A host–vector system for analysis and manipulation of rifamycin polyketide biosynthesis in *Amycolatopsis mediterranei*

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Modular polyketide synthases (PKSs) are a large family of multifunctional enzymes responsible for the biosynthesis of numerous bacterial natural products such as erythromycin and rifamycin. Advanced genetic analysis of these remarkable systems is often seriously hampered by the large size (>40 kb) of PKS gene clusters, and, notwithstanding their considerable fundamental and biotechnological significance, by the lack of suitable methods for engineering non-selectable modifications in chromosomally encoded PKS genes. The development of a facile host–vector strategy for genetic engineering of the rifamycin PKS in the producing organism, *Amycolatopsis mediterranei* S699, is described here. The genes encoding all 10 modules of the rifamycin PKS were replaced with a hygromycin-resistance marker gene. In a similar construction, only the first six modules of the PKS were replaced. The deletion hosts retained the ability to synthesize the primer unit 3-amino-5-hydroxybenzoic acid (AHBA), as judged by co-synthesis experiments with a mutant strain lacking AHBA synthase activity. Suicide plasmids carrying a short fragment from the 5' flanking end of the engineered deletion, an apramycin-resistance marker gene, and suitably engineered PKS genes could be introduced via electroporation into the deletion hosts, resulting in the integration of PKS genes and biosynthesis of a reporter polyketide in quantities comparable to those produced by the wild-type organism. Since this strategy for engineering recombinant PKSs in *A. mediterranei* requires only a selectable single crossover and eliminates the need for tedious non-selectable double-crossover experiments, it makes rifamycin PKS an attractive target for extensive genetic manipulation.

Keywords: rifamycin, polyketide synthase, *Amycolatopsis*

INTRODUCTION

Polyketides are a large family of microbial natural products especially abundant in actinomycetes. Medically important polyketides produced by species belonging to the genus *Amycolatopsis* include rifamycin (Sensi *et al.*, 1959), vancomycin (Barna & Williams, 1984), balhimycin (Nadkarni *et al.*, 1994) and dethymycin (Ueno *et al.*, 1992). Among these, rifamycin and its derivatives are of particular interest, as they can be used to treat major infectious diseases such as tuberculosis

and leprosy (Cole, 1996). They are also becoming increasingly important in the control of infections occurring in organ implant and AIDS patients. The glycopeptide antibiotics vancomycin and balhimycin have antibacterial activity against methicillin-resistant *Staphylococcus aureus* strains, whereas dethymycin is an immunosuppressant with a mode of action different from cyclosporin, FK506, rapamycin and other such immunosuppressants.

The mechanism of action of rifamycin against bacteria and viruses (Lowder & Johnson, 1987; Riva *et al.*, 1972) as well as its structure–activity relationships (Arora & Main, 1984; Arora, 1985; Bacchi *et al.*, 1998; Bartolucci *et al.*, 1995) have been extensively studied. More

Abbreviations: AHBA, 3-amino-5-hydroxybenzoic acid; PKS(s), polyketide synthase(s).

Fig. 1. Rifamycin PKS gene cluster. The three cosmids comprising the rifamycin PKS gene cluster, cloned in this study, are shown at the top. Beneath them are the genes encoding the rifamycin PKS pathway, together with their inferred biosynthetic intermediates. The product of the rifamycin PKS pathway is believed to be proansamycin X, although this remains to be directly confirmed. Recently it has been suggested that cyclization of the polyketide intermediate to generate the naphthalene functionality occurs between the tetraketide and pentaketide stages on the PKS-bound intermediate (Yu *et al.*, 1999). KS, Ketosynthase; AT, acyltransferase; DH, dehydratase; KR, ketoreductase; ACP, acyl carrier protein.

