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

Yirong Zhang, Linquan Bai and Zixin Deng

Laboratory of Microbial Metabolism and School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200030, China

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/candidicin 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.

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/candidicin from Streptomyces sp. FR-008/IMR3570 (Gil & Hopwood, 1983; Hu et al., 1994), aureothin from Streptomyces thioluteus (He & Hertweck, 2003), neoaureothin from Streptomyces orinoci (Traitecheva et al., 2007), pristinamycin 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.
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), neoaureothin (norG) (Traitcheva et al., 2007) and pristinamycin (papA) (Blanc et al., 1997). Even though PABA is the immediate precursor for aureothin and neoaureothin 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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant phenotype and/or characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH10B</td>
<td>recA</td>
<td>GibcoBRL</td>
</tr>
<tr>
<td>ET12567/pUZ8002</td>
<td>recE dam dcm hsdS Cm' Str' Tet' Km'</td>
<td>Paget et al. (1999)</td>
</tr>
<tr>
<td>BW25113</td>
<td>K12 derivative, ΔaraBAD ΔrhaBAD</td>
<td>Gust et al. (2003)</td>
</tr>
<tr>
<td>ZYR-9</td>
<td>BW25113-derived pabC mutant</td>
<td>This study</td>
</tr>
<tr>
<td>ZYR-9/pJTU927</td>
<td>ZYR-9 complemented with pJTU927 harbouring full-length FR-008 pabC-1</td>
<td>This study</td>
</tr>
<tr>
<td>ZYR-9/pJTU928</td>
<td>ZYR-9 complemented with pJTU928 harbouring full-length E. coli pabC</td>
<td>This study</td>
</tr>
<tr>
<td>ZYR-9/pJTU3295</td>
<td>ZYR-9 complemented with pJTU3295 harbouring full-length FR-008 pabC-2</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Streptomyces sp.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FR-008</td>
<td>Wild-type producer of antibiotic FR-008</td>
<td>Hu et al. (1994)</td>
</tr>
<tr>
<td>HJ-5</td>
<td>Mutant with the whole antibiotic FR-008 gene cluster deleted</td>
<td>Unpublished</td>
</tr>
<tr>
<td>ZYR-6</td>
<td>FR-008 derivative generated by replacement of a 437 bp internal to pabC-1 with 1.4 kb aac(3)IV</td>
<td>This study</td>
</tr>
<tr>
<td>ZYR-7</td>
<td>FR-008 derivative with pabC-1/pabC-2 double mutation</td>
<td>This study</td>
</tr>
<tr>
<td>ZYR-8</td>
<td>FR-008 derivative generated by deletion of a 675 bp DNA fragment internal to pabC-2</td>
<td>This study</td>
</tr>
<tr>
<td>ZYR-7/pJTU949</td>
<td>ZYR-7 complemented with pJTU949 harbouring full-length pabC-1</td>
<td>This study</td>
</tr>
<tr>
<td>ZYR-7/pJTU3283</td>
<td>ZYR-7 complemented with pJTU3283 harbouring full-length pabC-2</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescript KS(-)</td>
<td>Phagemid, bla lacZ</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pMD18 T-vector</td>
<td>pUC18 derivative</td>
<td>TaKaRa</td>
</tr>
<tr>
<td>pRSET-B</td>
<td>P77 RBS 6xHis Xpress Epitope EK, bla</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pET28a</td>
<td>P77 His-Tag/thrombin/T7-Tag, kan</td>
<td>Novogen</td>
</tr>
<tr>
<td>pJTU1278</td>
<td>bla lacZ oriT ori(pJ101)</td>
<td>Unpublished</td>
</tr>
<tr>
<td>pJTU968</td>
<td>pRSET-B derivative bla PermE*</td>
<td>Unpublished</td>
</tr>
<tr>
<td>pPM927</td>
<td>tsr oriT int attP</td>
<td>Smokvina et al. (1990)</td>
</tr>
<tr>
<td>pSET152</td>
<td>aac(3)IV oriT(RK2) ori(pUC18) int(φC31), attP(φC31) lacZs</td>
<td>Bierman et al. (1992)</td>
</tr>
<tr>
<td>pJTU468</td>
<td>A 3.0 kb KpnI/BamHI fragment carrying pabC-1 cloned in pHGF9053</td>
<td>This study</td>
</tr>
<tr>
<td>pJTU469</td>
<td>Insertion of a 1.4 kb aac(3)IV gene fragment into the Apal site sandwiched between the 0.8 kb and 1.8 kb fragments in Apal-digested and religated pJTU468</td>
<td>This study</td>
</tr>
<tr>
<td>pJTU474</td>
<td>Cloning of a 4.0 kb BamHI/KpnI fragment from pJTU469 to pJTU1278, which contains a linked 0.8 kb (left arm), 1.4 kb aac(3)IV, and 1.8 kb (right arm) for pabC-1 inactivation</td>
<td>This study</td>
</tr>
<tr>
<td>pJTU927</td>
<td>pET28a carrying the 0.8 kb FR-008 pabC-1</td>
<td>This study</td>
</tr>
<tr>
<td>pJTU928</td>
<td>pET28a carrying the 0.8 kb E. coli pabC</td>
<td>This study</td>
</tr>
<tr>
<td>pJTU949</td>
<td>pPM927 carrying PermE* and FR-008 pabC-1</td>
<td>This study</td>
</tr>
<tr>
<td>pJTU3283</td>
<td>pPM927 carrying PermE* and FR-008 pabC-2</td>
<td>This study</td>
</tr>
<tr>
<td>pJTU3286</td>
<td>pSET152 with the 9.3 kb EcoRI fragment from cosmid 11A10</td>
<td>This study</td>
</tr>
<tr>
<td>pJTU3287</td>
<td>pSET152 carrying the 8.7 kb EcoRI fragment with 0.68 kb DNA fragment internal to pabC-2 deleted</td>
<td>This study</td>
</tr>
<tr>
<td>pJTU3288</td>
<td>Cloning of an 8.7 kb EcoRI fragment from pJTU3287 to pJTU1278, which contains a linked 5.6 kb (left arm), 3.1 kb (right arm) for pabC-2 inactivation</td>
<td>This study</td>
</tr>
<tr>
<td>pJTU3295</td>
<td>pET28a carrying the 0.8 kb FR-008 pabC-2</td>
<td>This study</td>
</tr>
<tr>
<td>pJTU3296</td>
<td>pET28a carrying the 1.4 kb E. coli pabB</td>
<td>This study</td>
</tr>
</tbody>
</table>

