Mutants of *Streptomyces roseosporus* that express enhanced recombination within partially homologous genes

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***Streptomyces roseosporus* mutants that express enhanced recombination between partially homologous (homeologous) sequences were isolated by selection for recombination between the bacteriophage ϕ C31 derivative KC570 containing the *Streptomyces coelicolor* glucose kinase (*glk*) gene and the *S. roseosporus* chromosome. The frequencies of homeologous recombination in the *ehr* mutants were determined by measuring the chromosomal insertion frequencies of plasmids containing *S. coelicolor glnA* or *whiG* genes. *S. roseosporus ehr* mutants showed 10^2 - to 10^4 -fold increases in homeologous recombination relative to *Ehr*⁺ strains, but no increase in homologous recombination. Southern hybridization analysis revealed single unique sites for the insertion of each of the plasmids, and the crossovers occurred in frame and in proper translational register, yielding functional chimeric *glnA* and *whiG* genes.**

Keywords: *Streptomyces roseosporus*, homeologous recombination, transposition mutagenesis, glutamine synthetase, sporulation sigma factor

INTRODUCTION

Homologous recombination by conjugation and by protoplast fusion is well documented in many streptomycetes (Chater & Hopwood, 1983; Baltz & Matsushima, 1983). Homeologous recombination (i.e. between partially homologous sequences) also occurs in different species of *Streptomyces*, but at greatly reduced frequencies relative to homologous recombination (Lomovskaya *et al.*, 1977; Schlegel & Fleck, 1980; Mazieres *et al.*, 1981; Baltz & Matsushima, 1983; Chater & Bruton, 1983; Gomi *et al.*, 1984). Homeologous recombination has been used to generate recombinants to produce novel antibiotics (Schlegel & Fleck, 1980; Gomi *et al.*, 1984), but the low frequencies of recombination have limited the implementation of this methodology in antibiotic discovery.

Homeologous recombination occurs at low frequencies between *Escherichia coli* and *Salmonella typhimurium*, which share about 80 % nucleotide sequence identity. However,

S. typhimurium strains defective in methyl mismatch repair form recombinants by conjugation with *E. coli* at ~ 1000-fold higher frequencies than strains proficient in mismatch repair (Rayssiguier *et al.*, 1989; Matic *et al.*, 1994; Worth *et al.*, 1994). Furthermore, a mutant defective in *uvrD*-encoded helicase activity showed enhancement in homologous and homeologous recombination (Feinstein & Low, 1986; Rayssiguier *et al.*, 1989). Zahrt *et al.* (1994) have also shown that the barrier to homeologous recombination between *S. typhimurium* and *Salmonella typhi* in transductional crosses is mismatch repair. In *Saccharomyces cerevisiae*, mismatch repair also acts as a barrier to homeologous recombination (Selva *et al.*, 1995). Since mismatch repair is observed in other prokaryotes (Claverys & Lacks, 1986; Le *et al.*, 1993), yeast (Reenan & Kolodner, 1992; Selva *et al.*, 1995) and human cells (Fishel *et al.*, 1993), we reasoned that mismatch repair is likely to be expressed by streptomycetes, and that mutation in mismatch repair genes might cause elevated homeologous recombination.

Here we describe the isolation of *Streptomyces roseosporus* mutants that express enhanced homeologous recombination but not enhanced homologous recombination, and show that homeologous recombination occurs within genes in the correct register to produce functional hybrid proteins.

Abbreviations: CTAB, cetyltrimethylammonium bromide; GS, glutamine synthetase.

The GenBank accession numbers for the nucleotide sequences reported in this paper are U57630 (chimeric *glnA*), U57631 (chimeric *whiG*), U58138 (*S. roseosporus glnA*) and U58281 (*S. roseosporus whiG*).

