Functional characterization of the first two actinomycete 4-amino-4-deoxychorismate lyase genes

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In some antibiotic producers, *p*-aminobenzoic acid (PABA) or its immediate precursor, 4-amino-4-deoxychorismate (ADC), is involved in primary metabolism and antibiotic biosynthesis. In *Streptomyces* sp. FR-008, a gene *pabC-1* putatively encoding a fold-type IV pyridoxal 5'-phosphate (PLP)-dependent enzyme was found within the antibiotic FR-008/candicidin biosynthetic gene cluster, whose inactivation significantly reduced the productivity of antibiotic FR-008 to about 20 % of the wild-type level. Its specific role in PABA formation was further demonstrated by the successful complementation of an *Escherichia coli* *pabC* mutant. Moreover, a free-standing gene *pabC-2*, probably encoding another fold-type IV PLP-dependent enzyme, was cloned from the same strain. Inactivation of *pabC-2* reduced antibiotic FR-008 yield to about 57 % of the wild-type level in the mutant, and the complementation of the *E. coli* *pabC* mutant established its involvement in PABA biosynthesis. Furthermore, a *pabC-1/pabC-2* double mutant only retained about 4 % of the wild-type antibiotic FR-008 productivity, clearly indicating that *pabC-2* also contributed to biosynthesis of this antibiotic. Surprisingly, apparently retarded growth of the double mutant was observed on minimal medium, which suggested that both *pabC-1* and *pabC-2* are involved in PABA biosynthesis for primary metabolism. Finally, both PabC-1 and PabC-2 were shown to be functional ADC lyases by *in vitro* enzymic lysis with the release of pyruvate. *pabC-1* and *pabC-2* appear to represent the first two functional ADC lyase genes identified in actinomycetes. The involvement of these two ADC lyase genes in both cell growth and antibiotic FR-008 biosynthesis sets an example for the interplay between primary and secondary metabolisms in bacteria.

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INTRODUCTION

p-Aminobenzoic acid (PABA) is widespread in bacteria, fungi, plants and some parasites, but absent in humans and animals (Basset *et al.*, 2004; Edman *et al.*, 1993; Goncharoff & Nichols, 1984; Triglia & Cowman, 1999). In primary metabolism, PABA is an important precursor of folates, including tetrahydrofolate (vitamin B₉) and its derivatives, which are essential cofactors for one-carbon transfer reactions and thus required for synthesis of methionine, purines, thymidylate, pantothenic acid and *N*-formylmethionyl-tRNA (Appling, 1991). Moreover, at least in actinomycetes, PABA or its immediate precursor 4-amino-4-deoxychorismate (ADC) is incorporated into many secondary metabolites, such as antibiotic FR-008/candicidin from *Streptomyces* sp. FR-008/

Streptomyces griseus IMRU3570 (Gil & Hopwood, 1983; Hu *et al.*, 1994), aureothin from *Streptomyces thioluteus* (He & Hertweck, 2003), neo-aureothin from *Streptomyces orinoci* (Traitcheva *et al.*, 2007), pristnamycin from *Streptomyces pristinaespiralis* (Blanc *et al.*, 1997) and chloramphenicol from *Streptomyces venezuelae* (Brown *et al.*, 1996) (Fig. 1a).

PABA is usually biosynthesized through the shikimate pathway in most organisms (Porat *et al.*, 2006). In the well-studied bacterium *Escherichia coli*, PABA is synthesized in two steps catalysed by two separate enzymes. In the first step, ADC synthase, a heterodimeric enzyme consisting of PabA and PabB, encoded by *pabA* and *pabB* respectively, catalyses the conversion of chorismate and glutamine to ADC and glutamate. In the second reaction, a pyridoxal 5'-phosphate (PLP)-containing ADC lyase, encoded by *pabC*, mediates elimination of pyruvate from ADC and aromatization to give PABA (Green & Nichols, 1991; Nichols *et al.*, 1989; Ye *et al.*, 1990).

Separate *pabA* and *pabB* genes have also been identified as being involved in primary metabolism of *Streptomyces*

Abbreviations: *aac(3)/IV*, apramycin-resistance gene; ADC, 4-amino-4-deoxychorismate; Apr, apramycin; LDH, lactate dehydrogenase; PABA, *p*-aminobenzoic acid; *PermE**, up-mutated promoter of erythromycin-resistance gene; PKS, polyketide synthase; PLP, pyridoxal 5'-phosphate.

Supplementary methods and two supplementary figures are available with the online version of this paper.

