Recombineering in *Streptomyces coelicolor*

Bertolt Gust[1,2], Sean O'Rourke[1], Nicholas Bird[1], Tobias Kieser[1] and Keith Chater[1]

[1]Department of Molecular Microbiology, John Innes Centre, Norwich Research Park, Colney, Norwich, NR4 7UH, United Kingdom.

Current address: [2]Pharmazeutsiche Biologie, Universitat Tubingen, 72076 Tubingen, Germany

Introduction

Recombineering (recombination-mediated genetic engineering) is a powerful method based on homologous recombination in *E. coli* using recombination proteins provided from λ phage or Rac prophage (general information at http://recombineering.ncifcrf.gov/). Many bacteria are not readily transformable with linear DNA because of the presence of the intracellular *recBCD* exonuclease that degrades linear DNA. However, the λ RED (*gam, bet, exo*) or the corresponding RecE/RecT functions promote a greatly enhanced rate of recombination when using linear DNA (Zhang *et al*., 1998).

The strategy of recombineering for mutagenesis of *Streptomyces coelicolor* is to replace a chromosomal sequence within a *S. coelicolor* cosmid (Redenbach *et al*., 1996) by a selectable marker that has been generated by PCR using primers with 39 nt homology extensions. The inclusion of *oriT* (RK2) in the disruption cassette allows conjugation to be used to introduce the modified cosmid DNA into *S. coelicolor*. Conjugation is much more efficient than transformation of protoplasts and it is readily applicable to many actinomycetes (Matsushima *et al*., 1994). The potent methyl-specific restriction system of *S. coelicolor* is circumvented by passaging DNA through a methylation-deficient *E. coli* host such as ET12567 (MacNeil *et al*., 1992). Vectors containing *oriT* are mobilisable *in trans* in *E. coli* by the self-transmissible pUB307 (Bennett *et al*., 1977, Flett *et al*., 1997) or the non-transmissible pUZ8002, which lacks a *cis*-acting function for its own transfer (Kieser *et al*., 2000).

To adapt the procedure of λ RED mediated recombination for *Streptomyces*, cassettes for gene disruptions were constructed that can be selected both in *E. coli* and in *Streptomyces*. A list of actual cassettes, sequences and a program to assist in the primer design and in the analysis of the mutants generated are available at http://streptomyces.org.uk/redirect/index.html (Gust *et al*., 2003; Gust *et al*., 2004).

Protocol

1. Strains and plasmids for recombineering in *Streptomyces coelicolor*

Strain/ Plasmid	**Relevant Genotype/Comments**[1]	**Source/ Reference**
Plasmids		
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
pIJ776	P1-FRT-*oriT-neo*-FRT-P2	Gust *et al*., 2004
pIJ777	P1-FRT-*neo*-FRT-P2	Gust *et al*., 2004
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
pIJ785	P1-FRT-*oriT-aac(3)IV*-FRT-P2-tipA	B. Gust
pIJ799	*bla-oriT-aac(3)IV-bla*	S. O'Rourke
pIJ10700	P1-FRT-*oriT-hyg*-FRT-P2	S. O'Rourke
pIJ10701	*bla-oriT-hyg-bla*	S. O'Rourke
pUZ8002	*tra*, *neo*, RP4	Paget *et al*., 1999
Supercos1	*neo*, *bla*	Stratagene
E. coli		
BW25113	K-12 derivative: Δ*araBAD*, Δ*rhaBAD*	Datsenko & Wanner 2000
ET12567	*dam, dcm, hsdM, hsdS, hsdR, cat, tet*	MacNeil *et al*., 1992
BT340	DH5α/pCP20	Cherepanov & Wackernage 1995
S. coelicolor		
M145	SCP1$^-$, SCP2$^-$	Bentley *et al*., 2002

[1)] P1, P2 left and right priming sites

Table 1. Strains and plasmids used for gene inactivations in *Streptomyces coelicolor*

2. Purification of the PCR template (cassette)

Using whole plasmids as templates for the PCR can result in a high proportion of antibiotic-resistant transformants without gene disruption. This is caused by traces of CCC DNA that compete with the linear PCR fragment and result in the occurrence of false positive transformants. Using gel-purified disruption cassettes as templates prevents the occurrence of false positives.

1. Digest ~ 10 µg plasmid DNA (see sequence files at http://streptomyces.org.uk/redirect/cassettes/index.html) with 50 U *Eco*RI and 50 U *Hin*dIII in 1 X buffer in a 100 µl reaction.
2. Run the digest on a 20 x 20 x 0.25 cm (100 ml) 1% TAE (1x) agarose gel at 5V/cm for 2 - 3 h in 1x TAE buffer.
3. Cut out the cassette band from the gel and purify using the QIAGEN gel extraction kit. The purified fragment is stored in 10 mM Tris HCl (pH 8) at a concentration of 100 ng / µl at –20°C.
4. Absence of plasmid DNA is tested by using 1µl (100 ng) of purified cassette DNA to transform highly competent *E. coli* DH5α cells (10^8/µg). Plate on LB agar containing 100 µg/ml carbenicillin. If transformants appear, repeat steps 2-4 with purified cassette DNA from the first round of purification.

