λ Red-Mediated Genetic Manipulation of Antibiotic-Producing *Streptomyces*

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I. Introduction

Streptomycetes are high G+C Gram-positive, antibiotic-producing, soil bacteria that undergo complex morphological differentiation (Chater, 2001). The 8.7 Mb *Streptomyces coelicolor* A3(2) linear chromosome sequence has recently been determined by using a series of overlapping inserts in cosmid vector Supercos1 that have proved very valuable in the technical developments that we describe in this article (Bentley *et al.*, 2002). The sequence analysis revealed 7,825 predicted open reading frames (ORFs), not including those of the liner 365 kb plasmid SCP1 and the 31 kb circular plasmid SCP2, which have been sequenced separately (Bentley *et al.*, 2004; Haug *et al.*, 2003). Recently the genome sequence of *Streptomyces avermitilis* has been published as well, comprising the 9 Mb chromosome and a 94 kb linear plasmid, SAP1 (Ikeda *et al.*, 2003; Omura *et al.*, 2001). Together

Abbreviations: $Carb^{R/S}$, carbenicillin-resistance/sensitivity; $Amp^{R/S}$, ampicillin-resistance/sensitivity; $Apra^{R/S}$, apramycin-resistance/sensitivity; $Cml^{R/S}$, chloramphenicol-resistance/sensitivity; $Kan^{R/S}$, kanamycin-resistance/sensitivity; *oriT*, origin of transfer

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these two genomes contain more than 50 gene clusters that are apparently concerned with secondary metabolites including antibiotics, siderophores, pigments, lipids, and other molecules with functions that are less well understood. The majority of these are present in only one of the two organisms. The further analysis of gene sets such as these, and of many other fascinating aspects of these complex bacteria, requires efficient tools for manipulation of their genomes.

Until recently, time-consuming restriction- and ligation-based techniques have been the principal route for introducing defined changes into the *Streptomyces* chromosome. Although useful, these methods are often inapplicable for large DNA fragments because suitable unique restriction sites are missing. This has engendered a growing demand for simple, one-step procedures for such purposes as gene disruptions, gene fusions, epitope tagging, or promoter replacements. Here we first describe the principles of some of the techniques recently developed by others. We then demonstrate how we have adapted and exploited these methods for *Streptomyces*, and provide some practical tips and discussion of common problems.

II. λ Red-Mediated Recombination in *E. coli*

In *Saccharomyces cerevisiae* and *Candida albicans*, the ability of linear DNA fragments to undergo recombination with the chromosome in the presence of homologies as short as 35–60 bp led to the development of PCR-based methods for gene replacement and modification, in which selectable antibiotic resistance genes are amplified by using PCR primers with 5′-ends homologous to sequences in the gene to be targeted (Baudin *et al.*, 1993; Wilson *et al.*, 1999). Unlike yeast, most bacteria are not readily transformable with linear DNA because the intracellular *recBCD* exonuclease (Exo V) degrades linear DNA. Efforts to circumvent this problem involved the use of mutants or conditions inhibiting Exo V (Dabert and Smith, 1997; El Karoui *et al.*, 1999; Figueroa-Bossi *et al.*, 2001; Russell *et al.*, 1989). However, these methods promoted homologous recombination with linear DNA only when the homology extension of the fragment exceeded a few hundred base pairs. More recently, it has been shown that expression of the *E. coli* RecE and RecT proteins or the corresponding recombination system of bacteriophage λ (the λ Red system) can greatly increase homologous recombination in *E. coli* (see reviews of Court *et al.*, 2002; Muyrers *et al.*, 2000; Poteete, 2001). Using this approach, it has been possible to achieve allelic replacement of genes located on the *E. coli* chromosome by PCR products containing ‘‘homology arms’’ of

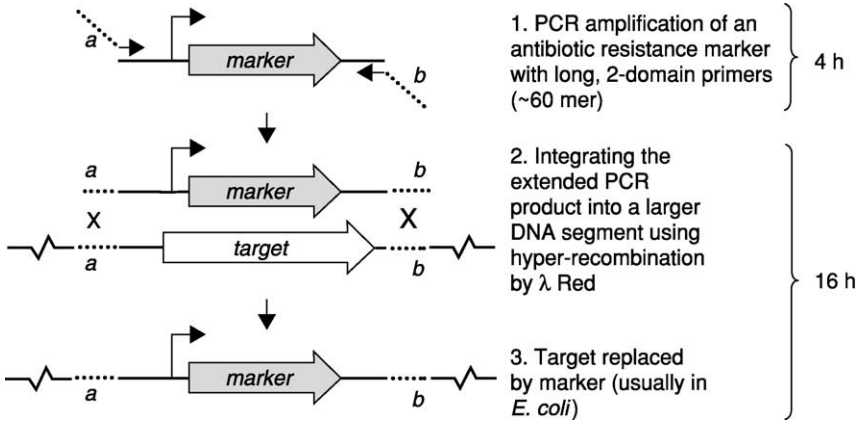


FIG. 1. Principle of λ Red-mediated replacement of a target gene with an antibiotic resistance marker. *a* and *b* (dotted lines) represent the 39 nt/bp extension sequences of the primer/PCR-product homologous to the adjacent sequence of the target gene. Starting with two gene-specific 59-nt primers, the entire procedure takes c.20 h.

36–50 bp, which form part of the commercially supplied customised oligonucleotide primers (Datsenko and Wanner, 2000; Murphy *et al.*, 2000; Yu *et al.*, 2000). Figure 1 shows a basic PCR-targeting strategy in *E. coli*.