Table 1. Bacterial strains, plasmids and phage

Strain or plasmid	Relevant characteristics	Source
<i>E. coli</i> strains		
S17-1	Contains RP4 integrated in chromosome	Simon <i>et al.</i> (1983)
XL1-blue MFR'	$\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173 \text{ end}A1$ <i>supE44 thi-1 recA1 gyrA96 relA1 lac</i> [F' <i>proAB lacI^qZAM15 Tn10 (Tet^r)</i>]	Stratagene
<i>S. roseosporus</i> strains		
A21978.6	Wild-type, produces daptomycin	ATCC 31568
TH101	A21978.6[chr::Tn5099-10] <i>ehr-1</i>	This work
TH104	A21978.6[chr::Tn5099-10] <i>ehr-2</i>	This work
TH109	A21978.6[chr::Tn5099-10] <i>ehr-3</i>	This work
TH114	A21978.6[chr::Tn5099-10] <i>ehr-5</i>	This work
TH116	A21978.6[chr::Tn5099-10] <i>ehr-6</i>	This work
TH117	A21978.6[chr::Tn5099-10] <i>ehr-7</i>	This work
TH119	A21978.6[chr::Tn5099-10]	This work
TH121	A21978.6[chr::Tn5099-10] <i>ehr-8</i>	This work
TH122	A21978.6[chr::Tn5099-10] <i>ehr-9</i>	This work
TH131	A21978.6[chr::Tn5099-10]	This work
TH134	A21978.6[chr::Tn5099-10]	This work
TH135	A21978.6[chr::Tn5099-10]	This work
TH101G	TH101::pRHB501	This work
TH104W	TH104::pRHB503	This work
TH104G1	TH104::pRHB501	This work
TH104G2	TH104::pRHB501	This work
TH104W1	TH104::pRHB503	This work
TH109G	TH109::pRHB501	This work
TH109W	TH109::pRHB503	This work
TH114G	TH114::pRHB501	This work
TH114W	TH114::pRHB503	This work
TH116W	TH116::pRHB503	This work
TH119G	TH119::pRHB501	This work
TH119W	TH119::pRHB503	This work
TH122G	TH122::pRHB501	This work
TH131G	TH131::pRHB501	This work
TH134W	TH134::pRHB503	This work
TH135W	TH135::pRHB503	This work
TH148	A21978.6 Sm ^R	T. Hosted & R. Baltz, unpublished
TH161	TH184 <i>glnA</i> ::Hm ^R	T. Hosted & R. Baltz, unpublished
TH503D	TH104::pRHB503	This work
TH503E	TH104::pRHB503	This work
TH503F	TH134::pRHB503	This work
SRG	A21978.6::pRHB501	This work
Plasmids/phage		
pBluescript II KS ⁻	Ap ^R , ColE1 <i>rep</i>	Stratagene
pCRII	3.9 kb TA cloning vector	Invitrogen
ϕ KC570	<i>c⁺ $\Delta attP$::tsr::vph::glk</i>	Fisher <i>et al.</i> (1987)
pKC1139	<i>rep^{ts} oriT</i>	Bierman <i>et al.</i> (1992)
pRHB501	8.8 kb, pKC1139 containing <i>S. coelicolor glnA</i>	This work
pRHB503	8.7 kb, pKC1139 containing <i>S. coelicolor whiG</i>	This work
pRHB534	pCRII containing 1.46 kb <i>S. roseosporus glnA</i> PCR fragment cloned in TA cloning site	This work

Table 1. (cont.)

Strain or plasmid	Relevant characteristics	Source
pRHB685	8.0 kb, pKC1139 containing <i>S. roseosporus</i> <i>glnA</i>	This work
pRHB596	4.2 kb derivative of pRHB501 containing chimeric <i>glnA</i>	This work
pRHB597	11.0 kb derivative of pRHB503 containing chimeric <i>whiG</i>	This work
pDELTAI	Tet ^R Ap ^R Kan ^R ColE1 <i>rep</i>	BRL
pLEW3	pIC19H containing the <i>S. coelicolor</i> <i>glnA</i> on a 2.3 kb <i>SacI</i> fragment	Wray & Fisher (1988)
mp18:: <i>whiG</i>	MP18 containing <i>S. coelicolor</i> <i>whiG</i> on a 2.0 kb <i>NcoI</i> fragment	Chater <i>et al.</i> (1989)
pRHB520	pBluescript II KS ⁻ containing 12 kb <i>PstI</i> fragment with Tn5099-10 and flanking DNA from TH111	This work
pRHB521	pDELTAI containing a 12 kb <i>MluI</i> fragment with Tn5099-10 and flanking DNA from TH119	This work
pRHB522	pBluescript II KS ⁻ containing a 6 kb <i>ClaI</i> fragment with Tn5099-10 and flanking DNA from TH129	This work
pRHB523	pBluescript II KS ⁻ containing a 14 kb <i>KpnI</i> fragment with Tn5099-10 and flanking DNA from TH104	This work
pRHB524	pDELTAI containing a 11 kb <i>MluI</i> fragment with Tn5099-10 and flanking DNA from TH122	This work

METHODS

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

Media and growth conditions. *S. roseosporus* strains were grown in CSM liquid medium (30 g Trypticase soy broth, 3 g yeast extract, 2 g MgSO₄, 5 g glucose and 4 g maltose per litre deionized H₂O) at 29 °C or 39 °C and were fragmented into individual colony-forming units by homogenization followed by ultrasonic vibration as described previously (Baltz, 1978). Colony-forming units were determined by plating diluted mycelial fragments in nutrient soft agar overlays (Cox & Baltz, 1984) on B agar (Solenberg & Baltz, 1991), and incubating at 29 °C or 39 °C for 6 d then counting. *E. coli* strains were grown in either TY medium (Sambrook *et al.*, 1989) or Circlegrow medium (Bio101). Antibiotics were added to appropriate media for streptomycete strains at 30 µg ml⁻¹ for apramycin (Am), 200 µg ml⁻¹ for hygromycin (Hm), 50 µg ml⁻¹ for nalidixic acid (Nal) and 50 µg ml⁻¹ for thiostrepton (Ts). For *E. coli* strains, antibiotics were added to agar at 100 µg ml⁻¹ for Am or Hm.

Glutamine synthetase (GS) assay. *S. roseosporus* strains were grown in CSM medium to stationary phase, permeabilized by cetyltrimethylammonium bromide (CTAB) as described by Penyige *et al.* (1994), and GS activity was determined using the γ -glutamyltransferase assay described by Bender *et al.* (1977). One unit of specific activity equals 1 mmol γ -glutamyl-hydroxamate formed min⁻¹ (mg protein)⁻¹ at 37 °C.

DNA techniques. Standard methods were used for plasmid isolation, restriction enzyme digestion, random priming and Southern hybridization analysis (Sambrook *et al.*, 1989). Restriction endonucleases and other enzymes were used according

to the manufacturer's recommendations. DNA fragments used for subcloning and radiolabelling were isolated from 0.8% SeaKem agarose (FMC) gels following electrophoresis using GeneCleanII (Bio101). *S. roseosporus* genomic DNA was isolated as described by Hopwood *et al.* (1985). DNA sequence was determined using a Taq Dye Deoxy Terminator Cycle sequencing kit and a model 373A DNA sequencing system (Applied Biosystems).

