Gene disruption and replacement in the rapamycin-producing *Streptomyces hygroscopicus* strain ATCC 29253

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A system for gene disruption and replacement based on a streptomycete temperate phage vector was developed to introduce DNA in the rapamycin-producing *Streptomyces hygroscopicus* strain ATCC 29253. This will be useful in attempts to produce, through genetic manipulation, novel forms of the therapeutically important immunosuppressive drug rapamycin. Recombinant phages were constructed from the ϕ C31 phage derivative KC515 (c^+ *attP*) carrying a thiostrepton or viomycin resistance gene along with segments of the *S. hygroscopicus* chromosome. Each of the cloned segments also contained the *aphII* neomycin/kanamycin resistance gene to enable gene replacement by loss of the phage-derived DNA. Specific deletion of the entire polyketide synthase (PKS) believed to govern rapamycin biosynthesis resulted in the loss of rapamycin production. In contrast, disruption or deletion of a region predicted to encode four PKS open reading frames, or another region predicted to encode another PKS plus a cytochrome P450 hydroxylase and ferredoxin, had no effect on the production of rapamycin or nigericin, a polyether antibiotic also produced by *S. hygroscopicus*. Therefore, *S. hygroscopicus* may have the capacity to produce polyketides additional to rapamycin and nigericin.

Keywords: nigericin, polyketide, modular polyketide synthase, *Streptomyces hygroscopicus*

INTRODUCTION

Streptomyces hygroscopicus ATCC 29253, the rapamycin producer, was originally isolated during screening for antifungal compounds (Vezina *et al.*, 1975). It was later found that rapamycin has valuable immunosuppressive activity (Melino & Sigal, 1990; Dumont & Su, 1996). Rapamycin, FK506 (Kino *et al.*, 1987) and FK520 (Hatanaka *et al.*, 1988) belong to a group of macrocyclic polyketides with greater immunosuppressive potency than cyclosporin A, the principal drug currently available. However, since rapamycin as well as cyclosporin A are not devoid of *in vivo* toxicity, a need still exists to discover less toxic immunosuppressive agents. One approach is to make *S. hygroscopicus* produce structural analogues of rapamycin through targeted mutation of the genes for rapamycin biosynthesis (Schwecke *et al.*, 1995). Structural ana-

logues of several polyketide antibiotics have been obtained in this way (Katz & Donadio, 1993; Hutchinson, 1994; Hutchinson & Fujii, 1995), most notably by targeted mutation of the polyketide synthase (PKS) genes for erythromycin biosynthesis (Donadio *et al.*, 1993; Kao *et al.*, 1994; Cortes *et al.*, 1995). The same approach is applicable to rapamycin since its biosynthesis appears to involve a modular (type I) PKS (Schwecke *et al.*, 1995), but this first requires a means to introduce DNA into the rapamycin-producing strain.

After discovering that the commonly used methods of transformation with plasmid DNA or conjugal transfer were unsuccessful with the rapamycin-producing strain (G. Meurer, N. Lomovskaya, N. Yang, X. Ruan & D. Stassi, unpublished), we examined vectors made from the temperate phage ϕ C31 (Lomovskaya *et al.*, 1972, 1980; Chater, 1986). Such vectors have been successfully used for cloning antibiotic biosynthetic genes (Chater & Bruton, 1983, 1985) and genes responsible for morphological development (Piret & Chater, 1985; Mendez

Abbreviation: PKS, polyketide synthase.

& Chater, 1987), as well as for disrupting RNA polymerase sigma factor and ppGpp synthetase genes (Buttner *et al.*, 1990; Buttner & Lewis, 1992; Chakraborty *et al.*, 1996), largely in *Streptomyces coelicolor* A3(2). Using the c^+ *attP*-deleted ϕ C31 derivative KC515 (Chater, 1986; Hopwood *et al.*, 1987) for both gene disruption and replacement, we studied the role of the modular PKS reported by Schwecke *et al.* (1995) in rapamycin biosynthesis as well as that of two other regions of *S. hygroscopicus* DNA containing apparent modular PKS genes. One region appears to encode a PKS consisting of four multifunctional proteins, and the other to encode portions of a PKS associated with genes for a cytochrome P450 hydroxylase and ferredoxin (X. Ruan, D. L. Stassi, N. Lomovskaya, L. Fonstein, C. R. Hutchinson & L. Katz, unpublished).

METHODS

Bacterial strains, plasmids and phages. *Escherichia coli* strain DH5 α (Sambrook *et al.*, 1989) and *E. coli* vectors pUC18 (Yanisch-Perron *et al.*, 1985), pGEM7Zf(–), pGEM9Zf(–) and pSP72 (Promega) were used for routine subcloning. The *Streptomyces* strains, plasmids and phages used in this study are listed in Table 1.

Biochemicals and chemicals. Thiostrepton was a gift from S. J. Lucania (E. R. Squibb & Sons, Princeton, NJ, USA), and rapamycin and nigericin were obtained from Jim McAlpine (Abbott Laboratories, Abbott Park, IL, USA). Restriction enzymes and other molecular biology reagents were from commercial sources.