3. Design of long PCR primers for recombineering

For each gene disruption, two long PCR primers (58 nt and 59 nt) are required. Each has at its 5′end 39 nt matching the *S. coelicolor* sequence adjacent to the gene to be inactivated, and at its 3′ end a 19- or 20-nt sequence matching the right or left end of the disruption cassette (all cassettes have the same priming sites P1 and P2, see table 1). The precise positioning of the 39 nt sequence as indicated in Fig. 1 is important for creating in-frame deletions by FLP recombinase-induced excision of the resistance marker (see section 10). The 5′- 39-nt sequence of the forward primer (upstream primer) must be from the coding strand of the gene of interest and its 3' end must be in the correct reading frame with respect to the replaced gene. The 5′- 39-nt sequence of the reverse primer (downstream primer) must be from the *complementary* strand. A program to assist in the primer design is available at http://streptomyces.org.uk/redirect/index.html.

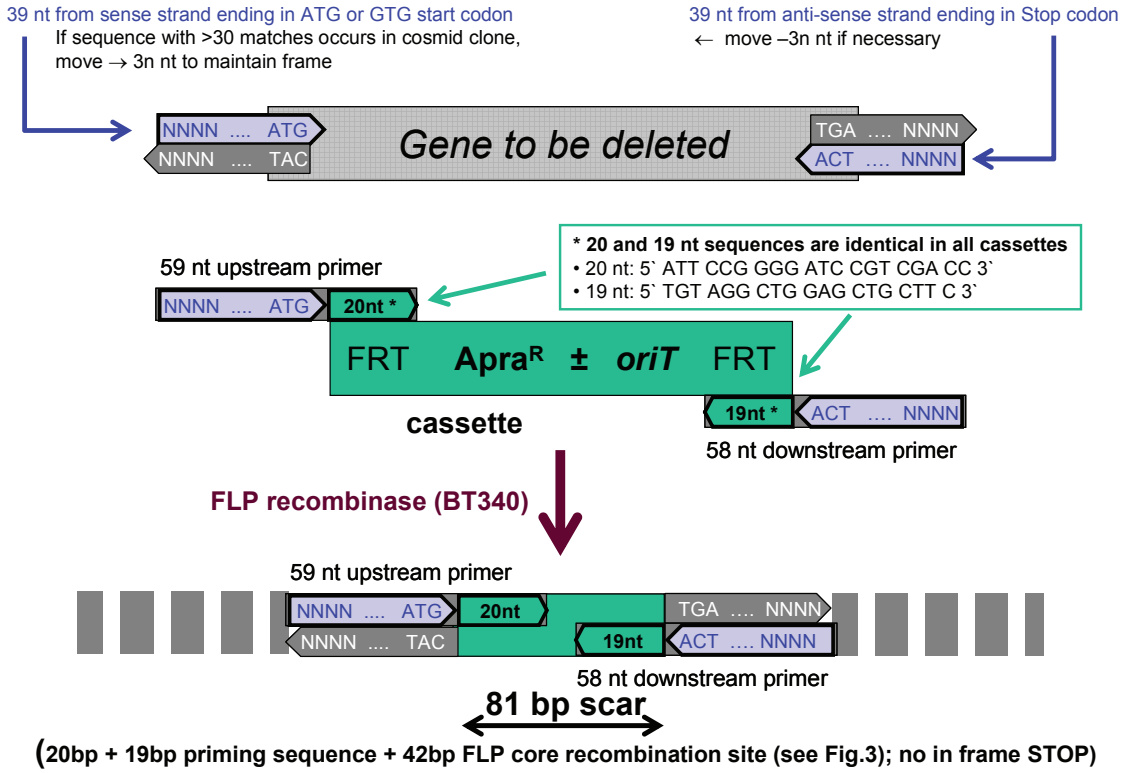

Fig.1: Designing PCR primers for making an in-frame deletion (the example illustrates a complete deletion) Details of the Flp recognition and scar sequences are given in section 10.