Fig. 1. PABA metabolism and ADC lyase gene homologue *pabC-1* in the antibiotic FR-008 biosynthetic gene cluster. (a) Metabolism of PABA in primary and secondary metabolisms. PABA- or ADC-derived moieties are boxed. ADC, 4-amino-4-deoxychorismate; ADPA, 4-amino-4-deoxyphenylpyruvate; PABA, p-aminobenzoic acid; PNBA, p-nitrobenzoate. (b) Gene organization of the upstream PKS region of the antibiotic FR-008 gene cluster. *pabAB* and *pabC-1* are highlighted with filled arrows.

lividans (Arhin & Vining, 1993) and *S. venezuelae* (Chang *et al.*, 2001) and found in many sequenced *Streptomyces* genomes (Bentley *et al.*, 2002; Omura *et al.*, 2001). In the biosynthetic pathways for secondary metabolism, however, the *pabAB* counterparts have been found to be fused in all cases, e.g. those for aureothin (*aurG*) (He & Hertweck, 2003), chloramphenicol (*cmlB*) (He *et al.*, 2001), antibiotic FR-008/candicidin (*pabAB*) (Chen *et al.*, 2003; Gil & Hopwood, 1983), neo-aureothin (*norG*) (Traitcheva *et al.*, 2007) and pristinamycin (*papA*) (Blanc *et al.*, 1997). Even though PABA is the immediate precursor for aureothin and neo-aureothin biosynthesis, so far only *aurG* and *norG*, encoding ADC synthase, have been localized, and the gene encoding ADC lyase has not been identified in these two biosynthetic gene clusters.

Antibiotic FR-008/candicidin is an antifungal heptaene macrolide, whose genetic information and biosynthetic pathway have been demonstrated to be nearly identical in *Streptomyces* sp. FR-008 and *S. griseus* IMRU3570. In *S. griseus*, the fused *pabAB*, located at one end of the polyketide synthase (PKS) genes, was demonstrated to be

essential for antibiotic production because the replacement of *pabAB* totally abolished antibiotic FR-008 production, which could be restored by feeding with exogenous PABA (Campelo & Gil, 2002). For antibiotic FR-008 production, a gene encoding an ADC lyase should be required to convert ADC to PABA (Fig. 1a).

In the work reported here, through *in vivo* inactivation, complementation and *in vitro* enzymic catalysis, we first confirmed an ADC lyase gene *pabC-1* within the biosynthetic gene cluster to be necessary for efficient antibiotic FR-008 biosynthesis. This was followed by the identification of another ADC lyase gene, *pabC-2*, which is involved in primary metabolism and in antibiotic FR-008 biosynthesis as well.

METHODS

Bacterial strains and plasmids. These are listed in Table 1.

General techniques. *E. coli* strains were cultivated at 37 °C in Luria-Bertani (LB) medium or on LB agar plates. *Streptomyces* strains

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant phenotype and/or characteristics	Reference or source
<i>E. coli</i>		
DH10B	<i>recA</i>	GibcoBRL
ET12567/pUZ8002	<i>recE dam dcm hsdS</i> Cm ^r Str ^r Tet ^r Km ^r	Paget <i>et al.</i> (1999)
BW25113	K12 derivative, Δ <i>araBAD</i> Δ <i>rhaBAD</i>	Gust <i>et al.</i> (2003)
ZYR-9	BW25113-derived <i>pabC</i> mutant	This study
ZYR-9/pJTU927	ZYR-9 complemented with pJTU927 harbouring full-length FR-008 <i>pabC-1</i>	This study
ZYR-9/pJTU928	ZYR-9 complemented with pJTU928 harbouring full-length <i>E. coli pabC</i>	This study
ZYR-9/pJTU3295	ZYR-9 complemented with pJTU3295 harbouring full-length FR-008 <i>pabC-2</i>	This study
<i>Streptomyces</i> sp.		
FR-008	Wild-type producer of antibiotic FR-008	Hu <i>et al.</i> (1994)
HJ-5	Mutant with the whole antibiotic FR-008 gene cluster deleted	Unpublished
ZYR-6	FR-008 derivative generated by replacement of a 437 bp internal to <i>pabC-1</i> with 1.4 kb <i>aac(3)IV</i>	This study
ZYR-7	FR-008 derivative with <i>pabC-1/pabC-2</i> double mutation	This study
ZYR-8	FR-008 derivative generated by deletion of a 675 bp DNA fragment internal to <i>pabC-2</i>	This study
ZYR-7/pJTU949	ZYR-7 complemented with pJTU949 harbouring full-length <i>pabC-1</i>	This study
ZYR-7/pJTU3283	ZYR-7 complemented with pJTU3283 harbouring full-length <i>pabC-2</i>	This study
Plasmids		
pBluescript KS(-)	Phagemid, <i>bla lacZ</i>	Stratagene
pMD18 T-vector	pUC18 derivative	TaKaRa
pRSET-B	P _{T7} RBS 6xHis Xpress Epitope EK, <i>bla</i>	Invitrogen
pET28a	P _{T7} His-Tag/thrombin/T7-Tag, <i>kan</i>	Novogen
pJTU1278	<i>bla lacZ oriT ori</i> (pIJ101)	Unpublished
pJTU968	pRSET-B derivative <i>bla Perme*</i>	Unpublished
pPM927	<i>tsr oriT int attP</i>	Smokvina <i>et al.</i> (1990)
pSET152	<i>aac(3)IV oriT</i> (RK2) <i>ori</i> (pUC18) <i>int</i> (ϕ C31), <i>attP</i> (ϕ C31) <i>lacZα</i>	Bierman <i>et al.</i> (1992)
pJTU468	A 3.0 kb <i>KpnI/BamHI</i> fragment carrying <i>pabC-1</i> cloned in pHGF9053	This study
pJTU469	Insertion of a 1.4 kb <i>aac(3)IV</i> gene fragment into the <i>Apal</i> site sandwiched between the 0.8 kb and 1.8 kb fragments in <i>Apal</i> -digested and religated pJTU468	This study
pJTU744	Cloning of a 4.0 kb <i>BamHI/KpnI</i> fragment from pJTU469 to pJTU1278, which contains a linked 0.8 kb (left arm), 1.4 kb <i>aac(3)IV</i> , and 1.8 kb (right arm) for <i>pabC-1</i> inactivation	This study
pJTU927	pET28a carrying the 0.8 kb FR-008 <i>pabC-1</i>	This study
pJTU928	pET28a carrying the 0.8 kb <i>E. coli pabC</i>	This study
pJTU949	pPM927 carrying <i>Perme*</i> and FR-008 <i>pabC-1</i>	This study
pJTU3283	pPM927 carrying <i>Perme*</i> and FR-008 <i>pabC-2</i>	This study
pJTU3286	pSET152 with the 9.3 kb <i>EcoRI</i> fragment from cosmid 11A10	This study
pJTU3287	pSET152 carrying the 8.7 kb <i>EcoRI</i> fragment with 0.68 kb DNA fragment internal to <i>pabC-2</i> deleted	This study
pJTU3288	Cloning of an 8.7 kb <i>EcoRI</i> fragment from pJTU3287 to pJTU1278, which contains a linked 5.6 kb (left arm), 3.1 kb (right arm) for <i>pabC-2</i> inactivation	This study
pJTU3295	pET28a carrying the 0.8 kb FR-008 <i>pabC-2</i>	This study
pJTU3296	pET28a carrying the 1.4 kb <i>E. coli pabB</i>	This study

