into four different classes according to the identity of the side group R1 attached at C-5 of the 1-(5′-amino-5′-deoxy-β-D-allofuranuronosyl) pyrimidine moiety. Different classes of polyoxins differ markedly in their activity spectra against plant pathogenic fungi (1, 10, 20).

It was deduced that the nucleoside moiety originated from the condensation of uridine with phosphoenolpyruvate (PEP) to generate octosyl acid as the intermediate (7, 12, 14). Then, a subsequent oxidative elimination of the two terminal carbons would create the nucleoside moiety (12). The detailed biosynthetic pathway of nucleoside moiety of polyoxins remains obscure. Our previous work showed that heterologous expression of the polyoxin biosynthetic gene cluster *pol* in *Streptomyces lividans* TK24 produces only thymine-derived polyoxin H (class III) (5). This prompted us to investigate the origin of a gene(s) related to the structural variation of polyoxins at the R1 site, which could be located outside the polyoxin biosynthetic gene cluster in the native producer.

The pyrimidine rings of polyoxins correspond one to one to the intermediates of the thymidine salvage pathway, which was characterized only for some fungi (see Fig. S1 in the supplemental material) (22). In the prime pathway of nucleotide metabolism, dUMP could be converted to dTMP under the catalysis of thymidylate synthase (TS) with tetrahydrofolic acid as a methyl donor while dTMP could not be converted back to dUMP reversibly (4, 17). In the 1970s, Shaffer et al. separated two enzymes, thymine-7-hydroxylase (THase; official name thymine dioxygenase) and isoorotate decarboxylase (IDCase),

from fungal sources which showed potential ability to convert thymine to uracil (22). THase is a trifunctional oxygenase and catalyzes three enzymatic reactions: thymine to 5-hydroxymethyluracil, 5-hydroxymethyluracil to 5-formyluracil, and 5-formyluracil to uracil-5-carboxylic acid (also designated isoorotate) (18, 26). IDCase catalyzes the subsequent decarboxylation reaction, and uracil-5-carboxylic acid is converted to uracil in the end (21). This process is catalyzed by THase, and IDCase is the core step of the thymidine salvage pathway in fungi.

BLAST analysis results with the amino acid sequence of THase from *Rhodotorula glutinis* and IDCase from *Neurospora crassa* as queries in the 25 sequenced *Streptomyces* strains (5 finished and 20 in process) of the genome database (from NCBI GenBank, accessed 24 July 2010) showed that the

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FIG. 1. Chemical structure of polyoxin complex. Class is determined by R1. The × indicates a different skeleton that does not have this particular residue.

Polyoxin	A	B	C	G	I	H	J
Class	I	I	I	I	I	III	III
R1	CH₂OH	CH₂OH	CH₂OH	CH₂OH	CH₂OH	CH₃	CH₃
R2	(azetidine-COOH)	OH	OH	OH	(azetidine-COOH)	(azetidine-COOH)	OH
R3	OH	OH	×	H	×	OH	OH

Polyoxin	D	E	F	K	L	M	N
Class	II	II	II	IV	IV	IV	×
R1	COOH	COOH	COOH	H	H	H	×
R2	OH	OH	(azetidine-COOH)	(azetidine-COOH)	OH	OH	OH
R3	OH	H	OH	OH	OH	H	OH