4. PCR amplification of the extended resistance cassette

All PCR amplifications are performed using the Expand high fidelity PCR system according to the manufacturer's instructions (Roche). Reaction conditions:

• Primers (100 pmoles/µl)	0.5 µl each	50 pmoles each
• Template DNA (100 ng/µl)	0.5 µl	50 ng ≈ 0.06 pmoles
• Buffer (10x)	5 µl	1 x
• dNTPs (10 mM)	1 µl each	50 µM each
• DMSO (100 %)	2.5 µl	5%
• DNA polymerase (2.5 U/µl)	1 µl	2.5 Units
• Water	36 µl	
• Total volume	50 µl	

Cycle conditions:

1. Denaturation:	94°C, 2 min	
2. Denaturation:	94°C, 45 sec	} 10 cycles
3. Primer annealing:	**50°C**, 45 sec	
4. Extension:	72°C, 90 sec	
5. Denaturation:	94°C, 45 sec	} 15 cycles
6. Primer annealing:	**55°C**, 45 sec	
7. Extension:	72°C, 90 sec	
8. Final extension:	72°C, 5 min	

5 µl of the PCR product is used for analysis by gel electrophoresis. The expected sizes are 78 bp larger than the sizes of the disruption cassettes (because of the 2 x 39 bp 5´-primer extensions). The remaining 45 µl of the PCR product is purified using the QIAGEN PCR purification kit according to the manufacturer's instructions. The PCR product is finally eluted from the columns with 12 µl of water (~200 ng/µl).

5. Introduction of a *S. coelicolor* cosmid clone into *E. coli* BW25113/pIJ790 (λ RED recombination plasmid) by electroporation

pIJ790 contains the resistance marker *cat* (chloramphenicol resistance) and a temperature sensitive origin of replication (requires 30°C for replication).

1. Grow *E. coli* BW25113/pIJ790 overnight at 30°C in 10 ml LB (Luria-Bertani medium; Sambrook *et al*., 1998) containing chloramphenicol (25 μg/ml).
2. Inoculate 100 μl *E. coli* BW25113/pIJ790 from overnight culture into 10 ml LB containing chloramphenicol (25 μg/ml).
3. Grow for 3-4 h at 30°C shaking at 200 rpm to an OD_{600} of ~ 0.4.
4. Recover the cells by centrifugation at 4000 rpm for 5 min at 4°C in a Sorvall GS3 rotor (or equivalent).
5. Decant medium and resuspend the pellet by gentle mixing in 10 ml ice-cold 10 % glycerol.
6. Centrifuge as above and resuspend pellet in 5 ml ice-cold 10 % glycerol, centrifuge and decant. Resuspend the cell pellet in the remaining ~ 100 μl 10 % glycerol.
7. Mix 50 μl cell suspension with ~ 100 ng (1-2 μl) of cosmid DNA. Carry out electroporation in a 0.2 cm ice-cold electroporation cuvette using a BioRad GenePulser II (or equivalent) set to: 200 Ω, 25 μF and 2.5 kV. The expected time constant should be 4.5 – 4.9 ms.
8. Immediately add 1 ml ice-cold LB to shocked cells and incubate shaking for 1h at 30°C.
9. Spread onto LB agar containing carbenicillin (100 μg/ml) and kanamycin (50 μg/ml) for selection of a *S. coelicolor* cosmid based on SuperCos1 (Stratagene), and chloramphenicol (25 μg/ml) for selection of pIJ790.
10. Incubate overnight at 30°C.
11. Transfer one isolated colony into 5 ml LB containing antibiotics as in (9) above.
12. Incubate with shaking overnight at 30°C. This culture will be used as a pre-culture for generating competent cells to be transformed with the extended resistance cassette (PCR product from section 4).

6. Gene inactivation on the *S. coelicolor* cosmid by recombineering

E. coli BW25113/pIJ790 containing a *S. coelicolor* cosmid is induced to express λ *red* genes and then electro-transformed with the extended resistance cassette. The example described uses the apramycin + *oriT* disruption cassette from pIJ773. Table **1** lists alternative cassettes and their resistance determinants.

1. Inoculate a 10 ml SOB culture (without $MgSO_4$) containing carbenicillin (100 μg/ml), kanamycin (50 μg/ml) and chloramphenicol (25 μg/ml) with 5% of the overnight culture of *E. coli* BW25113/pIJ790 containing the *S. coelicolor* cosmid. Add 100 μl 1M L-arabinose stock solution for induction of λ *red* genes (final concentration is 10 mM).
2. Grow for 3-4 h at 30°C shaking at 200 rpm to an OD_{600} of ~ 0.5.
3. Recover the cells by centrifugation at 4000 rpm for 5 min at 4°C in a Sorvall GS3 rotor (or equivalent).
4. Decant medium and resuspend the pellet by gentle mixing in 10 ml ice-cold 10% glycerol.
5. Centrifuge as above and resuspend pellet in 5 ml ice-cold 10 % glycerol, centrifuge and decant. Resuspend the cell pellet in remaining ~ 100 μl 10 % glycerol.
6. Mix 50 μl cell suspension with ~ 100 ng (1-2 μl) of PCR product. Carry out electroporation in a 0.2 cm ice-cold electroporation cuvette using a BioRad GenePulser II (or equivalent) set to: 200 Ω, 25 μF and 2.5 kV. The expected time constant is 4.5 – 4.9 ms.
7. Immediately add 1 ml ice cold SOC to shocked cells, and incubate with shaking for 1 h at 37°C (or 30°C if further gene disruptions will be made on the same cosmid; see below).
8. Spread onto LB agar containing carbenicillin (100 μg/ml), kanamycin (50 μg/ml) and apramycin (50 μg/ml). If no further gene disruptions will be made on this cosmid, incubate overnight at 37°C to promote the loss of pIJ790. (If further disruptions are planned, propagate overnight at 30°C and include chloramphenicol (25 μg/ml) so that pIJ790 is retained).