were routinely grown at 30 °C on SFM solid medium (2 % agar, 2 % mannitol, 2 % soybean powder, pH 7.2) for conjugation between *E. coli* and *Streptomyces* and solid fermentation, or in TSBY liquid medium (3 % TSB, 1 % yeast extract, 10.3 % sucrose, pH 7.2) for mycelial growth. Total DNAs were isolated from *Streptomyces* strains as described by Kieser *et al.* (2000). *In vivo* generation of targeted mutations in *Streptomyces* was achieved by conjugation between *E. coli* and *Streptomyces* strains as described previously (Chen *et al.*, 2003). For *Streptomyces*, apramycin and thiostrepton were used at 30 μ g ml⁻¹ and 25 μ g ml⁻¹, respectively, in both SFM agar and liquid media. PABA (Sigma) was used at 5 μ g ml⁻¹ in SFM medium,

minimal medium (Kieser *et al.*, 2000) and M9 minimal medium (Sambrook *et al.*, 1989) for feeding experiments. The agar used for minimal medium and M9 minimal medium was purchased from Oxoid. Synthesis of oligonucleotide primers and DNA sequencing of PCR products were performed by Shanghai Sangon and Invitrogen. Extraction of DNA fragments from agarose gel slices was performed with a Gel Recovery kit (Tiangen).

Antibiotic FR-008 detection. Antibiotic FR-008 production by wild-type and mutant strains was determined as follows. Seed culture was prepared in 10.3 % TSBY medium and inoculated with a spore

suspension. After incubation for 24 h at 30 °C and 220 r.p.m., the culture was used to inoculate (at 2%, v/v) 250 ml flasks containing 50 ml YEME medium minus sucrose. Production of antibiotic FR-008 was determined after 5 days. Exogenous PABA (final concentration 5 µg ml⁻¹) was added to YEME (minus sucrose) cultures at 12 h after inoculation when necessary. The fermentation broth was extracted with n-butanol. After centrifugation, the n-butanol phase was collected and the A₃₈₀ in butanol was measured in a Perkin-Elmer spectrophotometer (Gil *et al.*, 1980). The blank control experiment was carried out with broth from the fermentation of mutant HJ-5, in which the whole antibiotic FR-008 biosynthetic gene cluster was deleted (unpublished data). Reproducibility of the results was confirmed by at least three independent experiments.

Inactivation of the *pabC-1* gene. A 3.0 kb *KpnI*/*Bam*HI fragment carrying *pabC-1* was ligated to *KpnI*/*Bam*HI-digested pHGF9053 (Minagawa *et al.*, 2007) to give pJTU468. Plasmid pJTU468 was digested with *Apa*I, blunted and inserted with a 1.40 kb *aac(3)IV* gene fragment from pHGF9827 (Chen *et al.*, 2003) to generate pJTU469. Subsequently, 4.0 kb of DNA containing the 0.80 kb fragment (left arm), the 1.40 kb *aac(3)IV* fragment and the 1.80 kb fragment (right arm) was transferred as a *Bam*HI/*Kpn*I fragment from pJTU469 to *Bam*HI/*Kpn*I-digested pJTU1278. The resultant plasmid pJTU744 was used for targeted replacement of a 437 bp DNA fragment internal to *pabC-1* with the 1.40 kb *aac(3)IV* fragment in the wild-type strain FR-008 via conjugation as previously described (Chen *et al.*, 2003). The oligonucleotide primers used for *pabC-1* mutant confirmation were PabC-Det-F (5'-GCGGCATGGAGGAATG-3') and PabC-Det-R (5'-CGAACTGGACGGGGAAC-3').