TABLE 1. Bacterial strains and plasmids used in this study

Strain, plasmid, or cosmid	Description	Reference(s)
Strains		
S. cacaoi subsp. *asoensis*	Polyoxin producer	5
S. coelicolor A3(2)	Wild-type strain	2
S. avermitilis NRRL8165	Wild-type strain	9
S. lividans TK24/m5A7	Recombinant strain for polyoxins heterologous producing	5
Streptomyces sp. strain SJTU5001	*S. lividans* TK24/m5A7 harboring pJTU5001	This study
Streptomyces sp. strain SJTU5003	*S. avermitilis* NRRL8165 harboring cosmid m5A7	This study
Streptomyces sp. strain SJTU5008	*S. avermitilis* NRRL8165 harboring pJTU5007	This study
Streptomyces sp. strain SJTU5009	*pyrC* mutation derived from *S. avermitilis* NRRL8165	This study
Streptomyces sp. strain SJTU5013	Strain SJTU5009 harboring pJTU5010	This study
Streptomyces sp. strain SJTU5014	Strain SJTU5009 harboring pJTU5011	This study
E. coli BW25113/pIJ790	Host for PCR targeting	8, 16
E. coli ET12567/pUZ8002	Host for *E. coli*/*Streptomyces* conjugation	16, 19
Plasmids and cosmids		
pJTU968	*E. coli* plasmid harboring a constitutive promoter of gene *ermE**	27
pPM927	*Streptomyces* integrating vector, *ori*(pBR322) *int*(pSAM2) *attP*(pSAM2) *addA tsr oriT* RK2	24
pOJ260	*E. coli* colon vector, *ori*(pUC18) *addC(3)IV oriT* RK2	3
pSET152	*Streptomyces* integrating vector, *ori*(pUC18) *int* ΦC31, *attP* ΦC31 *aac(3)IV oriT* RK2	3
pJTU1278	*Streptomyces* replicating vector, *ori* ColEI *rep*(pIJ101) *bla tsr oriT* RK2	9
pJTU5001	pPM927 harboring P*ermE**-SAV_4805 MfeI/EcoRI fragment	This study
pJTU5005	pOJ260 harboring a 6.7-kb PstI DNA fragment which covers *pyrC* gene and the flank sequence	This study
pJTU5006	*pyrC* gene of pJTU5005 replaced by *nptII* gene	This study
pJTU5007	pJTU1278 harboring 8.1-kb EcoRI/SpeI fragment (covering *nptII* gene and the flank sequence) from pJTU5006	This study
pJTU5010	pSET152 harboring P*ermE**-SCO_6305 MfeI/EcoRI fragment	This study
pJTU5011	pSET152 harboring P*ermE**-*pyrC* MfeI/EcoRI fragment	This study
Cosmid m5A7	*S. cacaoi* genome library cosmid covers polyoxin biosynthetic gene cluster *pol*	5
Cosmid 20E4	*S. avermitilis* NRRL8165 genome library cosmid covers *pyrC* gene	9

THase and IDCase gene homologs are distributed extensively in *Streptomyces*, such as open reading frame (ORF) SAV_4805 (sharing 27% identity and 45% similarity with the THase gene) from *S. avermitilis* strain MA4680 and SCO_6305 (sharing 25% identity and 44% similarity with the IDCase gene) from *S. coelicolor* strain A3(2) (2, 11, 23). The results indicated a potential thymidine salvage pathway in *Streptomyces*, and the salvage pathway provides alternative nucleoside monophosphates as precursors for polyoxin biosynthesis.

ORF SAV_4805 enhanced the structural diversity of polyoxins. To investigate the deduced influence of this potential thymidine salvage pathway to the component variation of polyoxins, heterologous expression of the *pol* cluster in *S. avermitilis* strain NRRL8165 was conducted (Table 1). Cosmid m5A7 bearing the *pol* gene cluster was transferred into *S. avermitilis* strain NRRL8165 to give strain SJTU5003 by the method described in our previous work (5) (Table 1). *Streptomyces* was cultured in liquid fermentation medium [containing 20 g soy flour, 15 g corn flour, 10 g glucose, 10 g yeast extract, 4 g CaCO$_3$, 2 g KH$_2$PO$_4$, 2 g NaCl, and 0.08 g FeSO$_4$ · (NH$_4$)$_2$SO$_4$ · 6H$_2$O per liter] at 30°C for polyoxin production. Fermentation supernatant was harvested at 72 h. The polyoxin complex was purified by IRC-50 cation exchange resin (Sigma) from the fermentation supernatant of recombinant strains and detected by liquid chromatography-mass spectrometry (LC-MS) as described previously (5). In contrast to the result with *S. lividans* TK24/m5A7, polyoxin A (hydroxymethyl at the R1 site) was detected from the fermentation supernatant of recombinant strain SJTU5003 apart from polyoxin H (methyl at R1 site) (Fig. 2, panel 3). This result indicated that

some enzyme(s) in *S. avermitilis* NRRL8165 must have played a role in polyoxin structural variation at the R1 site.

ORF SAV_4805 was amplified with primer pair 480501/480502 (GC**CATATG**AGTGACGCCCGTACCC/GG**AATTC**ACGAAAGTTGCCCGCTGA; underlining indicates engineered sites introduced into the primers) with *S. avermitilis* strain NRRL8165 genome DNA used as a template and then digested with NdeI/EcoRI and inserted into the NdeI/EcoRI site of plasmid pJTU968 (a pRSET B derivative with P*ermE** promoter and a polylinker) (27). The P*ermE**-SAV_4805 MfeI/EcoRI fragment was separated out and inserted into the EcoRI site of plasmid pPM927 to create plasmid pJTU5001 (24). Then, plasmid pJTU5001 was transferred into *S. lividans* TK24/m5A7 to give strain SJTU5001 (Table 1). In the supernatant of P*ermE**-SAV_4805 and the *pol* gene cluster-coexpressing strain SJTU5001, polyoxin A was additionally detected, although its relative content was lower than that of *S. cacaoi* (Fig. 2, panel 6).