7. Confirmation of gene inactivation by restriction analysis

After 12 – 16 h of growth at 37°C different colony-sizes are observed. Cultivating for longer time results in an increased background of small colonies, which are false positives. It is important to note that at this stage wild-type and mutant cosmids often co-exist within one cell. The transformation with a PCR product and its integration in the cosmid DNA by homologous recombination will **not** occur in all copies of the cosmid molecules in one cell. One copy of a cosmid containing the incoming resistance marker is sufficient for resistance to this antibiotic. Normally, the larger the size of a colony, the more copies of mutagenised cosmids are present.
Alkaline lysis followed by phenol/chloroform extraction produces cosmid DNA suitable for restriction analysis and PCR.

Isolation of cosmid DNA

1. Inoculate a large colony in 5 ml LB liquid containing carbenicillin (100 µg/ml), kanamycin (50 µg/ml) and apramycin (50 µg/ml).
2. Grow at 37°C, shaking at 200 rpm, to an OD_{600} of ~ 0.6 (within 4-6 h; *E. coli* BW25113 without pIJ790 grows very fast).
3. Recover the cells from 1.5 ml of culture by centrifugation at 13000 rpm for 1 min at 4°C in a micro centrifuge.
4. Resuspend the cell pellet by vortexing in 100 µl solution I (50 mM Tris/HCl, pH 8; 10 mM EDTA).
5. Immediately add 200 µl solution II (200 mM NaOH; 1% SDS) and mix by inverting the tubes 10x.
6. Immediately add 150 µl of ice-cold solution III (3 M potassium acetate, pH 5.5) and mix by inverting the tubes 5x.
7. Spin at full speed in a micro centrifuge for 5 min at 4°C.
8. Immediately extract supernatant with 400 µl phenol/chloroform, vortex 2 min and spin at full speed in a micro centrifuge for 5 min.
9. Transfer the upper phase and add 600 µl 2-propanol. Leave the tubes on ice for 10 min.
10. Spin as above and wash the pellet with 200 µl 70% ethanol.

11. Spin as above and leave the tube open for 5 min at room temperature to dry the pellet. Resuspend the pellet in 50 µl 10mM Tris/HCl (pH 8) and use 10 µl for restriction digest.

Note: Omitting the phenol/chloroform extraction step results in degradation of the cosmid DNA. Use of miniprep-columns without including a phenol/chloroform extraction is not recommended.

8. Confirmation of gene inactivation by PCR

Verification of positive transformants by PCR requires an additional pair of 18 – 20 nt test primers which anneal 100 – 200 bp upstream and downstream of the 39 bp recombination region. (These primers can also be used later to verify the FLP-mediated excision of the resistance cassette.)

• Primers (100 pmoles/µl)	0.2 µl each	20 pmoles each
• Template DNA (~50 ng/µl)	1 µl	50 ng
• Buffer (10x)	5 µl	1 x
• dNTPs (10 mM)	1 µl each	50 µM each
• DMSO (100 %)	2.5 µl	5%
• DNA polymerase (2.5 U/µl)	1 µl	2.5 Units
• Water	36.1 µl	
• Total volume	50 µl	

Cycle conditions:

1. Denaturation:	94°C, 2 min	
2. Denaturation:	94°C, 45 sec	} 30 cycles
3. Primer annealing:	55°C, 45 sec	
4. Extension:	72°C, 90 sec	
5. Final extension:	72°C, 5 min	

5 µl of the PCR product is used for gel electrophoresis.

9. Transfer of the mutant cosmids into *Streptomyces*

If the target *Streptomyces* for mutagenesis carries a methyl-sensing restriction system (as is the case for *S. coelicolor* and *S. avermitilis*), it is necessary to passage the cosmid containing an apramycin resistance-*oriT* cassette through a non-methylating *E. coli* host. To achieve this, it is introduced by transformation into the non-methylating *E. coli* ET12567 containing the RP4 derivative pUZ8002. The cosmid is then transferred to *Streptomyces* by intergeneric conjugation (see Table 2 for resistance markers). If the target *Streptomyces* for mutagenesis does **not** carry a methyl-sensing restriction system (as is the case for *S. lividans*), common *E. coli* strains such as DH5α containing pUZ8002 can be used instead.