Nucleotide sequence and data analysis. Derived amino acid sequences were analysed using the GCG package (version 8.0) (Devereux *et al.*, 1984). Amino acid sequence homology searches were performed using the BLAST network service and non-redundant protein sequence databases (Altschul *et al.*, 1990).

Transformation, electroporation, conjugation, transduction and transposition. Plasmids were introduced into *E. coli* XL1-blue MFR' by electroporation using a BioRad GenePulser electroporator. Electrocompetent cells were prepared as described by Speyer (1990). *E. coli* S17-1 was transformed as described by Sambrook *et al.* (1989). Plasmids pRHB501 and pRHB503 were introduced into *S. roseosporus* strains by conjugation from *E. coli* S17-1. *S. roseosporus* was grown from a frozen (-70 °C) stock in CSM medium for 16 h, and *E. coli* was grown from a 1% (v/v) inoculum in TY broth for 3-5 h at 37 °C. *S. roseosporus* mycelia were homogenized and mated in 1:9, 1:1 and 9:1 ratios (100 µl total volume) with *E. coli* S17-1 containing the appropriate plasmid, and spread on B agar plates using a glass spreader. B agar plates were incubated for 15 h at 29 °C and then overlaid with nutrient soft agar containing Nal and Am to give 50 µg ml⁻¹ and 30 µg ml⁻¹ final bottom agar concentrations, respectively. Transconjugants appearing in 4-6 d were picked, homogenized and grown in CSM medium

containing 50 µg Nal ml⁻¹ and 30 µg Am ml⁻¹. *S. roseosporus* Tn5099-10 transposon libraries were constructed as previously described (Solenberg & Baltz, 1994). A library of *S. roseosporus* Tn5099-10 mutants was grown in CSM medium at 29 °C; 12 ml (~1.2 × 10⁹ cells) were transduced with KC570 at a multiplicity of infection of about 1 and plated on B agar. The plates were overlaid with nutrient soft agar containing Ts to give a final bottom agar concentration of 50 µg ml⁻¹. Ts^R transductants arose in 3–5 d and were patched to B agar plates, homogenized and grown in CSM medium containing 250 µg Ts ml⁻¹ and 200 µg Hm ml⁻¹. Transductants were subsequently grown with Hm selection alone.

Plasmid constructions. Plasmids were constructed in *E. coli* strains as follows. A 2.3 kb HindIII fragment containing the *S. coelicolor glnA* gene from pLEW3 (Wray & Fisher, 1988) was ligated with HindIII-cleaved pKC1139 (Bierman *et al.*, 1992) to give pRHB501. A 2.2 kb EcoRI–HindIII fragment containing *S. coelicolor whiG* from mp18::whiG (Chater *et al.*, 1989) was ligated with pKC1139 digested with EcoRI and HindIII to yield pRHB503. DNA from strain TH104G1 containing pRHB501 inserted in the chromosome was digested with ClaI, and self-ligated. Transformants were selected for Am^R to yield pRHB596. DNA from TH104W1 containing pRHB503 inserted in the chromosome was digested with BglII and self-ligated. Transformants were selected for Am^R to yield pRHB597. A 12 kb PstI DNA fragment from TH111 that hybridized to radiolabelled Tn5099-10 was separated by gel electrophoresis and ligated to PstI-digested pBluescript II KS⁻. Transformants of *E. coli* XL1-blue MFR' containing Tn5099-10 and flanking chromosomal DNA were selected for Hm^R to yield pRHB520. A 12 kb MluI DNA fragment from TH119 that hybridized to radiolabelled Tn5099-10 was isolated by gel electrophoresis and ligated to MluI-digested pDELTAI (BRL). Transformants of *E. coli* XL1-blue MFR' were selected for Hm^R, yielding pRHB521. A 6 kb ClaI DNA fragment from TH129 that hybridized to radiolabelled Tn5099-10 was isolated by gel electrophoresis and ligated to ClaI-digested pBluescript II KS⁻. Transformants of *E. coli* XL1-blue MFR' were selected for Hm^R, yielding pRHB522. A 14 kb KpnI DNA fragment from TH104 that hybridized to radiolabelled Tn5099-10 was isolated by gel electrophoresis and ligated to KpnI-digested pBluescriptII KS⁻. Transformants of *E. coli* XL1-blue MFR' were selected for Hm^R, yielding pRHB523. A 11 kb MluI DNA fragment from TH122 that hybridized to radiolabelled Tn5099-10 was isolated by gel electrophoresis and ligated to MluI-digested pDELTAI (BRL). Transformants of *E. coli* XL1-blue MFR' were selected for Hm^R, yielding pRHB524. A 1.46 kb fragment containing *S. roseosporus glnA* was PCR-amplified from *S. roseosporus* chromosomal DNA using primers PR170 (5' GCTGAGATGCCGCCCACCAC 3') and PR171 (5' ACGCCGCCCGCCTGGAGGTA 3') and ligated to pCRII (Invitrogen) to yield pRHB534. The 1.46 kb EcoRI fragment containing *glnA* was ligated with EcoRI-digested pKC1139 to yield pRHB685.