Cloning of *pabC-2* from *Streptomyces* sp. FR-008. To clone the ADC lyase gene homologues from the *Streptomyces* sp. FR-008 genome, degenerate primers PabB-J1 (5'-GATCGTGGACCTSGTC-CGSAACGA-3'; S=G/C) and PabC-J8 (5'-GAAGATCCGCATSG-CYTTSGCSGT-3'; S=G/C, Y=C/T) were used to amplify a 1.28 kb fragment from FR-008 genomic DNA, which was sequenced. Another pair of degenerate primers PabB-J1 and PabC-J9 (5'-GCCGTCG-CCCACSGTSAGNCCRTG-3'; S=G/C, R=A/G, N=A/T/G/C) were used to screen the cosmid library of *Streptomyces* sp. FR-008. A 9.30 kb *Eco*RI fragment from a positive cosmid, 11A10, carrying *pabC-2* was cloned into the vector pSET152 to generate pJTU3286.

Deletion of *pabC-2* in wild-type FR-008 and *pabC-1* mutant ZYR-6. Plasmid pJTU3286 was digested with *Bgl*II and self-ligated to generate pJTU3287. The shortened 8.70 kb *Eco*RI fragment from pJTU3287 was then transferred to the shuttle vector pJTU1278. The resultant plasmid pJTU3288 was used for deletion of a 675 bp DNA fragment internal to *pabC-2* in mutant ZYR-6 or wild-type FR-008 via conjugation as previously described (Chen *et al.*, 2003). The *pabC-1/pabC-2* double mutant (ZYR-7) and the *pabC-2* single mutant (ZYR-8) were screened and confirmed by PCR using primers PabB-J1 and PabC-J8.

Complementation of *pabC-1/pabC-2* mutant ZYR-7 with cloned *pabC-1*. The *pabC-1* gene was amplified with KOD-plus DNA polymerase using pJTU468 as template and primers PabC1-F (5'-CGGATCCCCATATGATCGAACTGGACG-3'; engineered *Bam*HI and *Nde*I sites underlined) and PabC1-R (5'-TAGAATTCAGAGCG-GGGAGCCGGGA-3'; engineered *Eco*RI site underlined). The PCR amplification was carried out under the following conditions: initial denaturation at 94 °C for 5 min, then 25 cycles of 45 s at 94 °C, 45 s at 60 °C and 1 min at 68 °C. A final elongation step was performed at 68 °C for 5 min. The resultant PCR product was purified and ligated into *Eco*RV-digested pBluescript KS(-) to generate pJTU903, which was sequenced to validate the inserted DNA sequence. A 0.80 kb *Nde*I/*Eco*RI fragment from pJTU903 was ligated to *Nde*I/*Eco*RI-digested pJTU968 to give pJTU948. Then the 1.10 kb *Mfe*I/*Eco*RI fragment, containing the

*Perme** promoter and *pabC-1*, was cleaved from pJTU948 and ligated into *Eco*RI-digested pPM927 to give pJTU949. The plasmid was introduced into ZYR-7 via conjugation, and the exconjugants were selected with thiostrepton and confirmed by PCR using primers PabC1-F and PabC1-R.

Complementation of *pabC-1/pabC-2* mutant ZYR-7 with cloned *pabC-2*. The *pabC-2* gene was amplified with Taq DNA polymerase using pJTU3286 as template and primers PabC2-F (5'-AGGGATCCTCATATGAAGATCTGGCTCG-3'; engineered *Bam*HI and *Nde*I sites underlined) and PabC2-R (5'-GTTTCTGAC-GAATTCAGGGGTCGATGT-3'; engineered *Eco*RI site underlined). The PCR amplification was carried out under the following conditions: initial denaturation at 94 °C for 5 min, then 25 cycles of 45 s at 94 °C, 45 s at 60 °C and 1 min at 72 °C. A final elongation step was performed at 72 °C for 5 min. The resultant PCR product was purified and ligated into pMD18 T-vector (TaKaRa) to generate pJTU3293, which was sequenced to validate the inserted DNA sequence. A 0.80 kb *Nde*I/*Eco*RI fragment from pJTU3293 was ligated to *Nde*I/*Eco*RI-digested pJTU968 to give pJTU3282. Then the 1.10 kb *Mfe*I/*Eco*RI fragment, containing the *Perme** promoter and *pabC-2*, was cleaved from pJTU3282 and ligated into *Eco*RI-digested pPM927 to give pJTU3283. The plasmid was introduced into ZYR-7 via conjugation, and the exconjugants were selected with thiostrepton and confirmed by PCR using primers PabC2-F and PabC2-R.

Complementation of *E. coli pabC* mutant ZYR-9 with FR-008 *pabC-1* or *pabC-2*. The *pabC* gene of *E. coli* BW25113 was replaced with *aac(3)IV* using the λ-Red-mediated recombination system (for details, see the supplementary material available with the online version of this paper). The *pabC-1* and *pabC-2* genes were subsequently subcloned using the *Nde*I and *Eco*RI restriction sites into pET28a (Novagen) to generate pJTU927 and pJTU3295, respectively. The mutant ZYR-9 was transformed with pJTU927 or pJTU3295, and the transformants were cultivated on M9 minimal medium (plus or minus PABA) for 24 h at 37 °C. The derivatives of mutant ZYR-9 transformed with pJTU928 (see supplementary material) or the vector pET28a were used as the controls.