Media and growth conditions. *E. coli* strains carrying plasmids were grown in Luria–Bertani medium (Sambrook *et al.*, 1989), and were selected with ampicillin (100 μ g ml^{–1}). *S. hygroscopicus* and *S. lividans* TK24 (Hopwood *et al.*, 1983) strains were grown on MOM medium [containing, per litre: bacto-oatmeal agar ISP3 (Difco), 11 g; cerelose, 4 g; N-Z casamino acids, 0.2 g; molasses, 1 g; NaCl, 1 g; Difco agar, 16 g] and on R2YE medium (Hopwood *et al.*, 1985) without sucrose for sporulation, and at 30 °C in R2YE liquid medium (Hopwood *et al.*, 1985) for preparation of protoplasts and isolation of chromosomal DNA. ϕ C31 and its derivatives were propagated as described by Hopwood *et al.* (1985) or in liquid medium containing, per litre: nutrient broth (Difco), 8 g; NaCl, 9 g. The following nutrients were added after autoclaving: 1 M MgSO₄·7H₂O, 10 ml; 0.8 M Ca(NO₃)₂, 10 ml, and a 50% solution of glucose, 20 ml. MOM agar medium was adapted for infection of *S. hygroscopicus* with ϕ C31 derivatives by adding, per litre, NaCl, 4.5 g, and (after autoclaving) 1 M MgSO₄·7H₂O, 10 ml, 0.8 M Ca(NO₃)₂, 10 ml. The minimal medium (MM) used was from Hopwood *et al.* (1985).

Isolation and *in vitro* manipulation of DNA. Plasmid DNA was isolated from bacterial cells with the Bio101 RPM kit. ϕ C31 phage DNA was isolated with the Qiagen lambda kit. Total *S. hygroscopicus* DNA was isolated by the protocol of Hopwood *et al.* (1985). Restriction endonuclease digestions and ligations used standard techniques (Sambrook *et al.*, 1989). DNA fragments for labelling and subcloning were isolated with the Qiaex (Qiagen) gel extraction kit. The conditions for phage DNA transfection were as described by Hopwood *et al.* (1985).

Southern blot analysis. *Streptomyces* chromosomal DNA was digested with restriction enzymes for 4 h, electrophoresed in a 0.8% agarose gel overnight and blotted to Hybond N membranes (Amersham) by capillary transfer (Sambrook *et al.*, 1989). Labelling, hybridization and detection were carried out with the Genius 1 non-radioactive DNA labelling kit (Boehringer Mannheim) according to the manufacturer's instructions.

Cloning of *S. hygroscopicus* DNA segments by PCR methods.

To create recombinant phage phWHM275, the 1.2 kb *Pst*I–*Xba*I fragment 4 (Fig. 2b) was amplified by the PCR method using the following two primers, 5'-GCGCTGCAGCCGGA-AACTTCCTCAGCTG-3' and 5'-GCGTCTAGAGCGTC-ATCCTGCCGCCATC-3'. Similarly, phWHM276 was constructed from the 1.68 kb *Bam*HI–*Xba*I fragment 6 (Fig. 3a) using the following two primers, 5'-GCGGGATCCATCAG-CGTCTTCGGGTGAAT-3' and 5'-GCGTCTAGATTCAA-GGATTTGGGTGTGGA-3'; and 1.33 kb *Xba*I–*Pst*I fragment 7 (Fig. 3a) using the following two primers, 5'-GCGTCTAGATCGTGGATGACACCCTCTAC-3' and 5'-GCGCTGCAGTCTTATTCGTTCTGCTCCT-3'. To confirm the gene replacements in the WMH1651 and WMH1652 strains the following primers were used: 5'-GCGGGATCCCTGCCATTTTATTCAGAACCGA-3' and 5'-GCGAAGCTTATCAGAGTGGTGACGTTTTC-3'. All PCR mixtures contained thermo DNA poly react buffer (Promega), 2.5 mM MgCl₂, 50 ng chromosomal DNA and 0.5 μ g of each pair of primers in a total volume of 91 μ l. The PCR reaction was carried out in a model 480 DNA Thermal Cycler (Perkin-Elmer Cetus). The reaction mixture was overlaid with mineral oil and incubated at 100 °C for 5 min, then cooled to 70 °C (3 min), and 2.5 U of *Taq* polymerase (Promega) and 60 μ M dCTP and dGTP, and 40 μ M dATP and dTTP were added. Amplification was achieved with 25 cycles of denaturation at 97 °C for 1.5 min followed by annealing and extension at 70 °C for 3.5 min, then the mixture was incubated for 10 min at 72 °C. The resulting PCR fragments were purified with the Qiagen PCR purification kit, digested with *Pst*I and *Xba*I (fragment 4), *Bam*HI and *Xba*I (fragment 6) or *Xba*I and *Pst*I (fragment 7), recovered from a 0.8% agarose gel after electrophoresis and cloned into plasmid vectors.

Construction of KC515-derived phages for insert-directed homologous recombination in *S. hygroscopicus*. For the first construct, phWHM261 (ϕ C31 derivatives in this work are prefixed with 'ph'), a 4.2 kb *Eco*RI fragment, which included the sequence encoding the putative coenzyme A ligase domain of the first ORF shown in Fig. 1(a), was subcloned from cosmid pAL58 into pUC18 to create pAL4.2E. A 2.2 kb *Xho*I–*Bam*HI fragment of pWHM253 containing the 1.1 kb *Xho*I–*Kpn*I internal fragment 1 from the coenzyme A ligase domain (Fig. 1a) and the *aphII* gene from pFDNeoS (Denis & Brzezinski, 1991) was ligated with *Xho*I- and *Bam*HI-digested KC515 to give phWHM261 (Fig. 1b) via plasmids pWHM249, pWHM252 and pWHM253, as described in Table 1.