RESULTS

Selection for *S. roseosporus ehr* mutants

We prepared a Tn5099-10 (Solenberg & Baltz, 1994) transposition mutant library of *S. roseosporus*, introduced the bacteriophage ϕC31 derivative KC570 (Fisher *et al.*, 1987) into the library, and screened for Ts^R transductants.

Since KC570 contains no plasmid replication functions and lacks the ϕC31 attP sequence, it can form recombinants only by inserting into the chromosome. KC570 contains the *gk* gene from *S. coelicolor*, so recombinants might be formed in *S. roseosporus* by homeologous recombination into the *S. roseosporus gk* gene. Several Ts^R transductants were obtained, and Southern analysis of transductants showed a single band that hybridized to the *tsr* gene (data not shown). Subsequently, transductants were grown with Hm selection alone, and hybridization to the *tsr* gene was lost, presumably due to loss of KC570 by recombinational excision and curing. Most of the putative *ehr* mutants contained Tn5099-10 insertions in different sites in the chromosome, and four different confirmed *ehr* mutants (strains TH104, TH119, TH122 and TH129; see below) contained Tn5099-10 insertions in genes unrelated to mismatch repair or to other known recombination functions based upon DNA sequence analysis (data not shown). Thus the *ehr* mutants were probably due to spontaneous point mutations.

Analysis of spontaneous mutation frequencies in *S. roseosporus* mutants

Since *mutL* and *mutS* mutants of *E. coli* and *S. typhimurium* express elevated spontaneous mutation rates, we tested many of the putative *ehr* mutants for spontaneous mutation frequencies to rifamycin resistance (Rif^R) and to streptomycin resistance (Sm^R). Table 2 shows that none of a representative sample of mutants showed spontaneous mutation frequencies that deviated significantly from those of the *S. roseosporus* parent strain. Thus, spontaneous mutability was not associated with the apparent Ehr phenotype.

Analysis of recombination frequencies by plasmid insertions

We constructed two plasmids to directly measure homeologous recombination and one to measure homologous recombination in the *S. roseosporus* mutants. pRHB501 (Fig. 1, Table 1) contains the *S. coelicolor glnA* gene inserted into pKC1139, a plasmid that has temperature sensitive (*ts*) replication functions for streptomycetes and *oriT* for conjugal transfer from *E. coli* to streptomycetes. pRHB685 is similar to pRHB501 except that it has the *S. roseosporus glnA* gene inserted in pKC1139 (Table 1). pRHB503 is also a similar plasmid containing the *S. coelicolor whiG* gene (Fig. 2, Table 1). pRHB501 and pRHB503 were introduced into many different putative *ehr* mutants of *S. roseosporus* by conjugation from *E. coli* S17-1. Transconjugants were grown in CSM medium at 29 °C for 16 h, sonicated, diluted, plated on B agar and incubated at 29 °C and 39 °C. Recombinant frequencies were determined as the ratio of the number of colonies that grew at 39 °C to the number that grew at 29 °C in the presence of Am. Table 2 shows that most of the putative *ehr* mutants gave recombinant frequencies of 10⁻⁵ to 10⁻⁴ with both plasmids. The *S. roseosporus* parental strain gave recombinant frequencies 10⁻⁷ or less. Similarly, two random Tn5099-10 transposition mutants, TH134 and

Table 2. Spontaneous mutation and recombination frequencies in *S. roseosporus* strains

Strain	Mutation frequency		Recombination frequency		
	Rif ^R	Sm ^R	pRHB501	pRHB503	pRHB685*
A21978.6 (wild-type)	5×10^{-8}	1×10^{-8}	$< 1 \times 10^{-7}$	1×10^{-7}	3×10^{-2} ; 4×10^{-2}
TH101	8×10^{-8}	ND	4×10^{-5}	ND	ND
TH104	7×10^{-8}	1×10^{-8}	7×10^{-5}	4×10^{-5}	ND
TH109	2×10^{-8}	ND	6×10^{-5}	6×10^{-5}	ND
TH114	ND	ND	4×10^{-5}	5×10^{-6}	ND
TH116	1×10^{-7}	2×10^{-8}	3×10^{-4}	9×10^{-5}	4×10^{-3} ; 3×10^{-3}
TH117	6×10^{-8}	9×10^{-9}	7×10^{-5}	1×10^{-5}	ND
TH119	9×10^{-9}	ND	2×10^{-4}	1×10^{-2}	ND
TH121	4×10^{-8}	4×10^{-9}	2×10^{-5}	2×10^{-5}	ND
TH122	7×10^{-8}	ND	1×10^{-3}	3×10^{-4}	3×10^{-3} ; 7×10^{-3}
TH131	3×10^{-8}	ND	2×10^{-7}	$< 1 \times 10^{-7}$	ND
TH134	ND	ND	$< 1 \times 10^{-7}$	$< 1 \times 10^{-7}$	2×10^{-2} ; 9×10^{-3}
TH135	ND	ND	$< 1 \times 10^{-7}$	$< 1 \times 10^{-7}$	ND

ND, Not determined.

* Recombination frequencies determined in two separate experiments.