Overexpression of PabB (*E. coli*), PabC (*E. coli*), PabC-1 and PabC-2. Overexpression of PabB (*E. coli*) in *E. coli* BL21(DE3)/pLysE harbouring pJTU3296 (see supplementary material) was induced by addition of 0.5 mM IPTG to LB medium at OD₆₀₀ 0.7. Incubation was continued for 12 h at 25 °C. The cell pellet was harvested, resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0), and disrupted by sonication. After centrifugation, the supernatant was passed through a nickel column and bound protein was eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). The final protein sample was dialysed against dialysis buffer (20 mM KH₂PO₄, pH 7.4, 50 mM KCl). Protein concentration was determined by Bradford assay (Sangon).

LB medium was used to culture *E. coli* BL21(DE3)/pLysE harbouring pJTU928 (for PabC), pJTU927 (for PabC-1) or pJTU3295 (for PabC-2) at 37 °C. Overexpression of target proteins was induced by addition of 0.5 mM IPTG at OD₆₀₀ 0.6. Incubation was continued for 16 h at 22 °C. The cell pellet was resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 20 µM PLP, pH 8.0) and disrupted by sonication. After centrifugation, the supernatant was passed through a nickel column, and the bound protein was eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, 20 µM PLP, pH 8.0). The enzyme was monitored by its yellow colour.

Enzymic analysis of PabC (*E. coli*), PabC-1 and PabC-2. Chorismate (barium salt), NADH and lactate dehydrogenase (LDH)

were purchased from Sigma. Ba^{2+} in the commercial chorismate was removed by addition of a slight excess of Na_2SO_4 before use. Enzymic catalysis was performed as previously described (He *et al.*, 2004). The pyruvate formation in the ADC lysis reaction was detected by a coupled LDH reaction, which oxidizes NADH to NAD. The consumption of NADH was continuously monitored at 340 nm in a Perkin-Elmer spectrophotometer using the time-drive programme. The 1 ml NH_4^+ -dependent assay contained 100 mM Bicine (pH 8.5), 100 mM $(\text{NH}_4)_2\text{SO}_4$, 5 mM MgCl_2 , 20 μM PLP, 5 mM chorismate (sodium salt), 200 μM NADH, 5 units LDH ml^{-1} , 16 μM PabB, and 11.3 μM PabC-1 or 22.8 μM PabC-2 or 38.2 μM *E. coli* PabC. All assays were performed at 37 °C. The negative controls were carried out with boiled PabB or PabC, or without chorismate.

RESULTS

The *pabC-1* gene, encoding a fold-type IV PLP-dependent enzyme, affects antibiotic FR-008 biosynthesis

Beyond the four putative regulatory genes (Fig. 1b) the gene *pabC-1* is located, encoding a fold-type IV PLP-dependent enzyme; these enzymes include D-amino acid aminotransferases, branched-chain amino acid aminotransferases and ADC lyases. Since the gene *pabAB* was previously shown to encode the ADC synthase involved in antibiotic FR-008/candicidin biosynthesis (Campelo & Gil, 2002), *pabC-1* was assumed to be an ADC lyase gene for providing the PABA starter unit. PabC-1 showed 27 % and 33.1 % similarities with PabCs from *E. coli* and *Bacillus subtilis*, respectively. *In silico* analysis of PabC-1 identified the conserved residues believed to be essential for catalysis (Lys130) and pyridoxal 5'-phosphate (PLP) binding (Arg44, Lys130, Glu163) for ADC lyase (see supplementary Fig. S2).

The possible involvement of *pabC-1* in antibiotic FR-008 biosynthesis was examined through gene replacement. In the pJTU1278-derived plasmid pJTU744, the apramycin-resistance gene *aac(3)IV* was inserted between the 0.80 kb left-flanking and the 1.80 kb right-flanking sequence of the 437 bp DNA fragment to be replaced (Fig. 2a). Two thiostrepton-sensitive, apramycin-resistant ($\text{Thio}^S \text{Apr}^R$) recombinant mutants (ZYR-6-a and ZYR-6-b) were obtained. Total DNAs from these two strains and from the wild-type were used as templates for PCR analysis with primers PabC-Det-F and PabC-Det-R. The wild-type gave a 0.85 kb PCR product, whereas the mutants gave a 1.80 kb PCR product (Fig. 2b), which confirmed that a 437 bp DNA fragment internal to *pabC-1* had been replaced by the 1.40 kb *aac(3)IV* cassette (Fig. 2a). Spectrophotometric quantification of the fermentation extract of the mutant ZYR-6 clearly showed that antibiotic FR-008 productivity was severely reduced, to approximately 20 % of the wild-type level. When exogenous PABA was added to the fermentation medium for ZYR-6, the antibiotic FR-008 production was restored to around 47 % of the wild-type level (Fig. 2c). This result indicated that *pabC-1* is involved in the biosynthesis of antibiotic FR-008.