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 medium (3% TSB, 1% yeast extract, 10.3% sucrose, pH 7.2) for conjugation between E. coli and 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., 2005) for Streptomyces, apramycin and thiostrepton were used at 30 μg ml⁻¹ and 25 μg ml⁻¹, respectively, in both SFM agar and liquid media. PABA (Sigma) was used at 5 μg 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 A260 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-3, 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/BamHI fragment carrying pabc-1 was ligated to KpnI/BamHI-digested pHG9053 (Minagawa et al., 2007) to give pTU468. Plasmid pTU468 was digested with Apal, blunted and inserted with a 1.40 kb aac(3)IV gene fragment from pHG9827 (Chen et al., 2003) to generate pTU469. 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 BamHI/KpnI fragment from pTU469 to BamHI/KpnI-digested pTU1278. The resultant plasmid pTU744 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′-GGGCGATGAGGAAGATTG-3′) and PabC-Det-R (5′-CGAATTCGGAGGGCGAAC-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′-GATCGTGGACCTGTCGSAACGA-3′; S=G/C) and PabC-J8 (5′-GAGATCCGAGCATGCTCGCTTTGCGSTG-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′-GCGGTCCGGCCACGTSAGNCGRTG-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 EcoRI fragment from a positive cosmid, 11A10, carrying pabc-2 was cloned into the vector pSET152 to generate pTU3286.