The second construct, phWHM262, contained fragments 2 and 3 (Fig. 1a) located 10.9 kb from each other in the cluster defined by cosmid pAL58 with the *aphII* gene inserted between them. This construct was expected to be able to generate neomycin-resistant clones of *S. hygroscopicus* due to a double recombination event that would delete 10.1 kb to eliminate parts of the putative ORF1 and ORF3 genes and all of ORF2 (Fig. 1a). A 4.4 kb *Xho*I–*Bam*HI fragment from pWHM259 containing the 1.55 kb *Sal*I fragment 2 and 1.7 kb *Eco*RI fragment 3 (Fig. 1a) with the *aphII* gene between them was

ligated with KC515 digested with *Bam*HI and *Xho*I to give phWHM262 (Fig. 1b) via plasmids pWHM254 to pWHM259, as described in Table 1.

The third construct, phWHM275, was intended to replace a portion of the second PKS cluster (Fig. 2a) and contained two fragments, 4 and 5, located 1.8 kb from each other in the gene cluster defined by cosmid pAL32 (Fig. 2a) and separated by the *aph*II gene. A 3.7 kb *Pst*I–*Sst*I fragment from pWHM285 containing 1.2 kb *Pst*I–*Xba*I PCR fragment 4 (Fig. 2a), the *aph*II gene and 1.4 kb *Kpn*I–*Apa*I fragment 5 (Fig. 2a) was ligated with KC515 digested with *Pst*I and *Sst*I to give phWHM275 (Fig. 2b) via plasmids pWHM281 to pWHM285, as described in Table 1.

The fourth construct, phWHM276, contained the 1.68 kb *Bam*HI–*Xba*I fragment 6 and 1.33 kb *Xba*I–*Pst*I fragment 7 (Fig. 3a), located 78.4 kb apart in the cluster of rapamycin biosynthesis genes (Schwecke *et al.*, 1995). The *aph*II gene was inserted between them to give the 4.1 kb *Bam*HI–*Pst*I fragment cloned in pWHM288, through the intermediate plasmids pWHM286 to pWHM288 (Table 1). This *Bam*HI–*Pst*I segment was moved into KC515, resulting in phWHM276 (Fig. 3b) by replacement of the 1.3 kb *Bam*HI–*Pst*I fragment of KC515 that contains the *vph* gene. phWHM276 enabled the isolation of neomycin-resistant *S. hygroscopicus* clones as a result of double crossover recombination, to produce a 78.4 kb deletion eliminating all of *rapA* and *rapP* and most of *rapB* and *rapC* (Fig. 3a).

Protoplasts of *S. lividans* TK24 were transfected with each of the phage constructs and plaques were observed after overnight incubation. The four different recombinant phages containing DNA cloned from pAL58, pAL32 or *S. hygroscopicus* (Table 1) were isolated by a convenient spot-test method we had developed (Lomovskaya *et al.*, 1996). Single plaques were transferred to 0.2 ml Difco nutrient broth and 10 µl from each sample was pipetted as spots on plates overlaid with soft R2YE agar containing a mixture of *S. lividans* TK24 and *S. lividans* (ϕC31) lysogen spores. After 17 h incubation at 30 °C the plates were overlaid with soft agar containing neomycin. Infection with phages carrying the *aph*II gene resulted in spots of neomycin-resistant growth after another overnight incubation at 30 °C. The recombinant phages were purified through a second round of the spot-test and the presence of the *aph*II and *vph* or *tsr* resistance markers was confirmed. High-titre phage stocks were then prepared and tested for the presence of the selectable resistance markers *tsr*, *vph* and *aph*II by the spot-test.

The recombinant phages were screened by selecting for neomycin (*aph*II) resistance with neomycin (10 µg ml^{−1}) added to R2YE growth medium. The presence or absence of the *aph*II, viomycin (*vph*) or thiostrepton (*tsr*) resistance genes in the phage vector was tested by adding neomycin (10 µg ml^{−1}), viomycin (200 µg ml^{−1}) or thiostrepton (30 µg ml^{−1}) to R2YE medium. In this way the recombinant phages phWHM261 and phWHM262 (Fig. 1b) were characterized as containing *aph*II and *vph* resistance genes, and phages phWHM275 (Fig. 2b) and pWHM276 (Fig. 3b) as containing *aph*II and *tsr* resistance genes. The presence of the cloned DNA was confirmed by restriction endonuclease digestion analysis.

Screening of *S. hygroscopicus* recombinant clones. *S. hygroscopicus* was infected with recombinant phages on supplemented MOM agar plates (5 × 10⁷ spores and 1 to 2 × 10⁸ phage) to allow phage multiplication and host sporulation. After 16 h the plates were overlaid with an aqueous neomycin solution to give a final concentration of 10 µg ml^{−1},

then after further growth for 7 d until sporulation, the clones were replica-plated on MM containing neomycin. Primary neomycin-resistant clones were isolated and their phenotype was determined after a second round of single colony isolation. Selection for phage vector integration or gene replacement was carried out on MM with neomycin (10 µg ml^{−1}), viomycin (30 µg ml^{−1}) or thiostrepton (30 µg ml^{−1}).