III. Adaptation of Hyper-Recombination Systems for Other Organisms

Datsenko and Wanner (2000) demonstrated the use of λ Red recombination to disrupt 40 genes on the *E. coli* chromosome. Importantly, a set of disruption cassettes (templates for the PCR reaction) and plasmids for expressing the λ Red proteins were made available by depositing them at the *E. coli* Genetic Stock Center at Yale University (http://cgsc.biology.yale.edu). This has greatly facilitated the adaptation of this technology to other organisms by other scientists. In *Salmonella enterica* and *Salmonella typhimurium*, mutants have been generated by transforming λ Red-proficient cells with linear DNA fragments (Boddicker *et al.*, 2003; Bonifield and Hughes 2003; Boucrot *et al.*, 2003; Bunny *et al.*, 2002). A two-step strategy was used for the filamentous fungi *Aspergillus nidulans* and *Aspergillus fumigatus*: a λ Red-expressing *E. coli* strain containing a genomic region of interest (on a cosmid clone) was first targeted with a PCR-product to replace the gene of interest within the cosmid. The genetic exchange in the fungus itself was then achieved by homologous recombination between

the chromosomal locus and the recombinant cosmid after transformation with cosmid DNA (Chaveroche *et al.*, 2000; Langfelder *et al.*, 2002). The same strategy was used by Pérez-Pantoja *et al.* (2003) and Stewart and McCarter (2003) to generate deletion mutants in *Ralstonia eutropha* and *Vibrio parahaemolyticus*, respectively. All of these two-step approaches exploited the inability of the *E. coli* cloning vector to replicate in the target organisms: a feature that we have also exploited in the work on *Streptomyces* reported here. RecE/T mutagenesis was used to disrupt the ORF gG of bovine herpesvirus (BHV-1) cloned in a bacterial artificial chromosome (BAC) in *E. coli* (Trapp *et al.*, 2003). The mutant virus could then be recovered after transfection into bovine kidney cells.

These examples show that the technology is rapidly gaining widespread currency, and we anticipate that this will lead to the development of further novel applications that will be of wide general use.

IV. PCR-Targeting in *Streptomyces*

The strategy for PCR-targeting of *S. coelicolor* is to replace DNA in a sequenced *S. coelicolor* cosmid insert (Bentley *et al.*, 2002; Redenbach *et al.*, 1996) by a selectable marker that has been generated by PCR with primers with 39 nt homology arms (Gust *et al.*, 2003). In designing primers for PCR amplification, we took into account the observation from Yu *et al.* (2000) that λ Red-mediated recombination frequencies approach their maximum levels with a 40 bp targeting sequence. We chose to use 39 bp because it involves an integral number of codons, slightly simplifying the primer design without significant reduction in recombination frequencies. The inclusion of *oriT* (RK2) in the disruption cassette allows RP4-mediated intergeneric conjugation to be used to introduce the PCR-targeted cosmid DNA into *S. coelicolor* from *E. coli*. Conjugation is usually much more efficient than transformation of protoplasts and it is readily applicable to many actinomycetes (Matsushima *et al.*, 1994). The potent methyl-specific restriction of *S. coelicolor* is circumvented by mating the PCR-targeted cosmid from a methylation-deficient *E. coli* host such as ET12567 (MacNeil *et al.*, 1992). Vectors containing *oriT* (Pansegrau *et al.*, 1994) are mobilisable *in trans* in *E. coli* by the self-transmissible pUB307 (Flett *et al.*, 1997) or by pUZ8002, which lacks a *cis*-acting function for its own transfer (Paget *et al.*, 1999). For *Streptomyces* work, we constructed cassettes that can be selected in both *E. coli* and *Streptomyces* (Table I). The λ Red recombination plasmid pKD20 (Datsenko and Wanner, 2000) was modified by replacing the ampicillin resistance gene *bla* with

TABLE I

STRAINS AND PLASMIDS USED IN THIS STUDY

Strain/ plasmid	Relevant genotype/comments[a,b]	Source/reference
Plasmids		
pCP20	FLP-Recombination Plasmid: *flp, bla, cat, rep101*^{ts}	Cherepanov and Wackernagel, 1995
pIJ790	λ-RED (*gam, bet, exo*), *cat, araC, rep101*^{ts}	Gust *et al.*, 2003
pIJ773	P1-FRT-*oriT-aac(3)IV*-FRT-P2	Gust *et al.*, 2003
pIJ775	P1-(*Swa*I, I-*Sce*I)-*oriT-aac(3) IV*-(I-*Sce*I, *Swa*I)-P2	This study
pIJ776	P1-FRT-*oriT-neo*-FRT-P2	This study
pIJ777	P1-FRT-*neo*-FRT-P2	This study
pIJ778	P1-FRT-*oriT-aadA*-FRT-P2	Gust *et al.*, 2003
pIJ779	P1-FRT-*aadA*-FRT-P2	Gust *et al.*, 2003
pIJ780	P1-FRT-*oriT-vph*-FRT-P2	Gust *et al.*, 2003
pIJ781	P1-FRT-*vph*-FRT-P2	Gust *et al.*, 2003
pIJ784	bla-*oriT-aac(3)IV*-bla	This study
pIJ785	*ptipA*-P1-FRT-*oriT-aac(3)IV*-FRT-P2	This study
pIJ786	*egfp*-P1-FRT-*oriT-aac(3)IV*-FRT-P2	This study
pIJ787	bla-*oriT-tet-attP-int*-bla	This study
pIJ8641	*egfp, oriT, aac(3)IV*	Jongho Sun, personal communication
pUZ8002	*tra, neo*, RP4	Paget *et al.*, 1999
Supercos1	*neo, bla*	Stratagene
E. coli		
BW25113	K-12 derivative: Δ*araBAD*, Δ*rhaBAD*	Datsenko and Wanner, 2000
ET12567	*dam, dcm, hsdM, hsdS, hsdR, cat, tet*	MacNeil *et al.*, 1992
BT340	DH5α/pCP20	Cherepanov and Wackernagel, 1995
S. coelicolor		
M145	SCP1$^-$, SCP2$^-$	Bentley *et al.*, 2002

[a]P1, P2 left and right priming sites.