TH135, gave recombinant frequencies of $< 10^{-7}$. Only one of the putative *ehr* mutants, TH131, was not confirmed to give enhanced homeologous recombination by this procedure. Another mutant (TH119) gave an unusually high frequency of recombination with pRHB503, which is probably not due to homeologous recombination (see below). To confirm that the recombinants contained pRHB501 or pRHB503 inserted into the chromosome, Am^R colonies were grown again in CSM broth plus Am at $30 \mu\text{g ml}^{-1}$. DNA was isolated, cleaved with *Hind*III, separated by agarose gel electrophoresis and analysed by Southern hybridization using pRHB501 or pRHB503 as probes. Fig. 1 shows that four individual transconjugants of strains TH101, TH109, TH114, TH119 and TH122 contained identical junction fragments and an internal pKC1139 fragment. Thus, all of the recombinants contained pRHB501 inserted into the same locus, presumably the *glnA* gene. The three rare recombinants obtained with the *ehr*⁺ strains TH131 and *S. roseosporus* A21978.6 gave identical patterns, indicating that recombinants formed in *ehr* mutants are identical to those formed in *ehr*⁺ strains.

Similarly, recombinants formed from strains carrying pRHB503 generally contained pRHB503 inserted into a unique locus, generating identical junction fragments (Fig. 2). Again, the rare recombinants formed in the *ehr*⁺ strains TH134 and TH135 gave the same pattern as the *ehr* mutants. The exception was strain TH119; this gave a totally different pattern, which included the 2.3 kb intact *S. coelicolor whiG* gene. The original strain TH119 was probed with pCZA185, and shown to contain plasmid sequences from the transposon delivery vector (data not shown). DNA sequence analysis of pRHB521, Tn5099-10 and flanking DNA from TH119 also confirmed that

plasmid sequences were present (data not shown). Thus, the high frequency of recombination of pRHB503 in TH119 (10^{-2} ; Table 2) is probably due to homologous recombination across plasmid sequences rather than to homeologous recombination across *whiG* sequences.

We also tested two of the *ehr* mutants and two control strains for homologous recombination within the *S. roseosporus glnA* using plasmid pRHB685. The control strains A21978.6 and TH134 gave homologous recombination frequencies of 1 to 4×10^{-2} , about 10^5 -fold higher than the homeologous recombination frequencies observed with pRHB501 and pRHB503. The *ehr* mutants TH116 and TH122 gave homologous recombination frequencies of 3 to 7×10^{-3} . These values are within an order of magnitude of the homeologous recombination frequencies observed with pRHB501 containing the *S. coelicolor glnA* gene. Interestingly, the homologous recombination frequencies were not enhanced in the *ehr* mutants, but reduced relative to controls. These data demonstrate that the *ehr* mutants are defective in a repair or recombination system that normally interferes specifically with homeologous recombination.

Analysis of crossover junctions in recombinants containing pRHB501 and pRHB503 inserted in the chromosome

Chromosomal DNA from strain TH104G1, containing pRHB501 inserted in the chromosome, was cleaved with *Cla*I, self-ligated and introduced into *E. coli* XL1-blue MFR' by electroporation. Am^R transformants contained plasmid pRHB596, comprised of pRHB501 and DNA flanking the original plasmid insertion site (Fig. 1). Similarly, DNA from strain TH104W1 containing

Fig. 1. Integration of pRHB501 into the *S. roseosporus* *glnA* gene and cloning of a *S. roseosporus* and *S. coelicolor* chimeric *glnA* junction fragment. (a) Map of pRHB501 and the corresponding chromosomal region of *S. roseosporus*. *SC glnA*, *S. coelicolor glnA*; *SR glnA*, *S. roseosporus glnA*; *rep^{pUC}*, pUC18 origin of replication; *rep^{ts}*, streptomycin temperature-sensitive origin of replication; *oriT*, conjugation origin of transfer; *Am^R*, apramycin resistance; arrows indicate the direction of transcription. (b) Map of the crossover of pRHB501 into the *S. coelicolor* chromosome. (c) Junction fragments and pKC1139 fragment generated by digestion with *Hind*III (not to scale). (d) Map of pRHB596 containing a *S. coelicolor* and *S. roseosporus* chimeric *glnA* gene prior to self-ligation. (e) Southern hybridization analysis of pRHB501 insertions into the *S. roseosporus* chromosome. The strains designated G1, G2, etc., represent independent insertions of pRHB501 in the parent strains (see Table 1). Chromosomal DNA was cleaved with *Hind*III, separated by agarose gel electrophoresis, blotted, and hybridized with radiolabelled pRHB501. Arrows indicate two crossover junction fragments (J1, J2), and a pKC1139 fragment.

pRHB503 was cleaved with *Bcl*II, self-ligated, and introduced into *E. coli* XL1-blue MFR' by electroporation. *Am^R* transformants contained plasmid pRHB597, comprised of pRHB503 and DNA flanking the insert (Fig. 2).

Portions of the putative recombinant *glnA* and *whiG* genes were sequenced, and compared to the *glnA* and *whiG* sequences of *S. coelicolor*. Fig. 3(a) shows that the *glnA* gene present in plasmid pRHB596 is chimeric. The left end of the chimeric gene from position 123 to 281 has 100% identity to the *S. roseosporus* sequence and 87% identity to the *S. coelicolor* sequence, whereas the right end from position 270 to 372 has 92% identity to the *S.*

roseosporus sequence and 100% identity to the *S. coelicolor* sequence. This indicates the crossover of pRHB596 into the chromosome occurred between positions 1198 and 2008 within the *S. roseosporus glnA* gene. These data confirmed that the crossover in strain TH104G1 occurred within the *glnA* structural gene, in frame, and in translational register (Fig. 3b) with the *glnA* gene of *S. coelicolor*.