Bioassays for rapamycin and nigericin production. The agar plug method was used to assay recombinant clones growing on MOM agar medium, using *A. niger* grown on Bacto dextrose agar and *S. aureus* grown on Bacto antibiotic medium 1 (Difco). To assay for rapamycin and nigericin specifically, MOM agar with 7-d-old *S. hygroscopicus* cultures was steeped in 0.5 vol. acetone for 2 h at room temperature. The resulting leachate was extracted with 0.5 vol. isopropyl acetate for 2 h and concentrated *in vacuo*. Rapamycin and nigericin were detected by TLC on silica gel using ethyl acetate/methanol/water (100:2.5:1, by vol.) as a solvent system, and visualized by spraying the air-dried plates with a solution containing, per litre, ceric sulfate, 10 g; ammonium molybdate, 25 g; concentrated H₂SO₄, 100 ml; water, to 1 l. Heating at 105 °C for 2 min developed the coloration. Alternatively, the dried TLC plates were bioassayed by overlaying them with Bacto potato dextrose agar or Bacto antibiotic medium 1 (Difco) containing *Aspergillus niger* spores or a *Staphylococcus aureus* overnight culture, then incubated overnight at 33 °C or 37 °C, respectively. TLC plates were examined for nigericin by a red blood cell lysis bioassay developed for the identification of polyether antibiotics (Ray Yao, Eli Lilly and Company, personal communication); in this assay, plates were overlaid with NaCl agar (0.85% NaCl and 1.5% agar) containing defibrinated horse blood (Colorado Serum Company) and incubated overnight at 37 °C to develop the zones of cell lysis.

RESULTS AND DISCUSSION

Interaction of ϕC31 and its derivatives with *S. hygroscopicus*

Wild-type ϕC31 phage infected *S. hygroscopicus* and formed plaques on a *S. hygroscopicus* lawn. A small reduction in efficiency of plating compared to *S. lividans* was observed when the phage was assayed on *S. hygroscopicus* and this did not depend on whether *S. lividans* TK24 or *S. hygroscopicus* was the previous host. In contrast, the attP⁺ ϕC31 derivatives ϕC31R3 and ϕC31R8 (Chater *et al.*, 1986) as well as attP ϕC31 derivatives KC400, KC500 and KC515 (Chater, 1986) did not give visible plaques on *S. hygroscopicus*.

Additional evidence of infection by these vectors was obtained by demonstrating the formation of *S. hygroscopicus* lysogens. After infection with ϕC31R3 attP⁺:*tsr*:*vph* or ϕC31R8 attP⁺:*tsr*, lysogens resistant to thiostrepton and viomycin (WMH1591) or thiostrepton alone (WMH1592) were obtained, respectively. The WMH1591 and WMH1592 lysogens released ϕC31R3 or ϕC31R8 phages at a rate of 3.7 × 10^{−6} to 8.3 × 10^{−7} as a result of spontaneous induction. When a WMH1592 lysogen was superinfected with the KC515 attP phage, double lysogens (WMH1593 is a representative) were obtained as a result of homologous recombination between the resident prophage and super-infecting phage.

Table 1. Bacterial strains, plasmids and phages

Strain, plasmid or phage	Genotype	Source*
Strains		
<i>S. hygroscopicus</i>		
ATCC 29253	Wild-type	ATCC
WMH1591	ϕ C31R3 $c^{+}attP^{+}::tsr::vph$ lysogen	
WMH1592	ϕ C31R8 $c^{+}attP^{+}::tsr$ lysogen	
WMH1593	ϕ C31R8 $c^{+}attP^{+}::tsr$ and ϕ C31KC515 $c^{+}\Delta attP::tsr::vph$ double lysogen	
WMH1597	<i>aphII vph</i> ; obtained after infection with phWHM261	
WMH1598	<i>aphII</i> ; obtained after infection with phWHM262	
WHM1651 to WHM1653	<i>aphII</i> ; obtained after infection with phWHM276	
<i>S. lividans</i>		
TK24	SLP1 ⁻ SLP2 ⁻ , <i>str</i>	Hopwood <i>et al.</i> (1983)
WMH1594	SLP1 ⁻ SLP2 ⁻ , <i>str</i> , ϕ C31 lysogen	
<i>Staphylococcus aureus</i>		
WMH1641	Wild-type	University of Wisconsin
<i>Aspergillus niger</i>	Wild-type	Tom Leonard, Madison, WI
Plasmids		
pFDNeoS	pUC18 containing the <i>aphII</i> gene	Denis & Brzezinski (1991)
pAL58	Cosmid 58 containing a 37 kb fragment of the <i>S. hygroscopicus</i> chromosome	
pAL4.2E	A 4.2 kb <i>EcoRI</i> fragment cloned from pAL58 into pUC18	
pWHM249	<i>XhoI</i> site eliminated from <i>aphII</i> gene of pFDNeoS	
pWHM252	A 1.1 kb <i>XhoI</i> – <i>KpnI</i> fragment cloned from pAL4.2E in pGEM7Zf(–)	
pWHM253	A 1.1 kb <i>BamHI</i> – <i>HindIII</i> fragment with <i>aphII</i> gene cloned from pWHM249 in pWHM252	
pWHM254	A 1.7 kb <i>EcoRI</i> fragment cloned from pAL58 in pGEM9Zf(–)	
pWHM255	<i>BamHI</i> site eliminated from pAL1.7E	
pWHM256	A 1.7 kb <i>EcoRI</i> fragment as <i>HindIII</i> – <i>XhoI</i> fragment cloned from pWHM255 in pGEM7Zf(–)	
pWHM257	A 1.55 kb <i>SalI</i> fragment cloned from pAL4.2E into pUC18	
pWHM258	A 1.55 kb <i>SalI</i> fragment as <i>BamHI</i> – <i>HindIII</i> fragment cloned from pWHM257 into pWHM256	
pWHM259	A 1.1 kb <i>BamHI</i> – <i>HindIII</i> fragment cloned blunt-ended from pWHM249 in the filled-in <i>HindIII</i> site of pWHM258	
pAL32	Cosmid 32 containing a 38 kb fragment of the <i>S. hygroscopicus</i> chromosome	
pWHM281	A 1.2 kb <i>PstI</i> – <i>XbaI</i> PCR fragment cloned from <i>S. hygroscopicus</i> chromosome into pSP72 with eliminated <i>SstI</i> site	
pWHM282	A 1.2 kb <i>PstI</i> – <i>XbaI</i> PCR fragment with eliminated <i>SstI</i> site cloned into pSP72	
pWHM283	A 1.8 kb <i>KpnI</i> – <i>PstI</i> fragment cloned from pAL32 into pSP72	
pWHM284	A 1.4 kb <i>KpnI</i> – <i>ApaI</i> fragment cloned from pWHM283 into pGEM7Zf(–), containing <i>aphII</i> gene between <i>SmaI</i> and <i>HindIII</i> sites	
pWHM285	A 2.5 kb <i>ApaI</i> – <i>PstI</i> fragment cloned from pWHM284 blunt-ended in filled-in <i>KpnI</i> site of pWHM282	
pWHM286	A 1.68 kb <i>BamHI</i> – <i>XbaI</i> PCR fragment cloned from <i>S. hygroscopicus</i> chromosome into pSP72	
pWHM287	A 1.33 kb <i>XbaI</i> – <i>PstI</i> PCR fragment cloned from <i>S. hygroscopicus</i> chromosome into pWHM286	
pWHM288	A 1.1 kb <i>SalI</i> –fragment containing <i>aphII</i> gene cloned from pWHM249 blunt-ended in filled-in <i>NruI</i> site of pWHM287	