recently, the biosynthesis of rifamycin has attracted attention. The polyketide chain starts with a 3-amino-5-hydroxybenzoic acid (AHBA) primer unit (Ghisalba & Nuesch, 1981; Ghisalba *et al.*, 1981; Hatano *et al.*, 1982). Initially, the gene encoding AHBA synthase, which catalyses the last step in AHBA biosynthesis, was cloned (Kim *et al.*, 1998); in turn, this led to cloning and sequencing of the whole gene cluster (Schupp *et al.*, 1998; Tang *et al.*, 1998). Encoded within the gene cluster is a 10-module polyketide synthase (PKS) presumably responsible for the biosynthesis of proansamycin X, the polyketide precursor of rifamycin B (Fig. 1). This multifunctional enzyme complex presents an attractive opportunity for engineering novel rifamycin analogues, as has been repeatedly demonstrated for the better-studied erythromycin PKS (Cane *et al.*, 1998). However, a central prerequisite for such genetic engineering is the availability of suitable genetic tools and methods.

Two basic strategies have been exploited for the genetic manipulation of erythromycin biosynthesis. One involves using the natural producer of erythromycin – *Saccharopolyspora erythraea*. Chromosomal mutants in the erythromycin PKS genes are generated through standard homologous recombination-mediated gene replacement methods. The principal advantage of this approach is that the system can make fully modified and often biologically active erythromycin derivatives (Donadio *et al.*, 1993; Ruan *et al.*, 1997; Marsden *et al.*, 1998). However, since homologous recombination occurs at a relatively low frequency, the method is tedious, especially where non-selectable double cross-overs are required. An alternative strategy takes ad-

vantage of a host–vector system that uses *Streptomyces coelicolor* CH999 as the host and pRM5 as the vector (McDaniel *et al.*, 1993). The erythromycin PKS genes or mutants thereof are cloned into pRM5 derivatives and introduced via transformation into CH999 to produce the expected reporter polyketides (Kao *et al.*, 1994, 1996; Bedford *et al.*, 1996). Whereas only aglycones are generated using this approach, it offers the convenience of shuttle-plasmid-borne genes, and is therefore more amenable to rapid mutagenesis. Recent work by Leadlay and coworkers has sought to combine features of these two approaches (Rowe *et al.*, 1998).

In *Amycolatopsis*, unlike *Streptomyces*, genetic tools and methods, such as transformation and transfection, and cloning vectors, are relatively undeveloped. Plasmids that replicate in *Streptomyces* cannot be maintained in *Amycolatopsis mediterranei* (Schupp & Diver, 1986; Pelzer *et al.*, 1997), although plasmids such as pIJ702 and pKC505 from *Streptomyces* could replicate in *Amycolatopsis orientalis* (Matsushima *et al.*, 1987). A few indigenous plasmids such as pMEA100 (Moretti *et al.*, 1985; Madon & Hutter, 1991), pMEA300 (Vrijbloed *et al.*, 1994) and pA387 (Lal *et al.*, 1991) have been found in *Amycolatopsis* species, but have not yet been fully developed into cloning and expression vectors. Consequently, genetic manipulation of the rifamycin PKS genes in *A. mediterranei* is very difficult, and primarily relies on the use of suicide delivery systems.

Here we describe the construction of specific deletion mutants of *A. mediterranei* S699 as hosts for PKS gene

expression. Engineered forms of the rifamycin PKS can be introduced into these hosts either on plasmid vectors or through single crossovers into the chromosome. We demonstrate the utility of this approach by reinserting the *rifA* gene into the chromosome to produce the expected tetraketide P8/1-OG.

METHODS

Bacterial strains and plasmids. The *Escherichia coli* strain used in this study was XL-1 Blue (Stratagene). *Amycolatopsis mediterranei* strains S699 and HGF003 were gifts from Heinz G. Floss (University of Washington, Seattle, USA). The former produces rifamycin B, whereas the latter is a null mutant in which the AHBA synthase gene has been inactivated (Kim *et al.*, 1998; Hunziker *et al.*, 1998). SuperCos 1 was used as the cosmid vector for constructing the genomic library. Plasmid pIY15 contains a 7.5 kb *Pst*I fragment from the rifamycin PKS gene cluster (August *et al.*, 1998), and was used as a probe to isolate clones containing *rif* genes from the cosmid library. Plasmid pSET152, which confers apramycin resistance both in *E. coli* and in *A. mediterranei*, was used as a cloning vector in this study (Flett *et al.*, 1997). (Note that although pSET152 contains the *att* integration site of the phage ϕ C31, its use here was solely based on cloning convenience. By itself, this vector is incapable of integrating into the genome of *A. mediterranei*.)