Unexpectedly, polyoxins harboring carboxyl at the R1 site (such as polyoxin F, shown in Fig. 2, panel 1) have not been detected from recombinant strain fermentation supernatants yet, which might be attributed to the weaker hydroxymethyl-to-carboxyl catalytic ability of enzyme encoded by SAV_4805 relative to that of THase from a fungal source.

The thymidine salvage pathway is not functional in *S. avermitilis*. UMP is *de novo* biosynthesized from carbamoylphosphate and aspartate in organisms (15). The thymidine salvage pathway is a UMP compensatory pathway which was characterized in fungi only, not in mammals or prokaryotic bacteria.
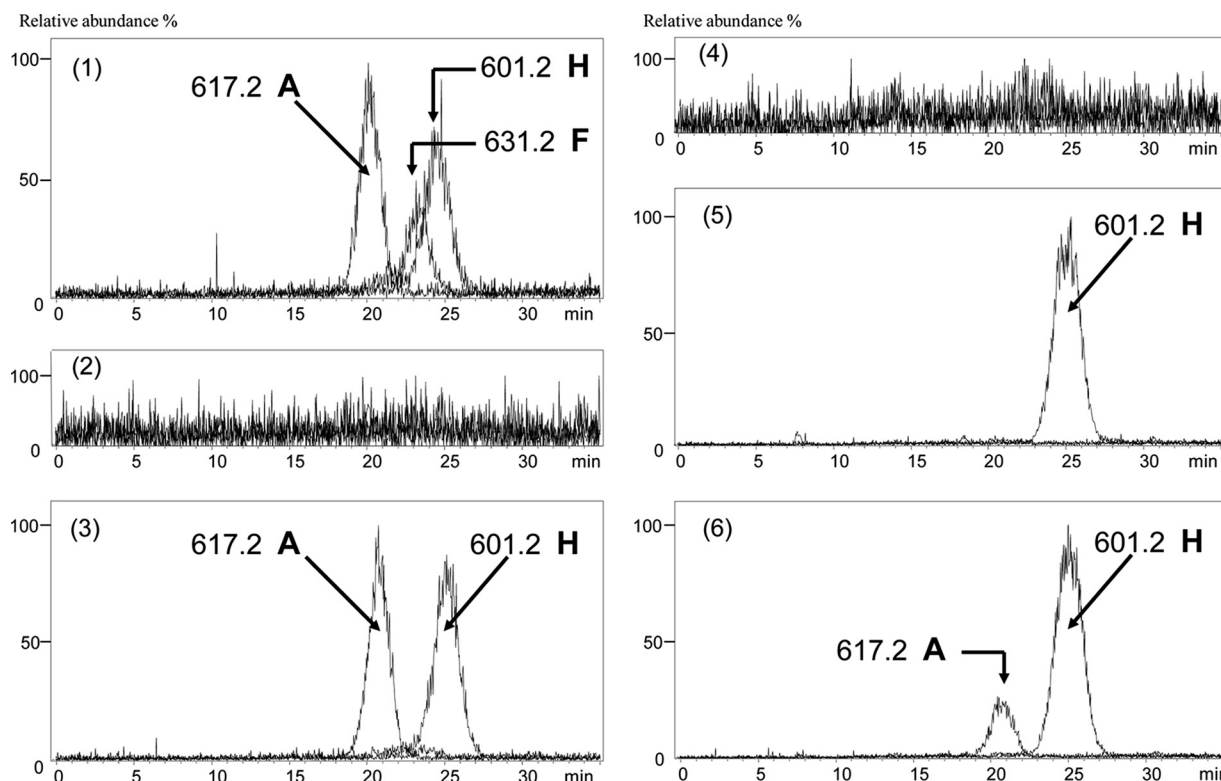
FIG. 2. Extracted ion graph from LC-MS analysis of polyoxin samples. Panels: 1, *S. cacaoi*; 2, *S. avermitilis* NRRL8165/pSET152; 3, *S. avermitilis* strain SJTU5003; 4, *S. lividans* TK24/pSET152; 5, *S. lividans* TK24/m5A7/pPM927; 6, strain SJTU5001. A, polyoxin A; H, polyoxin H; F, polyoxin F.