Table 1. cont.

Strain, plasmid or phage	Genotype	Source
Phages		
φC31	Wild-type, c^+attP^+	Lomovskaya <i>et al.</i> (1972)
KC400	$c^+ΔattP::vph$	Chater (1986)
KC500	$c^+ΔattP::tsr::vph$	Chater (1986)
KC515	$c^+ΔattP::tsr::vph$	Chater (1986)
φC31R3	$c^+attP^+::tsr::vph$	Chater <i>et al.</i> (1986)
φC31R8	$c^+attP^+::tsr$	Chater <i>et al.</i> (1986)
phWHM261	$c^+ΔattP::vph::2.2$ kb <i>XhoI</i> – <i>Bam</i> HI fragment:: <i>aphII</i> ; a 2.2 kb <i>XhoI</i> – <i>Bam</i> HI fragment cloned from pWHM253 in KC515	
phWHM262	$c^+ΔattP::vph::4.35$ kb <i>XhoI</i> – <i>Bam</i> HI fragment:: <i>aphII</i> ; a 4.35 kb <i>XhoI</i> – <i>Bam</i> HI fragment from pWHM259 cloned in KC515	
phWHM275	$c^+ΔattP::tsr::3.7$ kb <i>PstI</i> – <i>SstI</i> fragment:: <i>aphII</i> ; a 3.7 kb <i>PstI</i> – <i>SstI</i> fragment from pWHM285 cloned in KC515	
phWHM276	$c^+ΔattP::tsr::4.1$ kb <i>Bam</i> HI– <i>PstI</i> fragment:: <i>aphII</i> ; a 4.1 kb <i>Bam</i> HI– <i>PstI</i> fragment from pWHM288 cloned in KC515	

* Where no source or reference is listed, strains, plasmids and phages were derived in this study.

Fig. 1. (a) Organization of a gene cluster for the first modular PKS of unknown function in *S. hygroscopicus* ATCC 29253. The direction of gene expression and relative sizes of the ORFs are indicated by arrows. The three segments of the region in pAL58 that were used for cloning into the KC515 vector are shown beneath a restriction map showing only the sites of interest. (b) Structure of KC515 and the two phages constructed from it using segments F1, F2 and F3. $ΔattP$ and *cos* indicate the relative locations of the deletion in the attachment site and the cohesive ends of φC31; *aphII*, *tsr* and *vph* are defined in the text. (c) Southern blot hybridization analysis of the genomic DNA from the WMH1598 recombinant clone resulting from infection with phWHM262. WMH1598 DNA was digested with *KpnI*, *EcoO109I* or *XhoI* + *EcoRI* and probed with a fragment containing the *aphII* gene.

Characterization of *S. hygroscopicus* recombinant clones after infection with KC515 derivatives

Since the chemical structures of rapamycin and nigericin are consistent with the involvement of a modular PKS in their biosynthesis, DNA segments from the rapamycin PKS genes (Fig. 3a) (Schwecke *et al.*, 1995) and two other regions found to contain putative modular PKSs (Figs 1a and 2a) (X. Ruan, D. L. Stassi, N. Lomovskaya, L. Fonstein, C. R. Hutchinson & L. Katz, unpublished)

were cloned into the *attP* phage vector KC515 to perform gene-disruption and gene-replacement experiments. KC515 can transduce a host strain to drug resistance only when it carries an insert capable of homologous recombination with the host DNA.