Media, chemicals and growth conditions. *E. coli* strains were grown in Luria–Bertani (LB) medium at 37 °C. When necessary, 50 µg carbenicillin ml⁻¹ or 60 µg apramycin ml⁻¹ was present in the medium. *A. mediterranei* strains were grown in YMG (also called YM) (Lal *et al.*, 1998) medium at 30 °C, supplemented with 60 µg apramycin ml⁻¹ and/or 50 µg hygromycin ml⁻¹ when necessary. All antibiotics were purchased from Sigma; 3-hydroxybenzoic acid was obtained from Aldrich, whereas 3-amino-5-hydroxybenzoic acid (AHBA) was synthesized in this laboratory and verified by NMR.

DNA manipulation. DNA fragments for subcloning were purified with Qiaex I (for fragments smaller than 10 kb) or Qiaex II (for fragments larger than 10 kb) gel extraction kits (Qiagen). DNA blotting and Southern hybridization made use of protocols recommended for the DIG labelling and detection kit (Boehringer). All other DNA manipulations utilized standard methods (Sambrook *et al.*, 1989).

Construction of a genomic DNA library. Genomic DNA was isolated from *A. mediterranei* S699 and partially digested with *Sau*3A. The fraction containing 35–42 kb fragments was purified from a 0.3% agarose gel and ligated with *Bam*HI/*Xba*I-linearized SuperCos 1 (Stratagene). The ligation mixture was packaged using Gigapack III Gold packaging extract (Stratagene), and used to infect *E. coli* XL-1 Blue. Recombinants were selected on LB agar plates containing carbenicillin.

Purification of P8/1-OG. Spores of *A. mediterranei* were plated on Petri dishes containing 40 ml YMG medium to give lawns. Following growth at 30 °C for 3 d, the mycelium was overlaid with a solution of the starter unit AHBA in 10% DMSO diluted with water (pH adjusted to 7.2) to give 1.5 ml per plate. The agar in plates was allowed to dry for 45 min before use. After addition of the starter unit, incubation was continued for another 10 d. The medium was then homogenized and extracted three times with an equal amount of ethyl acetate containing 1% acetic acid. The crude extract was pre-purified on a short (5 cm) silica gel column using CHCl₃/CH₃OH 4:1

(containing 1% acetic acid) as an eluant. All fractions containing product were combined and evaporated. The residue was rechromatographed on a 10 cm column using the same solvent system; the chromatographic purification removed most of the contaminants except AHBA. P8/1-OG was further purified by a second chromatography (5% CH₃OH in ethyl acetate, 1% acetic acid overall content) after application to the column in a small amount of CHCl₃/CH₃OH 4:1. Fractions containing pure product were combined and evaporated to give a yellowish residue that was identified as P8/1-OG by: R_F = 0.41 (5% CH₃OH in ethyl acetate, 1% acetic acid), 0.37 (CHCl₃/CH₃OH 4:1, 1% acetic acid); HPLC (see below for conditions) t_r = 10.28 min; ¹H-NMR (400 MHz, CD₃OD) (refer to Ghisalba *et al.*, 1981 for ¹H-NMR data previously reported) – 6.24 (t, br, 1 H, J = 1.60 Hz), 6.16 (t, br, 1 H, J = 1.46 Hz), 6.13 (t, 1 H, J = 2.10 Hz), 6.04 (s, 1 H), 4.53 (d, 1 H, J = 8.85 Hz), 2.77 (qd, 1 H, J = 8.92, 7.09 Hz), 1.86 (s, 3 H), 0.97 (d, 3 H, J = 7.02 Hz) (br, broad; qd, quartet of doublets).