Identification of a second gene (*pabC-2*) encoding a fold-type IV PLP-dependent enzyme from *Streptomyces* sp. FR-008

The mutant with *pabC-1* inactivated still retained about 20 % of the wild-type level of antibiotic FR-008 production, suggesting the involvement of other ADC lyase gene(s) in the genome of *Streptomyces* sp. FR-008. Considering that the ADC lyase genes involved in folate biosynthesis could be conserved in *Streptomyces*, we searched for PabC-1 homologues in the genomes of *S. coelicolor* A3(2) (Bentley *et al.*, 2002), *S. avermitilis* (Omura *et al.*, 2001) and *S. griseus* (Ohnishi *et al.*, 2008). The best hits were SCO1546 (identity 25.6 %, similarity 34.4 %; putative type IV aminotransferase) (Bentley *et al.*, 2002), SAV6804 (identity 23.3 %, similarity 33.3 %; putative D-alanine aminotransferase) (Omura *et al.*, 2001) and SGR5991 (identity 21.2 %, similarity 29.7 %; putative aminotransferase) (Ohnishi *et al.*, 2008) from *S. coelicolor* A3(2), *S. avermitilis*, and *S. griseus*, respectively. Interestingly, each hit for a *pabC-1* homologue lies downstream of a putative anthranilate synthase, probably forming an operon in each strain. The information was used for the design of a pair of degenerate primers (PabB-J1, located in *pabB* homologues, and PabC-J8, residing in *pabC* homologues) for the cloning of a putative region spanning the putative *pabB-pabC* by PCR amplification using total DNA of the wild-type *Streptomyces* sp. FR-008 as template.

Indeed, a 1.28 kb PCR product was obtained and sequenced, which contained a 5' 0.49 kb DNA with significant similarity to SCO1547 and a 3' 0.78 kb similar to SCO1546 as expected. A pair of more efficient degenerate primers, PabB-J1 and PabC-J9, was then used to screen the cosmid library of *Streptomyces* sp. FR-008. Four positive cosmids (5E2, 11A10, 15F4 and 31D2) were obtained. Subcloning and sequencing of a larger 2.0 kb fragment containing the previous 1.28 kb sequence identified a complete gene encoding a fold-type IV PLP-dependent enzyme, with a downstream incomplete open reading frame similar to SCO1545 (GenBank accession number FJ496753). This complete gene was named *pabC-2*; its predicted encoded protein has only a weak homology (identity 25.6 %, similarity 35.9 %) with PabC-1, but contains the three conserved residues Arg50, Lys141, and Glu174 putatively essential for ADC lyase activity (see Supplementary Fig. S2).

pabC-2 contributes to antibiotic FR-008 biosynthesis

In order to identify the possible role of *pabC-2*, a 9.30 kb *EcoRI* fragment carrying this gene from one of the cosmids, 11A10, was cloned. For the generation of a mutant with a 675 bp DNA deletion internal to *pabC-2*, the pJTU1278-derived plasmid pJTU3288, with 5.60 kb left-flanking and 3.10 kb right-flanking sequence of the 675 bp DNA to be deleted, was introduced into the wild-type strain by

Fig. 2. Inactivation of *pabC-1*. (a) Schematic representation of *pabC-1* inactivation. (b) PCR analysis of wild-type and mutant ZYR-6 using PabC-Det-F and PabC-Det-R as primers. Lanes: 1, 1 kb ladder; 2, wild-type; 3, ZYR-6-a; 4, ZYR-6-b. (c) Spectrophotometric quantification of antibiotic FR-008 production in wild-type, ZYR-6, ZYR-6 fed with exogenous PABA, *pabC-1/pabC-2* double mutant ZYR-7 complemented with cloned *pabC-1*, and ZYR-7. Each column represents the mean value obtained from at least three independent experiments and the error bars indicate the standard deviation.

conjugation (Fig. 3a). Two suitable mutants (ZYR-8-a and ZYR-8-b) were obtained and confirmed by PCR using PabB-J1 and PabC-J8 as primers to carry a 0.6 kb PCR product, while the PCR fragment derived from the wild-type FR-008 was apparently about 1.30 kb (675 bp larger) (Fig. 3b). Spectrophotometric quantification of the fermentation extract indicated that the mutant strain (ZYR-8) produced only about 57% of the wild-type amount of antibiotic FR-008 (Fig. 3c).

***pabC-1/pabC-2* double mutation severely reduces antibiotic FR-008 production and renders the mutant PABA-auxotrophic**

To further validate the contribution of *pabC-2* to antibiotic FR-008 production in ZYR-6, a *pabC-1/pabC-2* double mutant (ZYR-7) was constructed by additional inactivation

of *pabC-2* in *pabC-1* mutant ZYR-6. The genotype of ZYR-7 was confirmed by PCR amplification as previously described for the confirmation of *pabC-2* mutant ZYR-8 (Fig. 3b). As expected, spectrophotometric quantification indicated that this double mutant produced antibiotic FR-008 at about 4% of the wild-type level, confirming that *pabC-1* and *pabC-2* are involved in antibiotic FR-008 biosynthesis (Fig. 3c).

When restreaked to minimal medium, the *pabC-1/pabC-2* double mutant ZYR-7 surprisingly showed very limited growth (Fig. 4). When exogenous PABA was added to minimal medium, the growth of the double mutant was restored (Fig. 4), and the antibiotic production recovered to 47% of the wild-type level (Fig. 3c). Therefore both *pabC-1* and *pabC-2* appear to be involved in PABA biosynthesis in primary metabolism.