The function of the genes encoding four modules of a putative type I PKS shown in Fig. 1(a) was examined first, by infection with phWHM261 and phWHM262. As expected, phWHM261 produced only clones re-

Fig. 2. (a) Organization of a gene cluster for the second modular PKS of unknown function in *S. hygroscopicus* ATCC 29253 together with the putative ferredoxin (Fd) and cytochrome P450 genes. The direction of PKS gene expression is indicated by an arrow. The two segments of the region in pAL32 that were used for cloning into KC515 are shown beneath a restriction map showing only the sites of interest. (b) Structure of the phWHM275 phage constructed from segments F4 and F5. All other abbreviations are as in Fig. 1.

Fig. 3. (a) Organization of the *rapABCP* PKS and *rapT* ketoreductase-dehydrogenase genes for rapamycin biosynthesis according to Schwecke *et al.* (1995). The direction of gene expression and relative sizes of the ORFs are indicated by arrows. The two segments of the region used to construct phWHM276 are shown beneath the ORFs that contain them and their ends are numbered according to the GenBank sequence file X86780. These two segments are not drawn to scale. (b) Structure of the phWHM276 phage constructed from segments F6 and F7. All other abbreviations are as in Fig. 1.

Table 2. Clones obtained after infection of *S. hygroscopicus* with recombinant phages

Recombinant phage	Phenotype of clones	No. of clones
phWHM261	Neomycin + viomycin resistant	111
	Neomycin resistant + viomycin sensitive	0
phWHM262	Neomycin + viomycin resistant	310
	Neomycin resistant + viomycin sensitive	91
phWHM275	Neomycin + thiostrepton resistant	168
	Neomycin resistant + thiostrepton sensitive	44
phWHM276	Neomycin + thiostrepton resistant	188
	Neomycin resistant + thiostrepton sensitive	30

sistant to both neomycin and viomycin due to the result of a single crossover recombination (Table 2). The presence of the integrated prophage had no obvious effect on morphological development and sporulation. After infection with phWHM262, two types of recombinant clones were obtained as a result of a single (neomycin/viomycin-resistant clones) or double (neomycin-resistant clones) crossover recombination (Table 2).

Southern analysis of the genomic DNA from representative neomycin-resistant WMH1598 (from phWHM262) as well as neomycin- and viomycin-

resistant WMH1597 (from phWHM261) strains was performed to confirm recombination between the cloned fragments and genomic DNA. The 1.1 kb *Pst*I–*Bam*HI fragment of pFDNeoS containing the *aphII* gene was used as a probe. When *Xho*I–*Nco*I (not shown), *Eco*RI–*Xho*I, *Kpn*I or *Eco*0109I-digested DNA from the WMH1598 strain was analysed, 3.25 kb *Xho*I–*Nco*I (not shown), 3.6 kb *Eco*RI–*Xho*I, 2.5 kb *Kpn*I and 3.4 kb *Eco*0109I fragments were detected with the *aphII* probe (Fig. 1c), as expected, confirming the presence of a 10.9 kb deletion between the *Sal*I and *Eco*RI sites as a result of a double crossover. This result indicated that replacement of the 14.1 kb fragment containing ORF2

Fig. 4. Biological activity of *S. hygroscopicus* recombinant clones obtained after infection with the phWHM276 phage. (a) 1, Recombinant clones resistant to neomycin did not produce zones of inhibition whereas ones resistant to neomycin and thiostrepton did when they were tested by the agar plug method against *Aspergillus niger*. 2, *S. hygroscopicus* ATCC 29253 control (first four plugs). (b) Bioautography of culture extracts for the wild-type strain and neomycin-resistant *S. hygroscopicus* clones with *A. niger*. Lane 1, rapamycin standard; lanes 2 and 6, *S. hygroscopicus* ATCC 29253; lanes 3 to 5, neomycin-resistant *S. hygroscopicus* WMH1651, WMH1652 and WMH1653, respectively.

and part of *ORF1* with the insert cloned in phWHM262 had taken place in the WMH1598 chromosome. Disruption of *ORF1* was demonstrated when *EcoRI*–*Bam*HI-, *Bgl*II- or *Mlu*I–*Bam*HI-digested genomic DNA from the WMH1597 strain was analysed. Since hybridizing bands of 3.0, 3.4 and 2.7 kb were identified (data not shown), the WMH1597 strain was produced as a result of single crossover between the cloned fragment and recipient chromosome within the 1.1 kb *Xho*I–*Kpn*I region of Fig. 1(a).

Similarly, the function of another set of *S. hygroscopicus* genes encoding a putative modular PKS plus a ferredoxin and a cytochrome P450 hydroxylase, shown in Fig. 2(a),

were examined by a gene replacement experiment. The genotype of the recombinant strains obtained with phWHM275 was not analysed, but the data above for the two other sets of recombinants suggest on the basis of the phenotypes given in Table 2 that part of the beginning of the PKS gene and all of the ferredoxin and P450 genes should have been deleted in these strains.

Since the *rapABCP* genes were proposed to govern assembly of the macrolide ring of rapamycin (Schwecke *et al.*, 1995), phage phWHM276 was designed for deletion of the *rapAP* genes and most of *rapB* and *rapC* (Fig. 3a). Following infection of *S. hygroscopicus* with phWHM276, clones resistant to neomycin as well as ones resistant to neomycin and thiostrepton were isolated (Table 2). PCR analysis of the chromosomal DNA from the representative WHM1651 and WHM1652 neomycin-resistant strains confirmed replacement of the 81.4 kb fragment containing the *rap* PKS genes with the 4.1 kb insert cloned in phWHM276 since the expected 1.5 kb fragment containing a portion of the 3' end of *rapB*, *aphII* and the 3' end of *rapC* was found and had the expected restriction map (data not shown).