Quantitative HPLC analysis of production levels. 3-Hydroxybenzoic acid (10 mg per plate) was fed to both *A. mediterranei* HGF003 and *A. mediterranei* HZ149 as described above. Following incubation, culture media were extracted with ethyl acetate containing 1% acetic acid. The extracts were evaporated *in vacuo* and the residue was redissolved in CH₃OH (2 ml per plate extracted). The resulting solution was analysed by HPLC [Beckman ULTRASPHERE C₁₈, 5 µ, 0.46 × 25 cm; flow rate, 1.0 ml min⁻¹; injection volume, 20 µl; UV detection at 254 and 306 nm; solvent gradient, 0–1 min: 100% A, 1–20 min: 100% A → 100% B, 20–25 min: 100% B (solvent A was 1% acetic acid in water and solvent B was 1% acetic acid in CH₃CN)]. The expected tetraketide eluted at t_r = 13.06 min.

RESULTS

Cloning the rifamycin PKS gene cluster

Although an earlier report (August *et al.*, 1998) has described the cloning and sequence analysis of the entire rifamycin gene cluster, most of the primary genomic fragments isolated in that study were relatively small (< 20 kb). Because this presented a problem for the genetic strategy described below, the rifamycin PKS genes were recloned as cosmid-sized inserts. When the 7.5 kb *Pst*I fragment from pIY5 was labelled with DIG and used to probe 2000 colonies from the library, 42 hybridizing clones were detected. Of these cosmid clones, cos2, cos6 and cos42 (Fig. 1) were shown by comparing their restriction sites with published sequences of the rifamycin PKS genes (Tang *et al.*, 1998) to cover the entire rifamycin PKS gene cluster.

Construction of a deletion host

DNA fragments A, B and C were subcloned from cosmids cos2, cos42 and cos6, respectively (Fig. 2). Fragment A starts at nt 651 and continues to nt 1438 (where nucleotides are numbered from the first base of the rifamycin PKS genes). Fragment B extends from nt 28464 to nt 29417, and includes the end of module 6 as well as the start of module 7. Fragment C contains the end of module 10 starting from nt 47393 and continues to nt 50159; it includes the 3' end of the *rifF* amide

Fig. 2. Construction of the deletion hosts HZ14 and HZ17. The relative positions of fragments A, B and C within the rifamycin PKS gene cluster (hatched) are shown. Position 1 refers to the first G of the start codon (GTG). In *A. mediterranei* HZ14 the first six modules were replaced by a *hyg* cassette, whereas in *A. mediterranei* HZ17 all 10 modules were replaced with the same *hyg* cassette.

Fig. 3. Southern hybridization of genomic DNA from the deletion hosts HZ14 and HZ17. The *hyg* gene fragment was used as the probe. Lanes: 0 and 6, 1 kb ladder (Gibco-BRL); 1, genomic DNA from *A. mediterranei* S699 digested with *EcoRI*+*XmnI*; 2 and 3, genomic DNA from two independent isolates of *A. mediterranei* HZ17 digested with *EcoRI*+*XmnI*; 4, genomic DNA from *A. mediterranei* S699 digested with *EcoRI*+*HindIII*; 5, genomic DNA from *A. mediterranei* HZ14 digested with *EcoRI*+*HindIII*. The expected bands from HZ14 had sizes of 3.2 and 7.8 kb, whereas the expected bands from HZ17 had sizes of 2.6 and 4.1 kb. An additional band from HZ17, at 6.7 kb, is presumably caused by partial digestion of the genomic DNA.