Deletion of pabc-2 in wild-type FR-008 and pabc-1 mutant ZYR-6. Plasmid pTU3286 was digested with BglII and self-ligated to generate pTU3287. The shortened 8.70 kb EcoRI fragment from pTU3287 was then transferred to the shuttle vector pTU1278. The resultant plasmid pTU3288 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 pTU468 as template and primers Pabc1-F (5′-CGGATCCCTATGATCGAAGACGGGCGAAC-3′; engineered BamHI and Ndel sites underlined) and Pabc1-R (5′-GAATTCGGAGGGCGAAC-3′; engineered EcoRI 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 EcoRV-digested pBluescript KS(−) to generate pTU903, which was sequenced to validate the inserted DNA sequence. A 0.80 kb Ndel/EcoRI fragment from pTU903 was ligated to Ndel/EcoRI-digested pTU906 to give pTU948. Then the 1.10 kb Ndel/EcoRI fragment, containing the Perme promoter and pabc-1, was cleaved from pTU948 and ligated into EcoRI-digested pPM927 to give pTU949. 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 pTU3286 as template and primers Pabc2-F (5′-AGGGATCCCTATGATCGAAGACGGGCGAAC-3′; engineered BamHI and Ndel sites underlined) and Pabc2-R (5′-GGTTCGCAGAATTCGAGGGCGAAC-3′; engineered EcoRI 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 pTU3293, which was sequenced to validate the inserted DNA sequence. A 0.80 kb Ndel/EcoRI fragment from pTU3293 was ligated to Ndel/EcoRI-digested pTU968 to give pTU3282. Then the 1.10 kb Ndel/EcoRI fragment, containing the Perme promoter and pabc-2, was cleaved from pTU3282 and ligated into EcoRI-digested pPM927 to give pTU3283. 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-2 mutant ZYR-9 with FR-008 pabc-for pabc-2. The pabc-2 gene of E. coli BW25113 was replaced with aac(3)IV using the i-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 Ndel and EcoRI restriction sites into pET28a (Novagen) to generate pTU927 and pTU3295, respectively. The mutant ZYR-9 was transformed with pTU927 or pTU3295, and the transformants were cultivated on M9 minimal medium (plus or minus PABA) for 12 h at 37 °C. The derivatives of mutant ZYR-9 transformed with pTU928 (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 pTU3296 (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 pTU928 (for Pabc), pTU927 (for Pabc-1) or pTU3295 (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 choromate was removed by addition of a slight excess of Na_{2}SO_{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 Bisenc (pH 8.5), 100 mM (NH_{4})_{2}SO_{4}, 5 mM MgCl_{2}, 20 μM PLP, 5 mM choromate (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 choromate.

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 24 % similarity to the pJTU1278-derived plasmid pJTU744, the apramycin-biosynthesis was examined through gene replacement. 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 675 bp DNA to be replaced (Fig. 2a). Two thiostrepton-sensitive, apramycin-resistant (Thio^{5} 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 cosmids 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...
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.
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 l-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).
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 NH₄⁺ 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 oxidization 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 A₃₄₀ dropped 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 A₃₄₀ dropped quickly, whereas a slow decrease in A₃₄₀ 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 candidicidin 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 cmIB 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. sphericoides, 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-
Streptomyces actinomycetes. The interplay between these two genes have been functionally identified for the first time from the antibiotic FR-008 biosynthesis, sets a good example of a genetic coordination, rather than uni-directional flow, between primary and secondary metabolisms.

ACKNOWLEDGEMENTS

We thank Dr Shuangjun Lin for helpful discussions and critical reading of our manuscript. This work received financial support from the National Science Foundation of China, the Ministry of Science and Technology (973 and 863 Programs), the Ministry of Education (NCET Program), the Shanghai Municipal Council of Science and Technology, and Shanghai Leading Academic Discipline Project B203.

REFERENCES


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

![Fig. 6. Biochemical analysis of PabC (*E. coli*), PabC-1 and PabC-2.](Image)

(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.
Streptomyces griseus. Gene aminobenzoic acid synthetase gene of the candicidin-producing. Purification of aminodeoxychorismate lyase and cloning of and chloramphenicol biosynthesis in Microbiology sets for a key enzyme, 4-amino-4-deoxychorismate synthase.