[b]bla represents 97 bp (left) and 301 bp (right) sequences matching left and right sequences of *bla* in Supercos1.

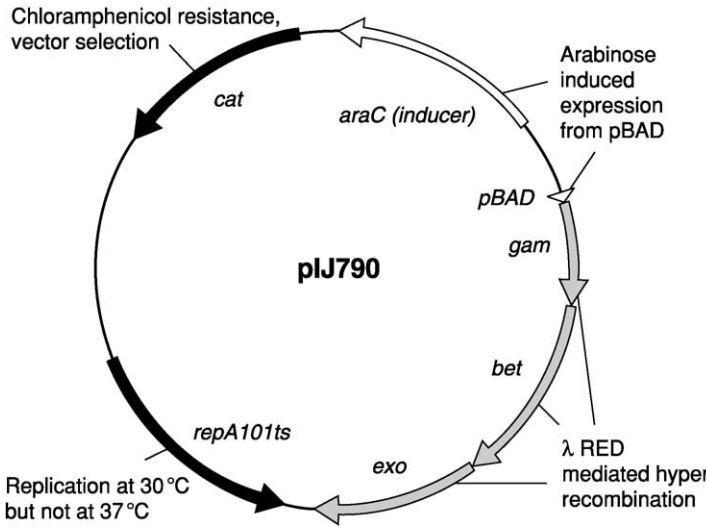


FIG. 2. λ Red recombination plasmid pIJ790.

the chloramphenicol resistance gene *cat*, generating pIJ790 (Fig. 2), to permit selection in the presence of Supercosl-derived cosmids (ampicillin and kanamycin resistant).

A. GENE DISRUPTIONS

A detailed protocol for generating gene disruptions in *Streptomyces* by λ Red-mediated PCR-targeting (Gust *et al.*, 2002) and a Perl Program (BMW) to assist in the primer design and in the analysis of the mutants generated are available at http://jic-bioinfo.bbsrc.ac.uk/S.coelicolor/redirect. Figure 3 shows the strategy for gene replacement in *Streptomyces*.

To demonstrate the high precision of the recombination occurring between 39 bp sequences, we initially chose to disrupt the *S. coelicolor* sporulation gene *whiI* (SCO6029) present in cosmid SC1C3. Many mutants defective in sporulation fail to produce the spore-specific grey pigment and appear white (Hopwood *et al.*, 1970), hence the designation of *whi* genes such as *whiI*. The *whiI* gene product is a response regulator-like protein (Aínsa *et al.*, 1999). Following transformation of the λ Red-expressing *E. coli* containing SC1C3 with a PCR-generated *whiI*-targeted $Apra^R$ disruption cassette, cosmid DNA samples of 50

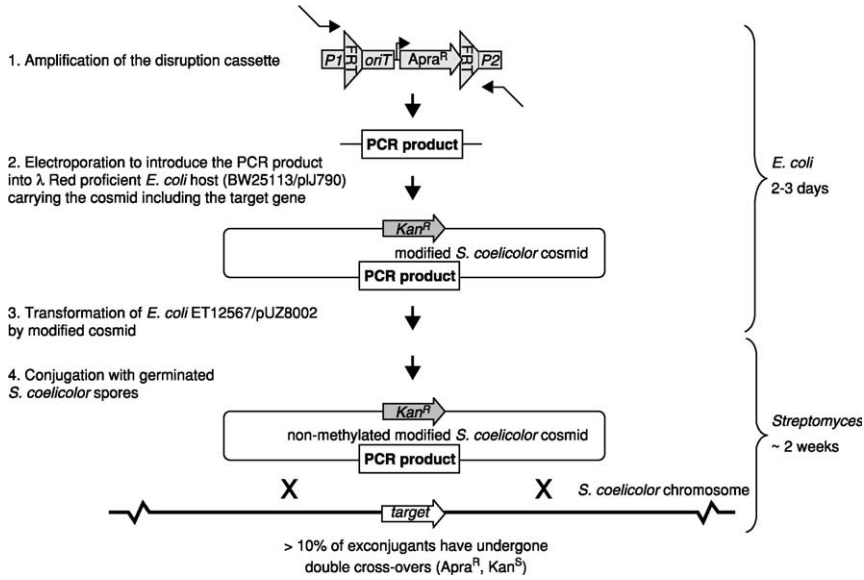


FIG. 3. Adaptation of PCR-targeting for *Streptomyces*. P1 and P2 represent the priming sites of the disruption cassette pIJ773. All disruption cassettes contain the same priming site for annealing of the long primers. FRT sites (FLP recognition targets) flanking the disruption cassette allow the elimination of the central part of the cassette to generate in-frame deletions (see below).

E. coli transformants were all shown to contain the desired gene replacement. The *whi*-disrupted cosmids were introduced into the methylation-deficient *E. coli* host ET12567 and mobilized into *S. coelicolor*. Apra^R exconjugants that had lost the Kan^R marker of the cosmid were readily obtained. They were all white in appearance (Fig. 4), and for four independent mutants the gene replacement was confirmed by Southern blot and PCR analysis.