Description	Name	Replication	$Carb^R$	Cml^R	Kan^R	Tet^R
S. coelicolor cosmid clones	Supercos 1		$Carb^R$		Kan^R	
λ Red plasmid	pIJ790	t^s		Cml^R		
FLP recombinase plasmid	BT340	t^s	$Carb^R$	Cml^R		
$OriT^-$ RP4 derivative	pUZ8002				Kan^R	
$OriT^+$ RP4 derivative	pUB307				Kan^R	
Non-methylating *E. coli*	ET12567			Cml^R		Tet^R

Table 2. Resistance markers of vectors, helper plasmids and strains (carbenicillin resistance ($Carb^R$), chloramphenicol resistance (Cml^R), kanamycin resistance (Kan^R), tetracycline resistance (Tet^R), temperature sensitive replicon (t^S)). See Table **1** for replacement cassettes.

1. Prepare competent cells of *E. coli* ET12567/pUZ8002 grown at 37ºC in LB containing kanamycin (25 μg/ml) and chloramphenicol (25 μg/ml) to maintain selection for pUZ8002 and the *dam* mutation, respectively. (ET12567 has a doubling time > 30 min.)

2. Transform competent cells with the *oriT*-containing cosmid clone, and select for the incoming plasmid using only apramycin (50 μg/ml) and carbenicillin (100 μg/ml).
3. Inoculate a colony into 10 ml LB containing apramycin (50 μg/ml), chloramphenicol (25 μg/ml) and kanamycin (50 μg/ml). Grow overnight with shaking at 37ºC.
4. Inoculate 300 μl overnight culture into 10 ml fresh LB plus antibiotics as above and grow with shaking for ~ 4 h at 37°C to an OD_{600} of 0.4.
5. Wash the cells twice with 10 ml of LB to remove antibiotics that might inhibit *Streptomyce*s, and resuspend in 1 ml of LB.
6. While washing the *E. coli* cells, for each conjugation add 10 μl (10^8) *Streptomyces* spores to 500 μl 2 × YT broth. Heat shock at 50°C for 10 min, then allow cooling by leaving at room temperature for 15 minutes.
7. Mix 0.5 ml *E. coli* cell suspension and 0.5 ml heat-shocked spores and spin briefly. Pour off most of the supernatant, and then resuspend the pellet in the c. 50 μl residual liquid.
8. Make a dilution series from 10^{-1} to 10^{-4} each step in a total of 100 μl of water.
9. Plate out 100 μl of each dilution on MS agar + 10mM $MgCl_2$ (without antibiotics) and incubate at 30°C for 16-20 h.
10. Overlay the plate with 1 ml water containing 0.5 mg nalidixic acid (20 μl of 25 mg/ml stock; selectively kills *E. coli*) and 1.25 mg apramycin (25 μl of 50 mg/ml stock). Use a spreader to lightly distribute the antibiotic solution evenly. Continue incubation at 30°C.
11. Replica-plate each MS agar plate with single colonies onto DNA plates containing nalidixic acid (25 μg/ml) and apramycin (50 μg/ml) with and without kanamycin (50 μg/ml). Double cross-over exconjugants are $kanamycin^S$ and $apramycin^R$. (DNA gives fast, non-sporulating growth of *S. coelicolor*.)
12. $Kanamycin^S$ clones are picked from the DNA plates and streaked for single colonies on MS agar (promotes sporulation) containing nalidixic acid (25 μg/ml) and apramycin (50 μg/ml).
13. Confirm kanamycin sensitivity by replica-plating onto DNA plates containing nalidixic acid (25 μg/ml) with and without kanamycin (50 μg/ml).

14. Purified kanamycin sensitive strains are then verified by PCR and Southern blot analysis.

Antibiotic	Stock mg/ml	µl for 1 ml overlay	Final conc. after flooding µg/ml	Concentration in	
				MS, DNA µg/ml	R2YE µg/ml
Apramycin	50	25	50	50	50
Hygromycin[1]	40	25	40	40	NA
Kanamycin	50	100	200	50	200
Spectinomycin	200	25	200	400	400
Streptomcyin	10	25	10	10	10
Viomycin	30	25	30	30	NA
Nalidixic acid	25 in 0.3 M NaOH	20	20	25	25

[1]Note that in liquid DNB cultures with hygromycin, selection is imposed with hygromycin (40µg/ml), carbenicillin (10µg/ml) and kanamycin (10µg/ml).

Table 3: Antibiotic concentrations for selection on *S. coelicolor* MS conjugation plates, DNA replica plates or R2YE protoplast regeneration plates (Note some small differences from Kieser *et al*., 2000).

10. FLP-mediated excision of the disruption cassette

The disruption cassettes are flanked by FRT sites (FLP recognition targets). Expression of the FLP-recombinase in *E. coli* removes the central part of the disruption cassette, leaving behind a 81 bp "scar" sequence which, in the preferred reading frame (bold in Fig. 2), lacks stop codons.