synthase gene. Two suicide plasmids were constructed in pSET152 (*NheI*–*EcoRI*) with the hygromycin-resistance gene (*hyg*) (Malpartida *et al.*, 1983) flanked by either fragments A and B or A and C in their natural orientations. These constructs, designated pHU130 and pHU131, respectively, were introduced into *A. mediterranei* S699 by electroporation. Approximately 10 *hyg*⁺ *apr*⁺ colonies were obtained from each transformation, presumably as a result of a single crossover in one of the homologous regions. One such transformant was randomly selected in each case, and

propagated through two rounds of subsequent growth on non-selective YMG agar. More than 200 single colonies were tested for resistance to apramycin and hygromycin on selective YMG plates. Two derivatives of pHU130::S699 and three derivatives of pHU131::S699 had lost their resistance to apramycin but were still resistant to hygromycin. Southern analysis of *XmnI* + *EcoRI*-digested genomic DNA from two of the three pHU131::S699 derivatives, and of *HindIII* + *EcoRI*-digested genomic DNA from one of the two pHU130::S699 derivatives, using the *hyg* gene as a probe, confirmed that all three colonies were derived from double-crossover events, as expected (Fig. 3). Randomly chosen derivatives of pHU130::S699 and pHU131::S699 were designated *A. mediterranei* strains HZ14 and HZ17, respectively (see Fig. 2).

Strains HZ14 and HZ17 were grown in YMG liquid medium for 10 d at 30 °C with constant shaking at 250 r.p.m. Supernatants from the cultures were extracted three times with ethyl acetate containing 1% acetic acid. The organic phases were evaporated and the crude extracts were resuspended in ethyl acetate. TLC analysis of these extracts using authentic AHBA as a reference revealed that both cultures had produced significant amounts of AHBA (the starter unit for rifamycin synthesis). This was confirmed by co-synthesis experiments using these strains as secretors and *A. mediterranei* HGF003 (which contains an inactive AHBA synthase gene) as a convertor strain. The mixed fermentations produced rifamycin B, detected by HPLC-UV analysis (data not shown). Together, these results showed that the biosynthesis of AHBA was not affected by the deletions in *A. mediterranei* HZ14 and HZ17.

Expression of a truncated form of the rifamycin PKS in a deletion host

The *XmnI* (position –651)–*RcaI* (position 14757) fragment from *cos2* was end-filled using T4 DNA polymerase and cloned into the *SmaI* site of pUC119 to give pHU42. The *XbaI*–*EcoRI* fragment from pHU42 was then ligated with *NheI* + *EcoRI*-digested pSET152 to give the plasmid pHU149. Plasmid pHU149 was introduced into the deletion host *A. mediterranei* strain HZ17 by electroporation. Two apramycin-resistant

Fig. 4. Construction and analysis of HZ149. The strategy for constructing strain HZ149 is shown. The *XmnI* (position –651)–*RcaI* (position 14757) fragment from *cos2* (Fig. 1) contains *rifA*, which encodes AHBA-CoA ligase and the first three modules of the rifamycin PKS. Integration of this fragment by a single crossover into the genome of HZ17 yielded HZ149, which was unable to produce either AHBA or P8/1-OG by itself, but produced P8/1-OG or its desamino analogue when supplemented with AHBA or 3-hydroxybenzoic acid, respectively. LD, Loading domain; *apr*, apramycin-resistance gene; *hyg*, hygromycin-resistance gene.

transformants, HZ149-1 and HZ149-3, were obtained (Fig. 4). Extraction with ethyl acetate containing 1% acetic acid and TLC comparison with a comparable extract from the deletion host HZ17 revealed no major metabolite in the fermentation medium. Moreover, no AHBA was produced by either transformant. Analysis by reverse-phase HPLC yielded the same result, leading us to suspect that HZ149-1 or HZ149-3 might be capable of producing tetraketides, but that tetraketide production was down-regulated due to attenuated biosynthesis of AHBA. Therefore, *A. mediterranei* strains HZ149-3, HZ17 and HGF003 were subjected to more detailed metabolite analysis.

Metabolite analysis of HZ149

A. mediterranei HZ149-3 was grown on YMG agar for 10 d in the presence of exogenous AHBA (10 mg per plate). The crude product obtained by extraction with

ethyl acetate containing 1% acetic acid was analysed for polyketide metabolites by TLC and HPLC. A major new product was isolated and identified as P8/1-OG (Fig. 4) from NMR spectroscopic data. Based on the amount of P8/1-OG recovered, the titre was calculated to be $\approx 30 \text{ mg l}^{-1}$.