Fig. 4(a) shows the sequence analysis of the chimeric *whiG* gene in plasmid pRHB597. In this case, there are two areas of 100% identity to the *S. coelicolor* sequence from positions 201 to 266 and 418 to 552 of the chimeric

Fig. 2. Integration of pRHB503 into the *S. roseosporus* *whiG* gene and cloning of the *S. roseosporus* and *S. coelicolor* chimeric *whiG* junction fragment. (a) Map of pRHB503 and the corresponding chromosomal region of *S. roseosporus*. SC *whiG*, *S. coelicolor whiG*; SR *whiG*, *S. roseosporus whiG*; other details as for Fig. 1(a). (b) Map of the crossover of pRHB503 into the *S. coelicolor* chromosome. (c) Junction fragments and pKC1139 fragment generated by digestion with *HindIII* and *EcoRI* (not to scale). (d) Map of pRHB597 containing *S. coelicolor* and *S. roseosporus* chimeric *whiG* prior to self-ligation. (e) Southern hybridization analysis of pRHB503 insertions into the *S. roseosporus* chromosome. The strains designated W1, W2, etc., represent independent insertions into the parent strains (see Table 1). Chromosomal DNA was cleaved with *EcoRI* and *HindIII*, separated by agarose gel electrophoresis, blotted, and hybridized with radiolabelled pRHB503. Arrows indicate two crossover junction fragments (J1, J2), a pKC1139 fragment, and a *whiG* fragment from a crossover in a pKC1139 sequence rather than in a *whiG* sequence in strain TH119W.

sequence. There are also two areas of 100% identity to the *S. roseosporus* sequence from positions 253 to 427 and 447 to 550. Similar to the above results with pRHB596, each region with 100% identity to either *S. roseosporus* or *S. coelicolor* shows less than 100% identity to the alternate sequence. These results indicate that the chimeric *whiG* gene was generated by three crossover events within a span of about 200 base pairs. As with the chimeric *glnA* gene, these recombinations occurred in frame and in proper translation register (Fig. 4b).

Functional analysis of the chimeric *glnA* and *whiG* gene(s)

Streptomyces express two glutamine synthetase (GS) activities, GSI and GSII (Behrmann *et al.*, 1990; Kumada

et al., 1990). GSI activity, encoded by the *glnA* gene (Fisher & Wray, 1989; Wray & Fisher, 1988), is heat stable, whereas GSII activity is heat labile.

To determine if chimeric *glnA* gene(s) generated by homeologous recombination are functional, we carried out GS assays with CTAB-permeabilized *S. roseosporus* strains before and after heat treatment. Table 3 shows that the *ehr* mutant TH104 and a random Tn5099 insertion mutant TH131 express primarily heat-stable GS activity. *S. roseosporus* TH104G1 and TH104G2, each containing two copies of chimeric *glnA* genes, expressed about twice as much GS activity as TH104 or TH131 before heat treatment, and comparable activities after heat treatment. Since a substantial amount of GS activity was retained after heat treatment, at least one of the chimeric *glnA*

(a)

GlnAX	GGACAAGAACGGCTCCAGGTCGAGCGGCAGCACCAGAGGTGGGCACGG	172
SrglnA	GGACAAGAACGGCTCCAGGTCGAGCGGCAGCACCAGAGGTGGGCACGG	1099
ScglnA	GGAGCGGTCGCGCTCCAGGTCGAGCGGCAGCACCAGAGGTGGGCACGG	1382
GlnAX	CGGGCCAGGGGAGATCAACTACAAGTTCAACACGCTGCTCGCCGCGGCC	222
SrglnA	CGGGCCAGGGGAGATCAACTACAAGTTCAACACGCTGCTCGCCGCGGCC	1149
ScglnA	CGGGCCAGGGGAGATCAACTACAAGTTCAACACGCTGCTCGCCGCGGCC	1432
GlnAX	GACGACCTGATGCTCTTCAAGTACATCGTGAAGAAGCTCGGCTGGCGCAA	272
SrglnA	GACGACCTGATGCTCTTCAAGTACATCGTGAAGAAGCTCGGCTGGCGCAA	1199
ScglnA	GACGACCTGATGCTCTTCAAGTACATCGTGAAGAAGCTCGGCTGGCGCAA	1482
GlnAX	CGGCAAGACGGCGACCTTCATGCGGAAGCCGATCTTCGGTGACAACGGCT	322
SrglnA	CGGCAAGACGGCGACCTTCATGCGGAAGCCGATCTTCGGTGACAACGGCT	1249
ScglnA	CGGCAAGACGGCGACCTTCATGCGGAAGCCGATCTTCGGTGACAACGGCT	1532
GlnAX	CGGGCATGACAGTCCACGAGTCGCTGTGGTGGGGCGGAGCGCGCTCTTC	372
SrglnA	CGGGCATGACAGTCCACGAGTCGCTGTGGTGGGGCGGAGCGCGCTCTTC	1299
ScglnA	CGGGCATGACAGTCCACGAGTCGCTGTGGTGGGGCGGAGCGCGCTCTTC	1582

(b)

GSX	DKNGLOVEROHHEVGTAGQAEINXKFNLLAAADDLMFKYIVKNVAVRN	
SRGS	DKNGLOVEROHHEVGTAGQAEINXKFNLLAAADDLMFKYIVKNVAVRN	246
SCGS	ERSGLOVEROHHEVGTAGQAEINXKFNLLAAADDLMFKYIVKNVAVRN	246
GSX	GKTATFMPKPIFGDNGSGMHVHQSLSWGGEPLF	
SRGS	GKTATFMPKPIFGDNGSGMHVHQSLSWGGEPLF	280
SCGS	GKTATFMPKPIFGDNGSGMHVHQSLSWGGEPLF	280