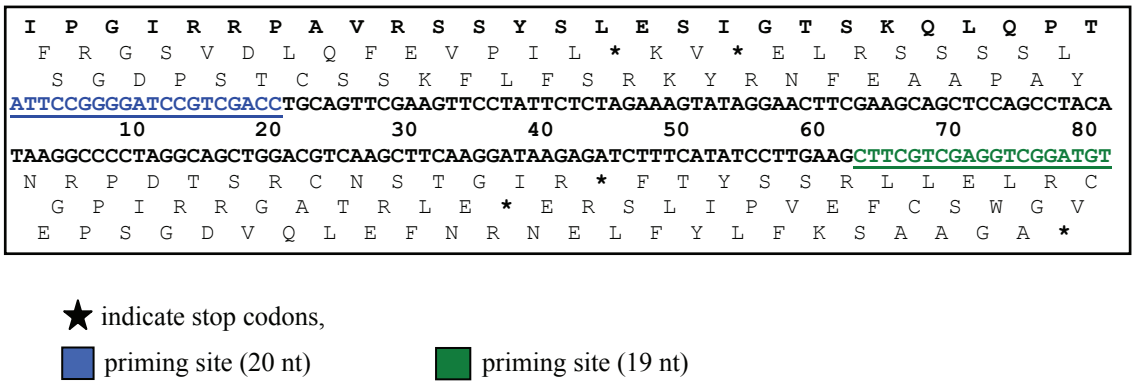

Fig.2: Sequence of the 81 bp "scar" sequence remaining after FLP-mediated excision of the disruption cassette. The translation of the preferred reading frame is printed bold. The 20 and 19 nt priming sites are underlined and printed in colour. (Fig. **1** explains the determination of the reading frame.)

This allows the generation of non-polar, unmarked in-frame deletions and repeated use of the same resistance marker for making multiple knock-outs in the same cosmid or in the same strain. *E. coli* DH5α cells containing the temperature sensitive FLP recombination plasmid BT340 (Datsenko and Wanner, 2000; can be obtained from the *E. coli* Genetic Stock Center: CGSC Strain# 7629) are transformed with the mutagenised cosmid DNA (obtained in section 6). BT340 contains ampicillin and chloramphenicol resistance determinants and is temperature-sensitive for replication (replicates at 30°C). FLP synthesis and loss of the plasmid are induced at 42°C (Cherepanov and Wackernagel, 1995).

1. Grow *E. coli* DH5α/BT340 overnight, with shaking at 30°C in 10 ml LB containing chloramphenicol (25 μg/ml).
2. Inoculate 100 μl *E. coli* DH5α/BT340 from overnight culture into 10 ml LB containing chloramphenicol (25 μg/ml).
3. Grow for 3-4 h at 30°C shaking at 200 rpm to an OD_{600} of ~ 0.4.
4. Recover the cells by centrifugation at 4000 rpm for 5 min at 4°C in a Sorvall GS3 rotor (or equivalent).
5. Decant medium and resuspend the pellet by gentle mixing in 10 ml ice-cold 10 % glycerol.
6. Centrifuge as above and resuspend pellet in 5 ml ice-cold 10 % glycerol, centrifuge and decant. Resuspend the cell pellet in remaining ~ 100 μl 10% glycerol.
7. Mix 50 μl cell suspension with ~ 100 ng (1-2 μl) of mutagenised cosmid DNA. Carry out electroporation in a 0.2 cm ice-cold electroporation cuvette using a BioRad GenePulser II (or equivalent) set to: 200 Ω, 25 μF and 2.5 kV. The expected time constant is 4.5 – 4.9 ms.
8. Immediately add 1 ml ice-cold LB to shocked cells and incubate shaking for 1 h at 30°C.
9. Spread onto LB agar containing apramycin (50 μg/ml) and chloramphenicol (25 μg/ml).
10. Incubate for 2 d at 30°C (*E. coli* DH5α/BT340 grows slowly at 30°C).
11. A single colony is streaked for single colonies on an LB agar plate without antibiotics and grown overnight at 42°C to induce expression of the FLP recombinase followed by the loss of plasmid BT340.
12. Make two masterplates by streaking 20 – 30 single colonies with a toothpick first on LB agar containing apramycin (50 μg/ml) and then on LB agar containing kanamycin (50 μg/ml).
13. Grow the masterplates overnight at 37°C. $Apramycin^S$ $kanamycin^R$ clones indicate the successful loss of the resistance cassette and are further verified by restriction, PCR or sequencing analysis.

11. Replacing resistance cassette inserts in *S. coelicolor* with the unmarked "scar" sequence

The chromosomal apramycin resistance cassette insert in *S. coelicolor* is replaced by the "scar" sequence. This is achieved by homologous recombination between the chromosome and the corresponding "scar cosmid" prepared in section 10. The procedure differs from section 9 because the cosmid lacks *oriT*, and the desired product is antibiotic sensitive. Therefore, it is necessary to introduce the scar cosmid into *Streptomyces* by protoplast transformation, and then select for kanamycin resistant *Streptomyces* containing the entire scar cosmid integrated by a single crossover. Restreaking to kanamycin-free medium, followed by screening for concomitant loss of kanamycin resistance and apramycin resistance, then identifies the desired *Streptomyces* clones. Note that if the target *Streptomyces* for mutagenesis carries a methyl-sensing restriction system (as is the case for *S. coelicolor* and *S. avermitilis*), it is necessary to passage the scar cosmid through the non-methylating *E. coli* host ET12567.