The technique has been used to disrupt more than 100 genes in *S. coelicolor* and has been successfully applied to different *Streptomyces* spp such as *S. spheroides* and *S. roseochromogenes* var. *oscitans* (Claessen *et al.*, 2003; Elliot *et al.*, 2003; Eustáquio *et al.*, 2003a,b; Gust *et al.*, 2003).

B. TIPS AND COMMON PROBLEMS

The most common problems we and others have encountered while using PCR-targeting in *Streptomyces* include the following:

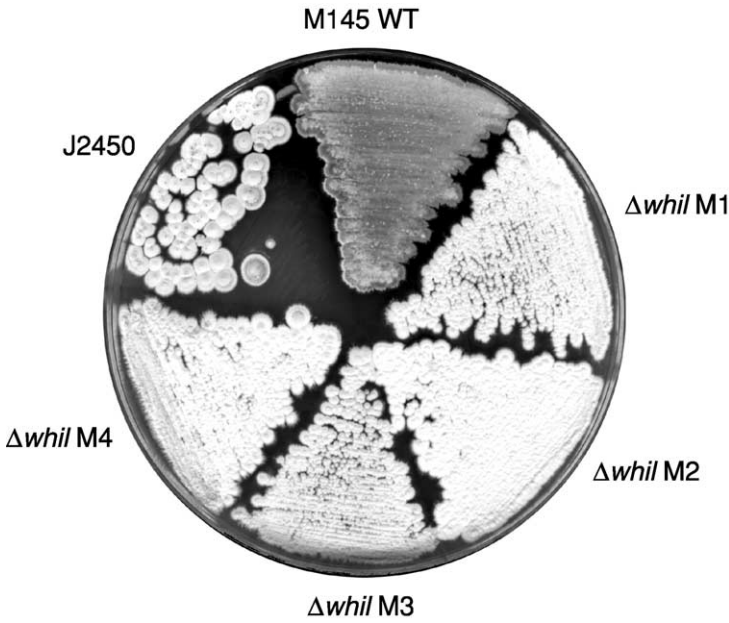


FIG. 4. Comparison of *whiI* mutants of *S. coelicolor* to the wildtype (M145) on MS agar. A previously, traditionally generated *whiI* null mutant, J2450 (Ainsa *et al.*, 1999) and four independent Δ*whiI* mutants (M1-M4), generated by PCR-targeting, all show the same white aerial mycelial phenotype.

1. *Little or no PCR-product is obtained*. The amount of template DNA is crucial for obtaining sufficient quantities of PCR-product for targeting. Approximately 100 ng of template should be used for the PCR reaction under the conditions given in the protocol (Gust *et al.*, 2002). Gene replacement was optimal with 200–300 ng of purified PCR-product.
2. *No transformants are obtained after PCR-targeting*. This common problem can mostly be resolved by using high-quality electrocompetent cells. It is important to start with the generation of competent cells immediately after the correct OD_{600} of 0.4–0.6 is reached without leaving the cultures on ice. Always keep the cells on ice between centrifugations. If no colonies are obtained after 16 h growth at 37 °C, repeat the experiment starting with a 50 ml SOB culture instead of 10 ml. Try to concentrate the cells as much as possible after the second washing step by removing all of the remaining 10% glycerol by using a pipette. Resuspend the pellet in the remaining drop of 10% glycerol (100–150 μl) and use this for electroporation.

3. *Different colony sizes are obtained after PCR-targeting*. After 12–16 hr growth at 37 °C different colony sizes are observed. It is important to note that, at this stage, wild-type and mutant cosmids co-exist within one cell, because, after transformation with a PCR product, not all copies in the cell will carry the disruption. One copy of a cosmid containing the incoming resistance marker is sufficient for resistance to the antibiotic, but nevertheless the larger the size of a colony, the higher the proportion of mutagenized cosmids. Cosmid copies lacking the disruption cassette will be lost during selection of the antibiotic resistance associated with the PCR cassette during subsequent transformation of the methylation-deficient *E. coli* host ET12567 containing the non-transmissible plasmid pUZ8002. This problem is not usually very important, because wild-type copies of the cosmid lack *oriT* and cannot be mobilised for conjugal transfer.
4. *Degradation of the isolated recombinant cosmid DNA*. This can easily be avoided by including a phenol/chloroform extraction step in the DNA isolation procedure even when using DNA isolation kits.
5. *The occasional presence of pseudo-resistant colonies on selective plates that fail to grow when transferred to liquid selective medium*. These can arise because of transient expression of the antibiotic resistance protein from the linear DNA (Muyrers *et al.*, 2000).
6. *No double cross-overs can be obtained in* Streptomyces. Typically, 5–70% of the exconjugants are double cross-over recombinants, if the gene of interest is not essential under the conditions of growth. The frequency of double cross-overs depends on the length of the flanking regions of homologous DNA on the cosmid. If <3 kb is present on one side of the disrupted gene, obtaining Kan^S double cross-over recombinants directly on the conjugation plates may be difficult. It may be necessary to streak out several exconjugants for single colonies or, more effectively, to harvest spores of Kan^R single cross-over recombinants and plate a series of dilutions on MS agar without antibiotics. After 3–5 days growth, the resulting colonies are replica-plated to nutrient agar with and without kanamycin, and screened for double cross-overs (Kan^S).

C. In-Frame Deletions

In the following two sections, we describe two different strategies to generate unmarked, non-polar in-frame deletions. The first includes site-specific recombination with the yeast FLP recombinase, which acts on FRT sites (*F*LP *r*ecognition *t*argets) flanking the disruption cassettes and allows removal of the antibiotic resistance and $oriT_{RK2}$.