If the SAV_4805 protein works like THase and the SCO_6305 protein works like IDCase, it is possible to construct a complete thymidine salvage pathway in *Streptomyces* by recombining these two ORFs in one host, such as *S. avermitilis* NRRL8165. The recombinant *S. avermitilis* should exhibit a normal phenotype when thymine is fed, even though its UMP *de novo* biosynthetic pathway is interrupted, since the thymidine salvage pathway could convert thymine to uracil *in vivo*.

To knock out gene *pyrC*, which encodes dihydroorotase, to interrupt the UMP *de novo* biosynthetic pathway in *S. avermitilis* NRRL8165, cosmid 20E4 was selected out by PCR screening with primer pair validate1/validate2 (TTGGCGGCAACGAACCC/TGAGGGTGGTGGTGGGAGA) from the genome library of *S. avermitilis* strain NRRL8165. According to the genome sequence of *S. avermitilis* strain MA4680, a 6.7-kb PstI DNA fragment which covers the *pyrC* gene and its flanking sequence (2.4 kb upstream and 2.9 kb downstream) was separated out from cosmid 20E4 and inserted into the PstI site of plasmid pOJ260 to give recombinant plasmid pJTU5005 (3). The *nptII* gene fragment with 39-nucleotide (nt) homology extensions (underlined sequence) amplified by primer pair targeting1/targeting2 (CGTCAGCCACACCCGTATCGAGGAGAAGTAAGACAGATGAGCTATTCCAGAAGTAGTGAGG/TCTGTCCGGCGGCAAGGTACGTCTGATGCAGTGATGTCAGCTCTGGATGCCGACGGATTTG) was used to target plasmid pJTU5005 in *Escherichia coli* strain BW25113/pIJ790 (8, 16). Recombinant plasmid pJTU5006 was created when the *pyrC* gene (from ATG to TGA) was replaced by the *nptII* gene. The 8.1-kb EcoRI/SpeI

fragment was separated out from plasmid pJTU5006 and inserted into the EcoRI/SpeI site of plasmid pJTU1278 to construct recombinant plasmid pJTU5007 (9). Plasmid pJTU5007 was transferred by conjugation into *S. avermitilis* strain NRRL8165 to give recombinant strain SJTU5008 (16, 19). After antibiotic-free culture and kanamycin selection, the double-crossover *pyrC* mutant strain SJTU5009 was picked up and confirmed by PCR with primer pair validate1/validate2 (Table 1).

The coding sequence of *pyrC* gene was amplified with primer pair dih1/dih2 (CACGACATATGAGCAAGATCCTGATCCGTGGTG/GGAATTCACTTCTGTCCGGCGGCAAGG), and fusion gene P*ermE**-*pyrC* was created similarly to the construction of P*ermE**-SAV_4805. The P*ermE**-*pyrC* MfeI/EcoRI fragment was inserted into the EcoRI site of plasmid pSET152 to create plasmid pJTU5011, and pJTU5011 was then introduced into strain SJTU5009 to give recombinant strain SJTU5014 (Table 1) (3).

ORF SCO_6305 was amplified by primer pair IDCase05/IDCase04 (GACATATGCCCACTCCAGCCGTGCCC/GCGAATTCAGTCGAGCTGCTTGTCGTAGTCC) with *S. coelicolor* strain A3(2) total DNA used as a template. Fusion gene P*ermE**-SCO_6305 was created and inserted into the EcoRI site of plasmid pSET152 to create plasmid pJTU5010; then pJTU5010 was introduced into strain SJTU5009 to give recombinant strain SJTU5013.