An alternative to introducing the scar cosmid by protoplast transformation is by using another targeting cassette that has been designed to target the *bla* gene in the Supercos backbone. Follow the same protocol as in sections 5 and 6 but substitute the amplified PCR product for the 1.3Kb restriction fragment of pIJ799 (*Eco*RI and *Hin*dIII digestion). Select overnight at 37°C using kanamycin (50µg/ml) and apramycin (50µg/ml). Now follow the protocol through all the remaining steps remembering that the single cross-over recombinants in *Streptomyces* will be apramycin and kanamycin resistant.

An alternative to FLP recombinase

It is possible to create unmarked in-frame deletion mutants using a restriction/ligation methodology. To do this requires the addition to the PCR primers of the restriction sites between the gene homology section of the long primers and the universal priming sites. It is recommended that the restriction enzymes are used in combination. There are four rare-cutting enzymes (i.e. for high GC DNA) that, when used in

combination, have the same single stranded overhang but once ligated together form a hybrid site that cannot be cut by either enzyme again (this is especially useful for the creation of multiple knockouts in the same cosmid). The 4 enzymes are *Spe*I, *Xba*I, *Nhe*I and *Avr*II (try to avoid using *Avr*II if possible due to an *Avr*II cut site in the Supercos backbone). Conventional use of the FLP recombinase is still available with such primers, but will create a 93-bp scar sequence instead of the conventional 81-bp scar.

Follow the protocol described for making knockouts in a cosmid, and once the knockout has been checked by restriction digestion the in-frame deletion can be constructed. It is advisable to use DNA from the BW25113 strain rather than from ET12567 for this process. Perform a double digest with the selected rare-cutting enzymes. Run out 5µl of the digest on an agarose gel to check digestion. A large band (approx 40kb) and a smaller band (1.4Kb) will be seen on the gel. Gel purify the larger band from the rest of the digest, and elute in 12µl. Set up a ligation using 3µl of the large fragment and incubate at 16°C overnight. Dialyse or precipitate the ligation and use it to transform DH5α competent cells. Plate onto LB containing kanamycin (50µg/ml) and carbenicillin (100µg/ml). Analysis of the colonies can be performed by making two masterplates, streaking 20 – 30 single colonies with a toothpick first on LB agar containing apramycin (50 µg/ml) and then on LB agar containing kanamycin (50 µg/ml). Grow the masterplates overnight at 37°C. Apramycin^{S} kanamycin^{R} clones indicate the successful loss of the resistance cassette and are further verified by restriction, PCR or sequencing analysis.
The scar transformants can then be introduced to *Streptomyces* by protoplast transformation or conjugation if the pIJ799 targeting cassette has been used.

Creation of gene knock-ins

The pIJ785 cassette has been designed to allow the introduction of the thiostrepton-inducible *tipA* promoter. The design of the primers follows the same rules as previously described except that one long primer includes homology to the *tipA*

promoter instead of the P2 priming site. All other steps of the protocol are as previously decribed.

12. Media

For more detailed information please see:

Kieser T, Bibb MJ, Buttner MJ, Chater KF and Hopwood DA (2000)

Practical Streptomyces Genetics, John Innes Foundation, Norwich Research Park, Colney, Norwich NR4 7UH, England

Mannitol Soya flour Medium (MS) Hobbs *et al.* (1989). Sometimes referred to as "SFM".

Agar	20 g
Mannitol	20 g
[1]Soya flour	20 g
Tap water	1000 ml

[1]Use soya flour from a health food shop or supermarket, not the expensive material from a laboratory supplier.

Dissolve the mannitol in the water and pour 200 ml into 250 ml Erlenmeyer flasks each containing 2 g agar and 2 g soya flour. Close the flasks and autoclave *twice* (115 ºC, 15 min), with gentle shaking between the two runs.

Difco nutrient agar (DNA)

Place 4.6 g Difco Nutrient Agar in each 250 ml Erlenmeyer flask and add 200 ml distilled water. Close the flasks and autoclave.

L agar

Agar	10 g
Difco Bacto tryptone	10 g
NaCl	5 g
Glucose	1 g
Distilled water	up to 1000 ml

Dissolve the ingredients, except agar, in the distilled water and pour 200 ml into 250-ml Erlenmeyer flasks each containing 2 g agar. Close the flasks and autoclave.