The second strategy (''oligo-targeting'') takes advantage of co-transforming a λ Red-proficient *E. coli* host with oligonucleotides and linear DNA molecules.

1. *Using FLP Recombinase*

The plasmid pCP20 shows temperature-sensitive replication and thermoinducible expression of the FLP recombinase, which acts on FRT sites (Cherepanov and Wackernagel, 1995). FLP synthesis and loss of the plasmid pCP20 are induced at 42 °C. Expression of the FLP recombinase removes the central part of the FRT-flanked disruption cassette from the disrupted gene, leaving behind an 81 bp ''scar'' sequence, which lacks stop codons in the 27 codons that are present in the preferred reading frame (Datsenko and Wanner, 2000; Gust *et al.*, 2003). The resulting in-frame deletions are expected to be free of polar effects on downstream genes in operons. In addition, multiple gene disruptions can be generated by repeated use of the same resistance marker. Figure 5 summarises the procedure for obtaining such in-frame deletions.

2. *Using Oligo-Targeting*

For some purposes it maybe desirable to generate ''scarless'' deletions. The scar sequence contains some rare codons for *Streptomyces* and therefore could reduce the translation level of downstream, co-translated proteins. It also contains a functional FRT site that may interfere with subsequent rounds of deletions in the same cosmid. The disruption cassette-containing plasmid pIJ775 was constructed for this purpose. Like pIJ773, it consists of the apramycin resistance gene *aac(3)IV* and $oriT_{RK2}$. The cassette is flanked by two *Swa*I restriction sites (ATTTAAAT). The genome of *S. coelicolor* has only one natural *Swa*I recognition site (at position 1,821,751 bp), and there are no sites on the Supercos1 vector used for the ordered cosmid library. Replacing a gene on a cosmid with the pIJ775 cassette (Table I) will therefore allow the linearisation of the entire cosmid by restriction with *Swa*I. After agarose gel purification to eliminate uncut circular DNA, the linearized cosmid DNA can then be co-electroporated with a 80mer oligonucleotide, which consists of two 40 nt sequences homologous to the upstream and downstream regions of the target gene (i.e., containing the desired deletion junction), into a λ Red-proficient *E. coli* strain. Kan^R and Amp^R colonies only occur after recircularisation of the cosmid brought about by a double cross-over between the 5′- and the 3′-ends of the oligonucleotide and the ends of the linearized cosmid DNA (Fig. 6). In addition to the *Swa*I site, an 18 bp I-*Sce*I recognition site is included in the pIJ775 disruption cassette to allow

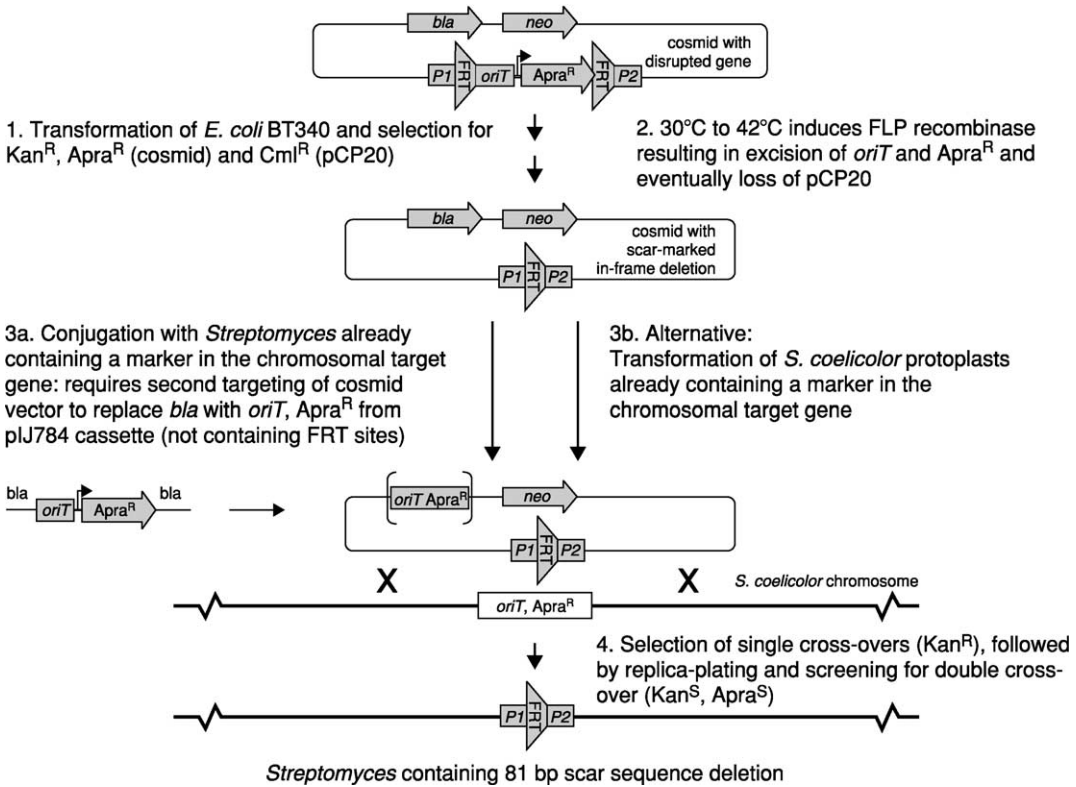


FIG. 5. Generating unmarked, in-frame deletions in *Streptomyces* by site-specific recombination with the yeast FLP recombinase. 3a and 3b are alternatives.