Biological activity of the recombinant clones

Representatives of the recombinant clones obtained from the above three sets of experiments were tested by the agar plug method for the presence of biological activity against *Aspergillus niger* (rapamycin production) and *Staphylococcus aureus* (nigericin production). All the clones obtained from phWHM276 (Fig. 3b) that were resistant to neomycin and 18 clones resistant to neomycin and thiostrepton were tested by the agar plug method for rapamycin production. The neomycin-resistant clones did not produce zones of inhibition but the clones resistant to both antibiotics were active against *A. niger* (Fig. 4a). Specific loss of rapamycin (Fig. 4b) but not nigericin production was established by bioautography of three representative culture extracts (data not shown). In contrast, all of the clones with disruptions or deletions in the two other PKS regions (Figs 1a and 2a) produced zones of inhibition of the two indicator strains, which suggested that the clones still made rapamycin and nigericin. This was confirmed by bioautography of culture extracts only for those clones whose genotype had been verified by Southern analysis; all clones exhibited antibiotic activity comparable to that of rapamycin against *A. niger*, as well as activity against *S. aureus* or red blood cells lysis corresponding to nigericin.

Specific deletion of the PKS predicted to be involved in rapamycin biosynthesis resulted in the loss of rapamycin production. In contrast, disruption or deletion of either of the two other PKS regions had no effect on the production of rapamycin or nigericin, a polyether antibiotic also produced by *S. hygroscopicus*, but it is not clear whether the production of some other compound was lost. The method described here for introducing DNA into ATCC 29253 should allow exploration

of the nature of the metabolites produced by the two other gene clusters as well as the possibility of manufacturing novel rapamycins.

Development of an effective system based on ϕ C31 for introducing DNA into *S. hygroscopicus* will greatly facilitate further characterization of the rapamycin biosynthesis genes (Schwecke *et al.*, 1995) and attempts to modify them for the production of novel forms of this important immunosuppressive agent. Despite the absence of a visible lytic response to phage infection, single and double lysogens of *S. hygroscopicus* ATCC 29253 were produced, and KC515 derivatives were shown to undergo homologous recombination via mechanisms resulting in gene disruption or replacement. Therefore, KC515 and other ϕ C31 derivatives will be useful for targeted mutation of the genes governing the biosynthesis of rapamycin and nigericin. These vectors can also be used in further investigations of the two other regions containing modular PKS genes, since current data suggest that these genes may be responsible for novel metabolites. Moreover, the results of our work support the wisdom of considering ϕ C31 derivatives for gene cloning and mutation in other *Streptomyces* spp. because of the wide host range and versatility of these vectors (Lomovskaya *et al.*, 1972, 1980; Chater, 1986; Hopwood *et al.*, 1987).