Fig. 3. Analysis of a segment of a chimeric *glnA* gene in pRHB596. (a) Homology between the nucleotide sequence of the chimeric *glnA* gene from pRHB596 (GlnAX) and the corresponding *glnA* sequence of *S. roseosporus* (SrglnA) and *S. coelicolor* (ScglnA). (b) Homology between the predicted amino acid sequence of the chimeric GS (GSX) and the corresponding amino acid sequence of *S. roseosporus* GS (SRGS) and *S. coelicolor* GS (SCGS). Nucleotide and amino acids with shading indicate identity with the corresponding chimeric sequence. Nucleotide and amino acid positions are indicated to the right of the corresponding sequences.

(a)

whiGX	GGGCGGATGATCGACGAACTCGGGGCGCTGGACTGGATCCCGGGTCCG	250
SrwhiG	GGGCGGATGATCGACGAACTCGGGGCGCTGGACTGGATCCCGGGTCCG	605
ScwhiG	GGGCGGATGATCGACGAACTCGGGGCGCTGGACTGGATCCCGGGTCCG	532
whiGX	TGCGGCAGAGGCGCGGAACGTGGAGCGCGGTACCGCCAGGCTGGAGGCA	300
SrwhiG	TGCGGCAGAGGCGCGGAACGTGGAGCGCGGTACCGCCAGGCTGGAGGCA	655
ScwhiG	TGCGGCAGAGGCGCGGAACGTGGAGCGCGGTACCGCCAGGCTGGAGGCA	582
whiGX	CAGTTCGGCGGTACGCCATCGGAGCGCGAGGTCGGCTCGGAGATGGGCAT	350
SrwhiG	CAGTTCGGCGGTACGCCATCGGAGCGCGAGGTCGGCTCGGAGATGGGCAT	705
ScwhiG	CAGTTCGGCGGTACGCCATCGGAGCGCGAGGTCGGCTCGGAGATGGGCAT	632
whiGX	CGCGCTGGAGGAAGTGCACGCGCTTTTCAGTCAGTTGTGCTGGCGAAGC	400
SrwhiG	CGCGCTGGAGGAAGTGCACGCGCTTTTCAGTCAGTTGTGCTGGCGAAGC	755
ScwhiG	CGCGCTGGAGGAAGTGCACGCGCTTTTCAGTCAGTTGTGCTGGCGAAGC	682
whiGX	TGTCGCCCTGGAGGAGTGTGTCACGCGGGCGCGGAGGGCGGGCGCGG	450
SrwhiG	TGTCGCCCTGGAGGAGTGTGTCACGCGGGCGCGGAGGGCGGGCGCGG	805
ScwhiG	TGTCGCCCTGGAGGAGTGTGTCACGCGGGCGCGGAGGGCGGGCGCGG	732
whiGX	CTGAGCCTGATGGACACCTGGAGGACACCGCCGCGGACAATCCGGTGGG	500
SrwhiG	CTGAGCCTGATGGACACCTGGAGGACACCGCCGCGGACAATCCGGTGGG	855
ScwhiG	CTGAGCCTGATGGACACCTGGAGGACACCGCCGCGGACAATCCGGTGGG	782
whiGX	GGTCGCCGAGGACCGCGAAGTGCAGAGACTCCTCGCGCGGGCCATCAACA	550
SrwhiG	GGTCGCCGAGGACCGCGAAGTGCAGAGACTCCTCGCGCGGGCCATCAACA	905
ScwhiG	GGTCGCCGAGGACCGCGAAGTGCAGAGACTCCTCGCGCGGGCCATCAACA	832

(b)

whiGX	GAMIDELRALDWIPRSVRQARNVERAYATLEAQRRTTPSEAFASEMGLAEELHAFVSQL	
SrwhiG	GAMIDELRALDWIPRSVRQARNVERAYATLEAQRRTTPSEAFASEMGLAEELHAFVSQL	170
ScwhiG	GAMIDELRALDWIPRSVRQARNVERAYATLEAQRRTTPSEAFASEMGLAEELHAFVSQL	172
whiGX	SLANVVALEELLHAGGEGGGRSLMDLTLEDTAADNPVEVAEDRELRLRLARANTLPDRE	
SrwhiG	SLANVVALEELLHAGGEGGGRSLMDLTLEDTAADNPVEVAEDRELRLRLARANTLPDRE	230
ScwhiG	SLANVVALEELLHAGGEGGGRSLMDLTLEDTAADNPVEVAEDRELRLRLARANTLPDRE	232

Fig. 4. Analysis of the segment of a chimeric *whiG* gene in pRHB596. (a) Homology between the nucleotide sequence of the chimeric *whiG* gene from pRHB596 (whiGX) and the corresponding *whiG* sequence of *S. roseosporus* (SrwhiG) and *S. coelicolor* (ScwhiG). (b) Homology between the predicted amino acid sequence of the chimeric σ^{whiG} (whiGX) and the corresponding amino acid sequence of *S. roseosporus* σ^{whiG} (SrwhiG) and *S. coelicolor* σ^{whiG} (ScwhiG). Nucleotide and amino acids with shading indicate identity with the corresponding chimeric sequence. Nucleotide and amino acid positions are indicated to the right of the corresponding sequences.

genes is functional in each strain. It is possible that both are functional, but somewhat more heat labile than the native GSI because of the chimeric structures. Further work is needed to clarify this point.