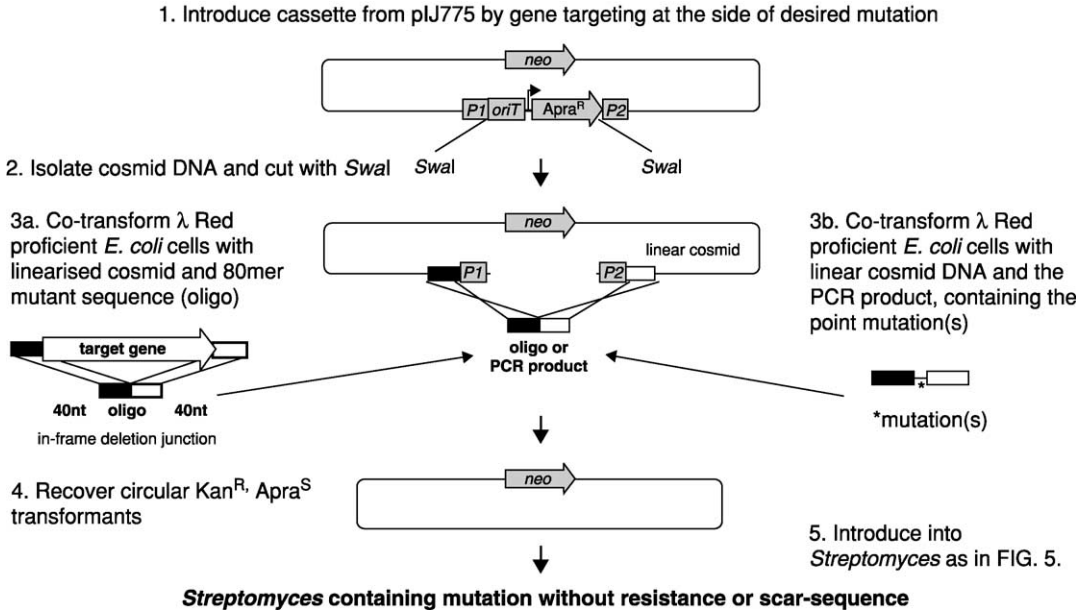


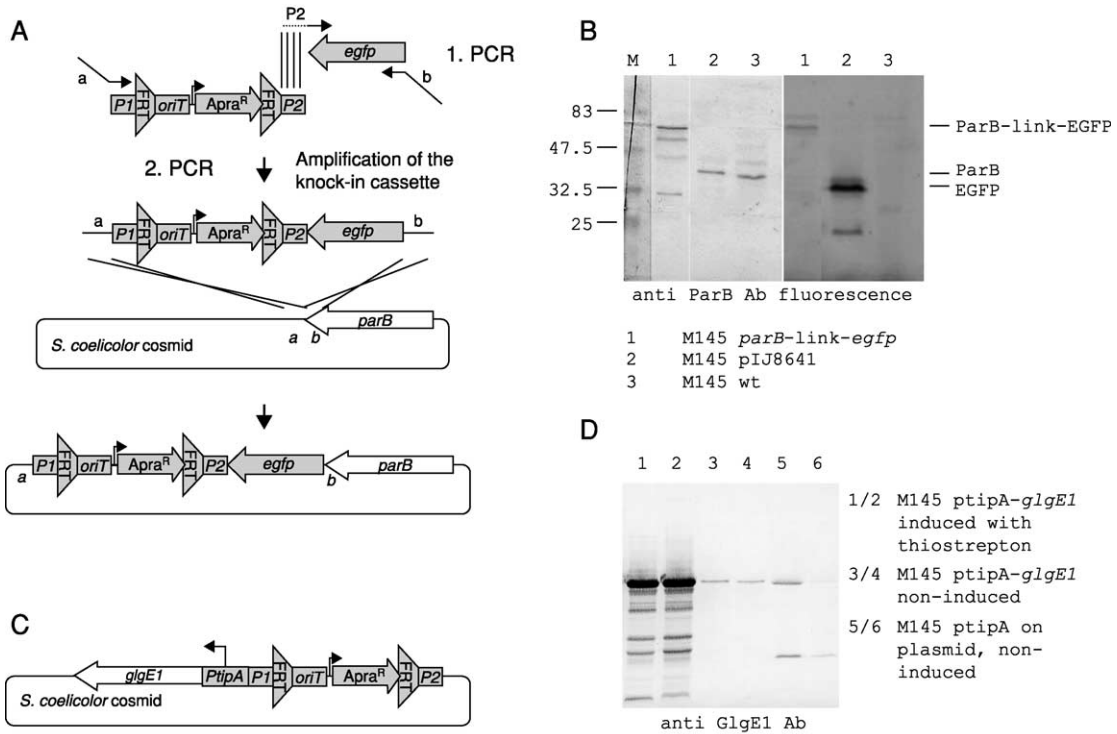
FIG. 6. Generating in-frame deletions or point mutations by co-transforming a λ Red-proficient *E. coli* strain with an 80-mer oligonucleotide (covering the deletion junction) or a PCR product (containing the point mutation) and linearized cosmid DNA. In the examples shown, *cyc2* deletion and *whiI* mutations were successfully introduced.

the generation of a scarless deletion *in vivo* by a double-strand repair mechanism. This complex strategy was used to engineer a reduced *E. coli* genome (Kolisnychenko *et al.*, 2002) and will not be further discussed in this work.