As shown in Fig. 3, the *pyrC* mutant strain SJTU5009 showed abnormal morphological differentiation under the growth conditions investigated while resuming a normal mor-
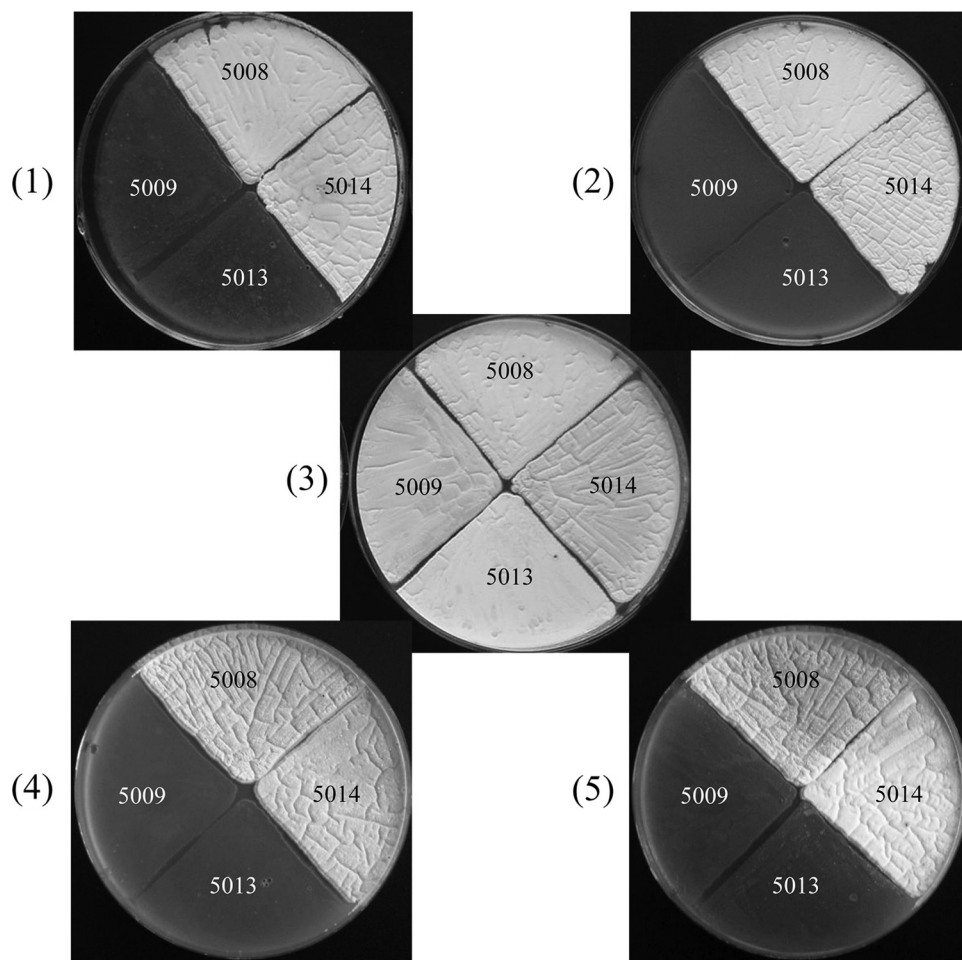
FIG. 3. The *pyrC* mutation restored by ORF SCO_6305, *pyrC*, and different pyrimidine derivatives. Panels: 1, SFM medium; 2, SFM medium with 5 mM thymine; 3, SFM medium with 5 mM uracil; 4, SFM medium with 5 mM 5-hydroxymethyluracil; 5, SFM medium with 5 mM uracil-5-carboxylic acid. 5008, recombinant *S. avermitilis* strain SJTU5008; 5009, recombinant *S. avermitilis* strain SJTU5009; 5013, recombinant *S. avermitilis* strain SJTU5013; 5014, recombinant *S. avermitilis* strain SJTU5014.

phology when exogenous uracil was supplied (Fig. 3, panel 3). Plasmid pJTU5011 bearing fusion gene P*ermE**-*pyrC* also absolutely reversed the mutant strain SJTU5009 back to the wild type (Fig. 3, panel 1). The mutation in SJTU5009 was used to test if the potential thymidine salvage pathway works in *Streptomyces*. Unfortunately, strain SJTU5013 still showed an abnormal phenotype when thymine was fed (Fig. 3, panel 2). In addition, neither 5-hydroxymethyluracil nor uracil-5-carboxylic acid could restore strain SJTU5013 to the normal phenotype (Fig. 3, panels 4 and 5). These results indicated that thymine and its derivatives could not be converted to uracil in strain SJTU5013. In other words, the constructed thymidine salvage pathway does not work in *S. avermitilis*, although SAV_4805 and SCO_6305 proteins shared certain sequence similarities with fungal THase and IDCase, respectively.

Despite the disability which might be due to the limited capability of ORF SCO_6305, it seems more likely that SAV_4805 protein is not a full THase and cannot catalyze the oxidation reaction from hydroxymethyl to carboxyl (attached on the C-5 of the pyrimidine ring), with the result that only polyoxins H (methyl at R1 site) and A (hydroxymethyl at R1

site), but not polyoxin F (carboxyl at R1 site), were detected when the *pol* gene cluster and P*ermE**-SAV_4805 fusion gene were coexpressed in polyoxin-nonproducing strain *S. lividans* TK24.

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