The *whiG* gene encodes a sigma factor σ^{whiG} required for sporulation in *S. coelicolor* (Chater *et al.*, 1989) and in *S. roseosporus* (T. Hosted & R. H. Baltz, unpublished). The

whiG gene is found in single copy in *S. coelicolor* and in other sporulating actinomycetes (Chater *et al.*, 1989; Soliveri *et al.*, 1993). We tested three recombinants, TH503D, TH503E and TH503F, containing pRHB503 inserted into the chromosome within the *whiG* gene, for sporulation on B agar. All produced normal amounts of aerial mycelium and spores, suggesting that at least one of the chimeric *whiG* genes is functional in each strain.

Table 3. GS activity in *S. roseosporus* strains

Strain	GS	GS specific activity (units)	
		Without heat treatment	With heat treatment*
TH104	Wild-type	8.8	8.5
TH131	Wild-type	10.2	7.5
TH104G1	Chimeric	19.0	6.1
TH104G2	Chimeric	18.4	9.8
TH161	GSI-defective	0.0	0.0

* Cells were incubated at 57 °C for 30 min prior to determining GS activity.

DISCUSSION

The *ehr* mutants isolated in the present study from a library of Tn5099-10 mutants did not appear to be caused by transposition mutations based upon sequence analysis of DNA flanking four different Tn5099-10 inserts. However, the selection for *ehr* mutants by demanding homeologous recombination between the *S. coelicolor glk* gene in KC570 and the *S. roseosporus glk* gene to obtain Ts^R transductants was successful. We confirmed that many different mutants expressed the Ehr phenotype by carrying out recombinational tests in two other genes, *glnA* and *whiG*. The *ehr* mutants generally showed 10²- to 10⁴-fold higher frequencies of recombination within these partially homologous genes, but did not show elevated homologous recombination. Somewhat surprisingly, the *ehr* mutants did not express a spontaneous mutator phenotype. This differs from the *mutL* and *mutS* genes in *S. typhimurium* and *E. coli*, which express elevated spontaneous mutation rates as well as enhanced homeologous recombination. On the other hand, *Saccharomyces cerevisiae* has multiple *mutS* homologues that seem to have diverged in function (Hollingsworth *et al.*, 1995; New *et al.*, 1993; Reenan & Kolodner, 1992; Selva *et al.*, 1995; Strand *et al.*, 1995). Mutations in the *S. cerevisiae MSH3* gene do not affect the rate of spontaneous mutation at several loci (Strand *et al.*, 1995), but cause enhanced homeologous recombination (Selva *et al.*, 1995). The *ehr* gene(s) in *Streptomyces roseosporus* may encode functions homologous or analogous to that provided by the *MSH3* gene in *S. cerevisiae*. The molecular cloning of *S. roseosporus ehr* gene(s) should shed light on this question.

Regardless of the precise function of the *ehr* gene(s), the *ehr* mutations caused enhanced homeologous recombination in frame and in proper translational register within the *glnA* and *whiG* structural genes, as confirmed by DNA sequence analysis. Furthermore, recombinants containing the hybrid *whiG* genes sporulated and recombinants containing hybrid *glnA* genes produced functional GS activity. It is known from studies in *S. coelicolor* that the *whiG* gene encodes a sigma factor required for sporulation (Chater *et al.*, 1989). The *whiG* gene in *S. roseosporus* is also required for sporulation (T. Hosted & R. Baltz, unpublished). Since the recombination of the *S. coelicolor whiG* gene with the *S. roseosporus whiG* gene

appears to have occurred by a triple crossover within a span of about 200 base pairs, presumably generating two hybrid (mosaic) genes, at least one of the hybrid genes must encode a functional σ^{whiG} . The recombinants containing two hybrid *glnA* genes produced GS activities at about twice the amount produced by the control strains before heat treatment and activities approximately equal to controls after heat treatment. Since GSI is a dodecameric enzyme arranged as two rings stacked on top of one another (Almassy *et al.*, 1986), recombinants containing two hybrid *glnA* genes may produce two different chimeric GSI subunits which in turn form mixed dodecameric structures. This suggests that both chimeric subunits may be active, and that the decreased heat stability may be due to the chimeric nature of the subunits.

These results suggest that *ehr* mutants may be useful for several applications. The *ehr* mutants can be used to generate hybrid genes and hybrid proteins at high frequencies *in vivo*. This provides a means to carry out protein engineering *in vivo*, and might provide a means to carry out recombination within related modular sequences found in type I polyketide synthase genes (Katz & Donadio, 1993; Schwecke *et al.*, 1995) and in peptide synthetase genes (Turgay *et al.*, 1992; von Döhren *et al.*, 1993; Stachelhaus *et al.*, 1995) to produce novel structures. The *ehr* mutants also provide an efficient means to insert known genes into partially homologous genes, facilitating physical mapping, gene disruption and cloning of related genes. Finally, *ehr* mutants may facilitate general recombination between diverse species of *Streptomyces* to enhance product yields or to produce novel secondary metabolites. The latter application has been demonstrated using conventional recombination techniques (Gomi *et al.*, 1984; Schlegel & Fleck, 1980).

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