Oligo-targeting was tested with the *S. coelicolor* cosmid SC9B1 carrying the *cyc2* gene, which has been shown previously to be involved in the biosynthesis of the soil odour geosmin (Gust *et al.*, 2003). An 80mer oligonucleotide was ordered which would delete the entire *cyc2* gene after co-electroporation with the linearised, phenol-chloroform extracted cosmid DNA. Kan^R Amp^R transformants were screened by colony PCR with test primers annealing 100 bp upstream and downstream of *cyc2*. From 96 transformants, 25 were identified as positive candidates, whereas 37 seemed to show no deletion of the pIJ775 cassette insertion. This can be explained by incomplete *Swa*I digestion, resulting in still circular cosmid molecules which could transform the λ Red-proficient *E. coli* cells efficiently. Surprisingly, 34 transformants failed to produce any product in the PCR test. Restriction analysis of DNA of these 34 transformants showed deletions of different sizes on both sites of *cyc2*, which included the priming sites of the test primers. This could either be due to illegitimate intramolecular recombination of the linear cosmid DNA or to mis-annealing of the oligonucleotide at different positions within the cosmid insert. However, all 25 positive candidates were verified as scarless deletions of *cyc2* by restriction analysis and sequencing of the PCR product generated with the test-primers.

D. Introducing Point Mutations

It is valuable to be able to introduce point mutations into genes at their proper chromosomal location. It has been shown previously that RecE/RecT mutagenesis or λ Red recombination can be used to introduce point mutations at any position in a gene of interest (Muyrers *et al.*, 2000). Here we have used a simplified variation of this procedure to introduce point mutations into the *whiI* gene of *S. coelicolor*. In our version, λ Red recombination was not used to generate the point mutation itself, but instead it was used to introduce PCR fragments containing the point mutations into the corresponding cosmid by co-transformation as described for oligo-targeting (Fig. 6). Integrating different point mutations in the same gene requires only one initial gene replacement with the disruption cassette derived from pIJ775. In principle, any unmarked DNA fragment can be used for targeting as long as it is


A
P2
egfp
1. PCR
a
b
FRT
P1
oriT
Apra^R
P2
2. PCR
Amplification of the knock-in cassette
parB
S. coelicolor cosmid
B
M 1 2 3 1 2 3
83
47.5
32.5
25
ParB-link-EGFP
ParB
EGFP
anti ParB Ab
fluorescence
1 M145 parB-link-egfp
2 M145 pIJ8641
3 M145 wt
C
glgE1
PtipA
D
1 2 3 4 5 6
1/2 M145 ptipA-glgE1 induced with thiostrepton
3/4 M145 ptipA-glgE1 non-induced
5/6 M145 ptipA on plasmid, non-induced
anti GlgE1 Ab

flanked by homologous sequences for λ Red recombination. Replacing chromosomal segments and module-swapping should therefore also be possible.

E. Promoter Replacements and Gene Fusions

Uzzau *et al.* (2001) have demonstrated the use of the Datsenko and Wanner procedure to introduce epitope tags into chromosomal genes in *Salmonella* by generating PCR templates containing a selectable marker and the epitope tag. Although this is straightforward and highly efficient, it relies on the construction of new template cassettes. Small tags like His-tags can be included directly into the long PCR-targeting primer sequence without the need to develop new cassettes. Here we describe how promoters can be replaced or gene fusions can be generated using the existing disruption cassettes.

The main idea is to use a two-step PCR, which in the first step generates a PCR product that contains the new promoter or the marker gene to be used for gene fusions. This PCR product is extended by choosing a primer sequence that allows annealing with the priming site of any of the disruption cassettes. For the second PCR reaction, the disruption cassette and first-round PCR-product serve as template and long PCR-targeting primers are used to amplify the complete ''knock-in'' cassette. The second-round PCR product can then be inserted directly at the desired position by λ Red-mediated recombination. Figure 7 shows two examples of such ''knock-in'' experiments, one a fusion of the enhanced green fluorescent protein gene *egfp* into the *parB* gene of *S. coelicolor* (Jakimowicz *et al.*, 2002) and the other a promoter replacement of the natural *glgEI* promoter by the thiostrepton-inducible promoter *ptipA* (Murakami *et al.*, 1989).

Fig. 7. Two examples of ''knock-in'' experiments. *A, egfp* is amplified in the first round PCR reaction to generate a PCR product flanked by sequences *b* and P2. The purified *egfp*-PCR fragment is then mixed with the disruption cassette and the knock-in cassette is generated by PCR amplification with the outside primers *a* and *b*. After λ Red-mediated targeting into the appropriate cosmid and introduction into *Streptomyces*, the ParB-EGFP fusion was analysed in *S. coelicolor* cell extracts using anti-ParB antibodies on Western blot and on the SDS-PAGE by phosphoimaging (*B*, left and right panels, respectively). *C*, Using a similar approach, the promoter of gene *glgE1* was replaced with the thiostrepton inducible promoter *ptipA*. Successful introduction of a functional construct into *Streptomyces* was confirmed by Western blot analysis of extracts prepared of cultures with and without induction by thiostrepton (*D*).

F. Heterologous Production of Antibiotics in *Streptomyces*

The genes for synthesis of any one antibiotic in streptomycetes are invariably clustered together on the chromosome (or sometimes on a plasmid). The availability of plasmid vectors which can efficiently carry stable large inserts into different *Streptomyces* spp. has been exploited in a number of laboratories to allow production in a heterologous host. Interspecies cloning of antibiotic biosynthesis genes or the corresponding resistance gene in non-producing hosts (often *S. lividans*) has been used to identify or confirm complete antibiotic gene clusters such as those for puromycin from *Streptomyces alboniger*, nikkomycin from *Streptomyces tendae* Tu901, kinamycin from *Streptomyces murayamaensis*, blasticidin S from *Streptomyces griseochromogenes*, complestatin from *Streptomyces lavendulae*, staurosporine from *Streptomyces sp.* TP-A0274, and rebeccamycin from *Saccharothrix aerocolonigenes* ATCC39243 (Bormann *et al.*, 1996; Chiu *et al.*, 2001; Cone *et al.*, 1998; Gould *et al.*, 1998; Lacalle *et al.*, 1992; Onaka *et al.*, 2002; Sanchez *et al.*, 2002). In addition, recombinant environmental libraries from soil for accessing microbial diversity were constructed as ''shotgun'' clones on an *E. coli—S. lividans* shuttle cosmid vector. New polyketide synthase genes were found with this approach in at least eight clones (Courtois *et al.*, 2003).