Earlier studies had shown that *A. mediterranei* HGF003 is capable of producing polyketides in high yields when fed a variety of exogenous starter units (Hunziker *et al.*, 1998). However, whereas AHBA is converted into rifamycin B by this strain, alternative starter units such as 3-hydroxybenzoic acid and 3,5-dihydroxybenzoic acid are processed only into tetraketide analogues of P8/1-OG. Therefore, the production levels of *A. mediterranei* strains HZ149-3 and HGF003 were compared by adding 3-hydroxybenzoic acid to growing cultures of both strains. Following incubation, culture extracts were analysed by HPLC for tetraketide (desamino P8/1-

OG) levels. Desamino P8/1-OG production by HZ149 was approximately 35% relative to HGF003. Purification of desamino P8/1-OG from the HZ149-3 extract gave 7.7 mg pure compound, which corresponded to a titre of approximately 50 mg l⁻¹. HGF003 has been shown previously to produce desamino P8/1-OG at 135 mg l⁻¹ (Hunziker *et al.*, 1998).

DISCUSSION

We describe the construction of two potentially useful hosts for manipulating rifamycin biosynthesis, and use one of them (the 10-module deletion host) to successfully express the *rifA* gene. We expect that the host-vector system described here will be valuable in further studies on the rifamycin PKS. There are several advantages to manipulating the rifamycin PKS in its original host, *A. mediterranei* S699. First, *A. mediterranei* produces a large amount of rifamycin (> 1 g l⁻¹) in stirred-tank fermentations. Since PKS manipulation often leads to reduced production of reporter polyketides, this enhanced productivity can facilitate isolating 'unnatural' natural products. Second, the rifamycin biosynthetic pathway includes several post-PKS tailoring steps, many of which are poorly characterized at the genetic and enzymological level. By using *A. mediterranei* one ensures that all downstream enzymes are available and that at least some modified cores can be converted into rifamycin B analogues. Third, the *A. mediterranei* derivatives described here offer relatively easy access to any combinatorial mutants in the 10 modules through the use of either the full 10-module deletion host (HZ17) or the six-module deletion host (HZ14). Finally, as other important biosynthetic gene clusters from *Amycolatopsis* species are cloned, the deletion hosts could also be useful for facile genetic engineering of heterologous biosynthetic pathways. For example, HZ14 and HZ17 could also be used to study genes from the recently described chloroeremomycin biosynthetic gene cluster from *A. orientalis* (Van Wageningen *et al.*, 1998).

Although *A. mediterranei* is taxonomically related to *Streptomyces*, methods for gene cloning in *A. mediterranei* are relatively underdeveloped. In particular, a suitable low-copy plasmid capable of holding large inserts is not yet available. Moreover, the absence of endogenous promoters suitable for polyketide gene expression is a disadvantage. Because our host-vector strategy uses a suicide vector to insert the desired gene(s) into the chromosome of *A. mediterranei* through a selectable single crossover, the expressed genes can use the same promoter that is presumably responsible for transcription of the rifamycin PKS genes. Although we were able to generate HZ149 in a single-step process, we were unable to deliver very large fragments (approx. 50 kb, carrying the first six modules of the rifamycin PKS gene cluster) into the chromosomes of the deletion hosts (data not shown). This may be due to either poor transfer of extremely large fragments into *A. mediterranei*, or the greater likelihood of their destruction by endogenous nucleases. As improved plasmids and pro-

motors are developed for *A. mediterranei*, we expect that the utility of our deletion hosts will increase further.

Finally, it is intriguing that production of P8/1-OG in HZ149 depends on the addition of exogenous AHBA. Since both wild-type *A. mediterranei* and its deletion derivatives can produce large amounts of AHBA, our results may suggest that one or more genes downstream of the rifamycin PKS operon are required for AHBA biosynthesis, and that integration of a suicide vector disrupts their transcription. Further experiments may help resolve this interesting conundrum.

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