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REFERENCES

- Aparico, J. F., Molnar, I., Schwecke, T., Konig, A., Haydock, S. F., Khaw, L. E., Staunton, J. & Leadlay, P. F. (1996). Organization of the biosynthetic gene cluster for rapamycin in *Streptomyces hygroscopicus*: analysis of the enzymatic domains in the modular polyketide synthase. *Gene* **169**, 9–16.
- Buttner, M. J. & Lewis, C. G. (1992). Construction and characterization of *Streptomyces coelicolor* A3(2) mutants that are deficient in the nonessential *hrd*-encoded RNA polymerase sigma factor. *J Bacteriol* **174**, 5165–5167.
- Buttner, M. J., Chater, K. F. & Bibb, M. J. (1990). Cloning, disruption, and transcriptional analysis of three RNA polymerase sigma factor genes of *Streptomyces coelicolor* A3(2). *J Bacteriol* **172**, 3367–3378.
- Chakraborty, R., White, J., Takano, E. & Bibb, M. J. (1996). Cloning, characterization and disruption of a (p)ppGpp synthetase gene (*relA*) of *Streptomyces coelicolor* A3(2). *Mol Microbiol* **19**, 357–368.
- Chater, K. F. (1986). *Streptomyces* phages and their application to *Streptomyces* genetics. In *The Bacteria*, vol. IX, *Antibiotic producing Streptomyces*, pp. 119–158. Edited by S. E. Queener & L. E. Day. Orlando, FL: Academic Press.
- Chater, K. F. & Bruton, C. J. (1983). Mutational cloning in *Streptomyces* and the isolation of antibiotic production genes. *Gene* **26**, 67–78.
- Chater, K. F. & Bruton, C. J. (1985). Resistance, regulatory and production genes for the antibiotic methylenomycin are clustered. *EMBO J* **4**, 1893–1897.
- Chater, K. F., Lomovskaya, N. D., Voeykova, T. A., Sladkova, I. A., Mkrtumian, N. M. & Muravnik, G. L. (1986). *Streptomyces* ϕ C31-like phages: cloning vectors, genome changes and host range. In *Biological, Biochemical and Biomedical Aspects of Actinomycetes. Proceedings of the Sixth International Symposium on Actinomycete Biology*, pp. 45–53. Edited by G. Szabo, S. Biro and M. GoodFellow. Budapest: Academic Kivado.
- Cortes, J., Wiesmann, K. E. H., Roberts, G. A., Brown, M. J. B., Staunton, J. & Leadlay, P. F. (1995). Repositioning of a domain in a modular polyketide synthase to promote specific chain cleavage. *Science* **268**, 1487–1489.
- Denis, F. & Brzezinski, R. (1991). An improved aminoglycoside resistance gene cassette for use in Gram-negative bacteria and *Streptomyces*. *FEMS Microbiol Lett* **81**, 261–264.
- Donadio, S., McAlpine, J. B., Sheldon, P. J., Jackson, M. & Katz, L. (1993). An erythromycin analog produced by reprogramming of polyketide synthesis. *Proc Natl Acad Sci USA* **90**, 7119–7123.
- Dumont, F. J. & Su, Q. (1996). Mechanism of action of the immunosuppressant rapamycin. *Life Sci* **58**, 373–395.
- Hatanaka, H., Kino, T., Miyata, S., Inamura, N., Kuroda, A., Goto, T., Tanaka, H. & Okuhara, M. (1988). FR-900520 and FR-900523, novel immunosuppressants isolated from a *Streptomyces*. II. Fermentation, isolation and physico-chemical and biological characteristics. *J Antibiot* **41**, 1592–1601.
- Hopwood, D. A., Kieser, T., Wright, H. M. & Bibb, M. J. (1983). Plasmids, recombination and chromosomal mapping in *Streptomyces lividans* 66. *J Gen Microbiol* **129**, 2257–2268.
- Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M. & Schempff, H. (1985). *Genetic Manipulation of Streptomyces: a Laboratory Manual*. Norwich: John Innes Foundation.
- Hopwood, D. A., Bibb, M. J., Chater, K. F. & Kieser, T. (1987). Plasmid and phage vectors for gene cloning and analysis in *Streptomyces*. *Methods Enzymol* **153**, 116–165.
- Hutchinson, C. R. (1994). Drug synthesis by genetically engineered microorganisms. *Bio/Technology* **12**, 375–380.
- Hutchinson, C. R. & Fujii, I. (1995). Polyketide synthase gene manipulation: a structure–function approach in engineering novel antibiotics. *Annu Rev Microbiol* **49**, 201–238.
- Kao, C. M., Luo, G., Katz, L., Cane, D. E. & Khosla, C. (1994). Engineered biosynthesis of a triketide lactone from an incomplete modular polyketide synthase. *J Am Chem Soc* **116**, 11612–11613.
- Katz, L. & Donadio, S. (1993). Polyketide synthesis: prospects for hybrid antibiotics. *Annu Rev Microbiol* **47**, 875–912.
- Kino, T., Hatanaka, H., Miyata, S., Inamura, N., Hishiyama, H., Yajima, T., Goto, T., Okuhara, M., Kohsaka, M., Aoki, H. & Ochiai, T. (1987). FK-506, a novel immunosuppressant isolated from a *Streptomyces*. II. Immunosuppressive effect of FK-506 *in vitro*. *J Antibiot* **40**, 1256–1265.
- Lomovskaya, N. D., Mkrtumian, N. M., Gostimskaya, N. C. & Danilenko, V. N. (1972). Characterization of temperate actinophage ϕ C31 isolated from *Streptomyces coelicolor* A3(2). *J Virol* **9**, 258–262.
- Lomovskaya, N. D., Chater, K. F. & Mkrtumian, N. M. (1980). Genetics and molecular biology of *Streptomyces* bacteriophages. *Microbiol Rev* **44**, 206–229.
- Lomovskaya, N., Hong, S.-K., Kim, S.-U., Fonstein, L., Furuya, K. & Hutchinson, C. R. (1996). The *Streptomyces peucetius* *drnC* gene encodes a UvrA-like protein involved in daunorubicin resistance and production. *J Bacteriol* **178**, 3238–3245.

- Melino, M. R. & Sigal, N. H. (1990).** Distinct mechanisms of suppression of murine T cell activation by the related macrolides FK-506 and rapamycin. *J Immunol* **144**, 251–258.
- Mendez, C. & Chater, K. F. (1987).** Cloning of *whiG*, a gene critical for sporulation of *Streptomyces coelicolor* A3(2). *J Bacteriol* **169**, 5715–5720.
- Pahe, A. & Keller, U. (1992).** FK506-binding proteins from streptomycetes producing immunosuppressive macrolactones of the FK-506 type. *J Bacteriol* **174**, 5888–5894.
- Piret, J. M. & Chater, K. F. (1985).** Phage-mediated cloning of *bldA*, a region involved in *Streptomyces coelicolor* morphological development, and its analysis by genetic complementation. *J Bacteriol* **163**, 965–972.
- Sambrook, J., Frisch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*. NY: Cold Spring Harbor Laboratory.
- Schwecke, T., Aparicio, J. F., Molnar, I., Konig, A., Khaw, L. E., Haydock, S. F., Oliynyk, M., Caffrey, P., Cortes, J., Lester, J. B., Bohm, G. A., Staunton, J. & Leadlay, P. F. (1995).** The biosynthesis gene cluster for the polyketide immunosuppressant rapamycin. *Proc Natl Acad Sci USA* **93**, 7839–7843.
- Vezina, C., Kudelski, A. & Sehgal, S. N. (1975).** Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *J Antibiotics* **28**, 721–726.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985).** Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13 mp18 and pUC19 vectors. *Gene* **33**, 103–119.
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