Many of the currently analysed antibiotic clusters are cloned on either cosmid or BAC vectors. Integrating these *E. coli* clones into *Streptomyces* chromosomes has the following advantages:

1. Introducing large DNA molecules into strains such as *S. coelicolor* or *S. lividans* has been established for a long time.
2. Using the methods described in this review, changes can be introduced into clusters within a few days and then rapidly integrated into the *Streptomyces* chromosome for further analysis.
3. Genetic tools have been widely established for some streptomycetes, making them desirable hosts for various genetic manipulations (Kieser *et al.*, 2000).
4. Once integrated, there is usually no need to maintain selection for the vector.
5. Where the clusters originate from streptomycetes or related high GC organisms, it is much more likely that they will express properly in a *Streptomyces* host than in *E. coli*.

To allow integration of any Supercos1-based cosmid into a *Streptomyces* chromosome, the targeting cassette in pIJ787 was constructed. It consists of a tetracycline resistance marker for selection in *E. coli* after

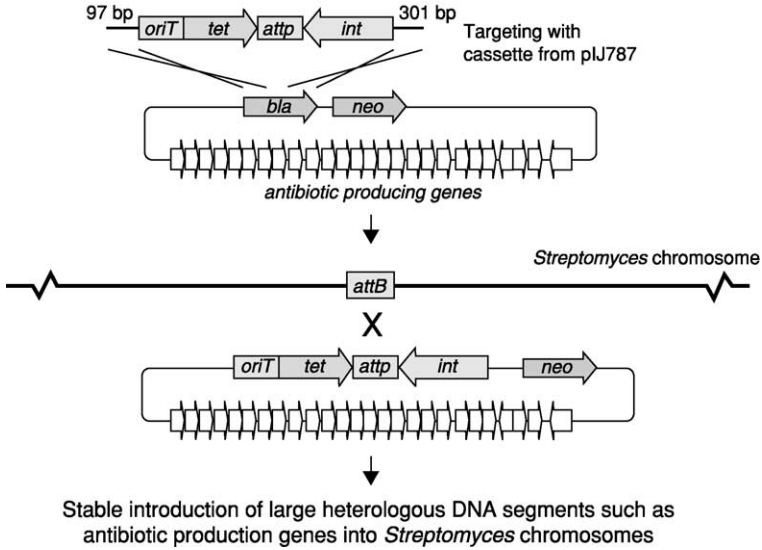


FIG. 8. Integrating cosmid clones into the chromosomal phiC31 attachment site present in *Streptomyces* species.

targeting, an $oriT_{RK2}$ for conjugal transfer and an *attP* site, and the integrase gene of phage phiC31 to allow integration into the chromosome by *attP*/*attB* recombination (Thorpe *et al.*, 2000). The cassette is flanked by sequences (97 bp at one end and 301 bp at the other end) homologous to the ampicillin resistance gene *bla* on the Supercos 1 vector backbone. This 4,990 bp cassette is cloned into Supercos1 and can be extracted as a *Dra*I/*Bsa*I restriction fragment, so that no PCR amplification is needed. The procedure to replace the ampicillin resistance gene *bla* on the cosmid vector by the pIJ787 cassette (Fig. 8) is the same as described for gene disruption (Fig. 3).

As a test system, we used cosmid C73 of the ordered SCP1 cosmid library (Redenbach *et al.*, 1998), which contains the functional methylenomycin biosynthesis cluster (O'Rourke, 2003). Because methylenomycin is encoded by the linear *S. coelicolor* plasmid SCP1 (Wright and Hopwood, 1976), the recombinant cosmid C73_787 was introduced into the non-producing *S. coelicolor* M145 strain, which lacks SCP1. Successful integration into the M145 chromosome was confirmed with a bioassay for methylenomycin production (O'Rourke, 2003).

V. Conclusions and Future Prospects

We have adapted and exploited the use of λ Red recombination for use in *Streptomyces*. This rapid and highly efficient method has made the generation of gene disruptions more precise and allows the construction of in-frame deletions. So far, more than 100 segments of the *S. coelicolor* genome ranging in size between 4 bp and over 7 kb have been replaced by PCR-targeting. The technique has also succeeded in other *Streptomyces* species (Eustáquio *et al.*, 2003a,b). Here, we have described the use of this technology for various other DNA modifications such as introducing point mutations, promoter replacements, and gene fusions. Combining the different approaches enables us to manipulate *Streptomyces* DNA more rapidly and precisely than using traditional techniques. The facile integration of whole antibiotic gene clusters into *Streptomyces* chromosomes makes high-throughput manipulation of the clusters possible. Since it has been possible to reconstruct large gene segments (>90 kb) starting from pre-existing, smaller fragments by co-integrate formation and resolution *in vivo* (Sosio *et al.*, 2001) or by combining overlapping BACs with the help of λ Red recombination (Zhang and Huang 2003), even large antibiotic clusters will be manageable in the future. In combination with ET-cloning (Zhang *et al.*, 2000), swapping modules, for example within a polyketide biosynthetic megagene, should be achievable.

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