Fig. 3. Inactivation of *pabC-2*. (a) Schematic representation of *pabC-2* inactivation. (b) PCR analysis of wild-type, *pabC-2* mutant ZYR-8, and *pabC-1/pabC-2* double mutant ZYR-7 with primers PabB-J1 and PabC-J8. Lanes: 1, 1 kb ladder; 2, wild-type; 3, ZYR-7-a; 4, ZYR-7-b; 5, ZYR-8-a; 6, ZYR-8-b. (c) Spectrophotometric quantification of antibiotic FR-008 production in wild-type, *pabC-2* mutant ZYR-8, *pabC-1/pabC-2* double mutant ZYR-7 complemented with cloned *pabC-2*, ZYR-7, and ZYR-7 fed with exogenous PABA. Each column represents the mean value obtained from at least three independent experiments and the error bars indicate the standard deviation.

Recovery of antibiotic FR-008 productivity in *pabC-1/pabC-2* mutant ZYR-7 with cloned *pabC-1* or *pabC-2*

To further confirm that *pabC-1* and *pabC-2* are involved in antibiotic FR-008 biosynthesis, plasmids pJTU949 and pJTU3283, harbouring *pabC-1* and *pabC-2* respectively, were introduced into the double mutant ZYR-7 by conjugation. Under the control of the strong constitutive *PerME** promoter, *pabC-1* and *pabC-2* complemented antibiotic FR-008 production in strain ZYR-7 to about 78 % (Fig. 2c) and 67 % (Fig. 3c), respectively, of the wild-type level.

Successful complementation of an *E. coli pabC* mutant with cloned *pabC-1* or *pabC-2*

The evidence that PabC-1 and PabC-2 were involved in the biosynthesis of PABA, the starter unit of antibiotic

FR-008, came from the complementation of an *E. coli pabC* mutant with cloned *pabC-1* and *pabC-2*, respectively. For this purpose, an *E. coli* BW25113 *pabC* mutant, ZYR-9, was constructed using the λ -Red-mediated recombination system (Gust *et al.*, 2003) (see supplementary material and Fig. S1). The genes *pabC-1* and *pabC-2* were cloned into *E. coli* expression vector pET28a, and the resultant constructs pJTU927 and pJTU928 were used to transform the mutant ZYR-9 separately. Expression of FR-008 PabC-1 or PabC-2 restored the ability of the mutant ZYR-9 to grow well without PABA supplementation in M9 minimal medium (Fig. 5). The mutant transformed with pJTU928 (harbouring *E. coli pabC*; see supplementary material) was used as the positive control. In the absence of PABA, the slight growth of mutant ZYR-9 and its derivative containing vector pET28a was due to a low rate of spontaneous PABA formation from ADC (Fig. 5).

Fig. 4. Growth of wild-type (I), *pabC-2* mutant ZYR-8 (II), *pabC-1* mutant ZYR-6 (III) and *pabC-1/pabC-2* double mutant ZYR-7 (IV) on minimal medium with or without PABA supplementation.

Enzyme assays confirm that PabC-1 and PabC-2 are functional ADC lyases

In order to examine the biochemical functions of the PabC-1 and PabC-2 proteins, the individual recombinant proteins were overexpressed and assayed *in vitro* for ADC lyase activity. For comparison, *E. coli* PabC was also overexpressed and purified. The recombinant *E. coli* PabB and NH_4^+ were used to convert chorismate to ADC, which is the substrate of ADC lyase. Subsequent cleavage of ADC by ADC lyase will release pyruvate, which is then reduced to lactate by LDH with coupled oxidation of NADH to NAD (He *et al.*, 2004; Ye *et al.*, 1990). The decrease of NADH concentration was monitored continuously at 340 nm (Fig. 6). The ADC lyase activity of *E. coli* PabC, as the positive control, was tested first. The A_{340} decreased dramatically within 4 min in the presence of chorismate, PabB and *E. coli* PabC. When *E. coli* PabC was omitted, the

decrease in NADH occurred very slowly. When the substrate chorismate was omitted, the coupled reactions also occurred slowly. The slow decreases in each control may have been due to contamination with enzymes which can oxidize NADH to NAD (Fig. 6a). The assays of FR-008 PabC-1 and PabC-2 proceeded in the same manner as that for *E. coli* PabC. When chorismate, PabB, and PabC-1 or PabC-2 were added, the A_{340} dropped quickly, whereas a slow decrease in A_{340} was observed in the absence of PabB, chorismate, PabC-1 (Fig. 6b) or PabC-2 (Fig. 6c). These results indicated that PabC-1 and PabC-2 need ADC as substrate to generate pyruvate, suggesting that both enzymes have ADC lyase activity.

DISCUSSION

The involvement of fused *pabAB* in candicidin biosynthesis was confirmed through gene inactivation and feeding with exogenous PABA (Campelo & Gil, 2002). The abolition of antibiotic production was nearly complete, suggesting that the deficiency of ADC or PABA could not be remedied via primary metabolism. Attempts to clone discrete ADC synthase genes from *Streptomyces* were made in *S. lividans* (Criado *et al.*, 1993) and *S. venezuelae* (Chang *et al.*, 2001), with successful complementation of an *E. coli* *pabB* mutant. In *S. venezuelae* (the chloramphenicol producer), besides the knockout of the ADC synthase gene *cmlB* for chloramphenicol biosynthesis, the inactivation of a second set of ADC synthase genes, *pabA/pabB*, did not affect growth on minimal medium, which suggested that additional unidentified ADC synthase genes might be present in the genome (Chang *et al.*, 2001).