Difco nutrient broth (DNB)

Difco Nutrient Broth Powder	8g
Distilled water	1000ml

L (Lennox) broth (LB)

Difco Bacto tryptone	10 g
Difco yeast extract	5 g
NaCl	5 g
Glucose	1 g
Distilled water	up to 1000 ml

2 X YT medium

Difco Bacto tryptone	16 g
Difco Bacto yeast extract	10 g
NaCl	5 g
Distilled water	up to 1000 ml

SOB (SOC)

Difco Bacto tryptone	20 g
Difco Bacto yeast extract	5 g
NaCl	0.5 g
KCl	0.186g

Adjust to pH7 with 10N NaOH.

Distilled water	up to 1000 ml

For preparation of SOC, add 20 ml of 1M glucose (sterile) after autoclaving.

13. Trouble-shooting

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

a) *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. Gene replacement was found to be optimal with 200-300 ng of purified PCR-product. It is advisable to carry out a control PCR reaction without the Taq polymerase. Run 5µl of this reaction alongside the other PCR reactions. This allows determination of amplification efficiency, and the expected size change of a successful PCR reaction will also be visible.

b) *No transformants are obtained after PCR-targeting*. This common problem can mostly be resolved by using high quality electrocompetent cells. 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 using a pipette. Resuspend the pellet in the remaining drop of 10% glycerol (100-150 µl) and use this for electroporation. Also a second induction of the λ *red* genes can be performed by adding another aliquot of L-arabinose (final concentration is now 20mM) 30 min before harvesting the cells.

c) *Different colony sizes are obtained after PCR-targeting*. After 12 – 16 h 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 mutagenised 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.

d) *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.

e) *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).

f) *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 kanamycin sensitive 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 kanamycin resistant 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).

14. Literature

Bennett, P.M., Grinsted, J. and Richmond, M.H. (1977) *Mol.Gen.Genet*., **154**, 205-211

Bentley, S. D., Chater, K. F., Cerdeno-Tarraga, A. M., Challis, G. L., Thomson, N. R., James, K. D., Harris, D. E., Quail, M. A., Kieser, H., Harper, D., Bateman, A., Brown, S., Chandra, G., Chen, C. W., Collins, M., Cronin, A., Fraser, A., Goble, A., Hidalgo, J., Hornsby, T., Howarth, S., Huang, C. H., Kieser, T., Larke, L., Murphy, L., Oliver, K., O'Neil, S., Rabbinowitsch, E., Rajandream, M. A., Rutherford, K., Rutter, S., Seeger, K., Saunders, D., Sharp, S., Squares, R., Squares, S., Taylor, K., Warren, T., Wietzorrek, A., Woodward, J., Barrell, B. G., Parkhill, J., and Hopwood, D. A. (2002). Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417**(6885)**,** 141-147.

Cherepanov , P.P and Wackernagel, W. (1995) *Gene*, **158**, 9-14

Datsenko, K.A. and Wanner, B.L. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 6640-6645

Flett, F., Mersinias, V. and Smith, C.P. (1997) *FEMS Microbiol.Lett*., **155**, 223-229

Gust, B., Challis, G. L., Fowler, K., Kieser, T., and Chater, K. F. (2003) *Proc Natl Acad Sci U S A* **100**(4)**,** 1541-1546.

Gust, B., Chandra, G., Jakimowicz, D., Tian, Y., Bruton, C.J. and Chater, K.F. (2004) *Advances in Applied Microbiology* **54**: 107-28 **1.893**

Hobbs G, Frazer CM, Gardner DCJ, Cullum JA, Oliver SG (1989) *Appl.Microbiol.Biotechnol*., **31**, 272-277.

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F. and Hopwood, D.A. (2000) *Practical Streptomyces Genetics*: John Innes Foundation, Norwich Research Park, Colney, Norwich NR4 7UH, UK

MacNeil, D.J., Gewain, K.M., Ruby, C.L., Dezeny, G., Gibbons, P.H. and MacNeil, T. (1992) *Gene*, **111**, 61-68

Matsushima ,P., Broughton, M.C., Turner, J.R. and Baltz, R.H. (1994) *Gene*, **146**, 39-45

Muyrers, J. P., Zhang, Y., and Stewart, A. F. (2000) *Genet Eng (N Y)* **22,** 77-98.

Paget, M. S., Chamberlin, L., Atrih, A., Foster, S. J., and Buttner, M. J. (1999). Evidence that the extracytoplasmic function sigma factor sigmaE is required for normal cell wall structure in *Streptomyces coelicolor* A3(2). *J Bacteriol* **181**(1)**,** 204-211.

Redenbach, M., Kieser, H.M., Denapaite, D., Eichner, A., Cullum, J., Kinashi, H. and Hopwood, D.A. (1996) *Mol. Microbiol*., **21**, 77-96

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Habor, NY.

Zhang, Y., Buchholz, F., Muyrers, J. P., and Stewart, A. F. (1998) *Nat Genet* **20**(2)**,** 123-128.