However, no attempt to identify an ADC lyase gene from *Streptomyces* has been reported. To the best of our knowledge, the gene *pabC-1* represents the first confirmed ADC lyase gene in actinomycetes; its inactivation resulted in an 80 % decrease of antibiotic FR-008 productivity (Fig. 2c). A putative ADC lyase gene was identified in the novobiocin biosynthetic gene cluster from *S. spheroides*, but no relevant function has been found (Steffensky *et al.*, 2000). In aureothin and neo-aureothin biosynthesis, the missing but required ADC lyase activity is most likely provided by the corresponding protein for PABA biosynthesis from primary metabolism. This assumption was supported by the successful heterologous expression of the aureothin gene cluster in *S. lividans* ZX1 (He & Hertweck, 2003). The result that the SCO1546 mutant of *S. coelicolor* required PABA for growth on minimal medium (unpublished data) encouraged us to search for equivalent genes in the *Streptomyces* sp. FR-008 genome. Indeed, a SCO1546 homologue, *pabC-2*, which shows 85 % identity with SCO1546, was cloned from the *Streptomyces* sp. FR-008 genome.

Double mutation of *pabC-1* and *pabC-2* almost completely abolished antibiotic FR-008 production (Fig. 3c), demonstrating that the remaining 20 % production of anti-

Fig. 5. Complementation of an *E. coli* *pabC* mutant with *pabC-1* or *pabC-2*. Cells of wild-type (WT) *E. coli*, *pabC* mutant ZYR-9 (ZYR-9), ZYR-9 transformed with vector pET28a alone (V), ZYR-9 with cloned *E. coli* *pabC* (V+E-*pabC*), ZYR-9 with cloned *pabC-1* (V+pabC-1), and ZYR-9 with cloned *pabC-2* (V+pabC-2) were cultivated on M9 minimal medium supplemented or not with PABA.

Fig. 6. Biochemical analysis of PabC (*E. coli*), PabC-1 and PabC-2. (a) PabC (*E. coli*): I, chorismate with boiled PabB and boiled PabC (*E. coli*); II, chorismate with PabB and boiled PabC (*E. coli*); III, chorismate with boiled PabB and PabC (*E. coli*); IV, PabB and PabC (*E. coli*), no chorismate; V, chorismate with PabB and PabC (*E. coli*). (b) PabC-1: I, chorismate with boiled PabB and boiled PabC-1; II, chorismate with PabB and boiled PabC-1; III, chorismate with boiled PabB and PabC-1; IV, PabB and PabC-1, no chorismate; V, chorismate with PabB and PabC-1. (c) PabC-2: I, chorismate with boiled PabB and boiled PabC-2; II, chorismate with PabB and boiled PabC-2; III, chorismate with boiled PabB and PabC-2; IV, PabB and PabC-2, no chorismate; V, chorismate with PabB and PabC-1. The recombinant *E. coli* PabB and NH_4^+ were used to convert chorismate to ADC, which is the substrate of ADC lyase. The pyruvate generated by ADC lyase was detected by an LDH-coupled assay, in which the decrease of NADH concentration was monitored continuously at 340 nm; kinetic traces within 10 min are presented. The slow decrease in each control may be due to contamination with enzymes which can oxidize NADH to NAD.

biotic FR-008 in ZYR-6 was mostly contributed by the product of an ADC lyase gene outside the biosynthetic gene cluster. Moreover, the retarded growth of the *pabC-1/pabC-2* double mutant on minimal medium (Fig. 4) assigned *pabC-1* and *pabC-2* also to PABA synthesis in primary metabolism. Even though the double mutant showed retarded growth on minimal medium, noticeable growth still occurred. This may be caused by the slow spontaneous conversion from ADC to PABA (Tewari *et al.*, 2002), low enzymic conversion by other ADC lyases in the mutant, or contamination by trace amounts of PABA in the medium.

Interestingly, PabC-2 shows high homology with its equivalent proteins from *S. coelicolor* (SCO1546, 81 % identity/89 % similarity), *S. avermitilis* (SAV6804, 80 % identity/90 % similarity) and *S. griseus* (SGR5991, 75 % identity/85 % similarity). Moreover, the three equivalent genes of *pabC-2* are all found to be sandwiched between an upstream putative anthranilate synthase gene and a downstream putative *N*-acetyltransferase gene in each genome. The degenerate primers used for cloning of *pabC-2* could also amplify specific PCR products of the expected size from some other *Streptomyces* studied in our lab (data not shown). This finding points to the probable common existence of *pabC-2* equivalent genes in other *Streptomyces*.

In conclusion, two ADC lyase genes, including one (*pabC-1*) from the antibiotic FR-008 biosynthetic gene cluster, have been functionally identified for the first time from actinomycetes. The interplay between these two genes in *Streptomyces* sp. FR-008, in both cell growth and antibiotic

FR-008 biosynthesis, sets a good example of a genetic coordination, rather than uni-directional flow, between primary and secondary metabolisms.

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