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Expression of Cre recombinase during transient phage infection permits efficient marker removal in *Streptomyces*

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ABSTRACT

We report a system for the efficient removal of a marker flanked by two *loxP* sites in *Streptomyces coelicolor*, using a derivative of the temperate phage ϕC31 that expresses Cre recombinase during a transient infection. As the test case for this recombinant phage (called Cre-phage), we present the construction of an in-frame deletion of a gene, *pglW*, required for phage growth limitation or Pgl in *S.coelicolor*. Cre-phage was also used for marker deletion in other strains of *S.coelicolor*.

INTRODUCTION

Bacteria in the genus *Streptomyces* are important producers of antibiotics and other pharmacologically active compounds. These bacteria are also important model organisms for bacterial development, as they have a mycelial growth habit and a sporulation phase. *Streptomyces* spp. are relatively easy to manipulate genetically, thanks to tried and tested methods and customized tools accumulated over the years (1–3). Recently the application of the λ Red recombination proteins to manipulate the ordered *Streptomyces coelicolor* cosmid library in *Escherichia coli* has greatly facilitated the construction of targeted mutants (2). Adaptations to the original process as described by Datsenko and Wanner (4) have tuned the materials and procedures for their specific use in *Streptomyces*, and this approach is known as the REDIRECT system (2). As with the original Datsenko and Wanner procedure many of the targeting cassettes for REDIRECT are constructed to allow the use of the FLP/*frt* recombination system for optional marker removal (4). All that is left after FLP/*frt* recombination is an 81 bp 'scar' containing one *frt* site and the cassette primer-binding sequences. The drawback in the REDIRECT system is that FLP/*frt* recombination currently has to be performed in *E.coli*, after which the unmarked cosmid is introduced into *Streptomyces* by protoplast transformation, or manipulated further to introduce an *oriT* for conjugation. Attempts to clone the FLP determinant into *Streptomyces* phage or plasmid vectors have not been successful (C. Bruton, personal communication), possibly due to the very high A+T content of the *FLP* gene.

We present here the use of the Cre-*loxP* system from bacteriophage P1 for marker removal in *Streptomyces*. In the new system, the Cre determinant is introduced into *Streptomyces* during infection by an engineered derivative ('Cre-phage') of ϕC31, a bacteriophage that has been widely studied as a model temperate phage of *Streptomyces*, and extensively exploited as a cloning vector (1). As a test case for this new tool we chose to construct an in-frame deletion in a gene, *pglW*, whose product is thought to be required for the phage growth limitation (Pgl) system in *S.coelicolor* A3(2) (5). The Pgl system is characterized by inability of phages of the ϕC31 family to form plaques on wild-type (i.e. Pgl$^+$) *S.coelicolor* including the M145 strain used here. This bacterium encodes two closely located operons, *pglWX* and *pglYZ*, that were shown previously to confer the Pgl phenotype (5). Currently the mechanism of Pgl is not understood, but bioinformatic analysis of the predicted gene products suggests that PglW is a serine-threonine protein kinase, PglX is a DNA adenine methyltransferase, PglY is an ATPase and PglZ is a protein of unknown function. As both *pglW* and *pglY* are the first genes in their respective two-gene operons, the availability of in-frame gene knockouts is a requirement for further analysis of this system.

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Table 1. Oligonucleotides

Oligonucleotide	Sequence
SL6	5′-GAAGCCCGGGTGAGCCGGGTTGCCGACTCCCTTACGGGTCCCTGAGAGCAACTACGAAGGGGAGTCAGTATGTCCAA TTTCCTGACCGTACACCAAAATTTG
SL5	5′-TTACGCGTTAACGGCTAATCGCCATCTTCCAGCAGGCGC
774FLOXP	5′-TGTAGGCTGGAGCTGCTTCGATAACTTCGTATAATGTATGCTATACGAAGTTATGGAATAGGAACTTATGAGCTC
774RLOXP	5′-ATTCCGGGGATCCGTCGACCCATAACTTCGTATAGCATACATTATACGAAGTTATGAAGTTCCCGCCAGCCTCGC
LJM5	5′-GCGGTCCTTGAAGGACACGAGGAGCGGGGAACAGGCATGATTCCGGGGATCCGTCGACC
LJM6	5′-GCTGCTTCAGGTCGTTCAACAGAGCCTTGCGGTCGATCATGTAGGCTGGAGCTGCTTC
LJM11	5′-CGGACGCCTATCCACTCACC
LJM12	5′-ACCTCATCGAGCGCCTTCAC

Although an in-frame deletion of part of the *pglY* ORF had been constructed previously (6), we had not been able to construct an in-frame deletion of *pglW* (5). The requirement for *pglW* in the Pgl system was inferred using an insertion in *pglW*, which could have also prevented expression of *pglX* due to polarity. Therefore, to complement the *pglX* requirement in the *pglW* insertion mutant, a second copy of *pglX* was introduced ectopically expressed from the *ptipA* promoter (5). Although this strain was Pgl^- there was still the possibility that the phage sensitivity was due to incomplete complementation of *pglX*. We show here, using Cre-phage for marker removal, that an in-frame deletion of *pglW* is indeed Pgl^- and can be complemented by addition of a wild-type allele of *pglW*.

MATERIALS AND METHODS

Bacterial strains

E.coli DH5α was used as a general cloning host (7) and *E.coli* BW25113(pIJ790) was used for the λ Red recombination reactions (2). *S.coelicolor* strain M145 was used as the parent strain for the generation of the Δ*pglW* ($SLMW_4$1-4) and Δ*pglY* ($SLMY_4$3 and $SLMY_4$4) mutants. J1929 [$pglY^-$; (6)] was used as an indicator strain for ϕC31 derivatives KC515 and Cre-phage.

Bacteriophages and plasmids

A derivative of the ϕC31-based cloning vector KC515 (1) was constructed that contained an allele of *cre* that had been manipulated for expression during phage infection. The *cre* gene was amplified by PCR using Expand High Fidelity Polymerase (Roche Molecular Biochemicals), according to the manufacturer's instructions and with p705-Cre (8) as a template. The annealing temperature was 55°C. The upstream primer, SL6 (Table 1), was designed to introduce a ribosome-binding site and a phage-specific promoter known to be activated during lytic growth (9). The downstream primer was SL5 (Table 1). The modified *cre* gene was cloned initially into the TA vector, pDK101, cut with XmnI (10) to form pSL36, and then excised via PstI and HpaI restriction sites and inserted into KC515 cut with PstI and ScaI to form Cre-phage. A high titre stock of Cre-phage was prepared using *S.coelicolor* J1929 as an indicator strain and using the plate soak out method as described in Kieser *et al.* (1).

To test the activity and efficiency of Cre-phage in marker removal via Cre-*loxP* recombination we constructed a mutant *pglW* using a novel cassette vector, pIJ774, for REDIRECT (2). This plasmid contains *loxP* sites flanking the *aac3(IV)* marker encoding apramycin resistance and the *oriT* site. The apramycin resistance marker *aac*(3)*IV* and the origin of transfer *oriT* from RK2 were jointly amplified from the 1383 bp EcoRI/HindIII pIJ773 disruption cassette (2) with primers 774FLOXP and 774RLOXP (Table 1). Amplification was performed as described previously (2). The resulting 1363 bp fragment was inserted into the EcoRV sites of pBluescript II SK(+), resulting in pIJ774. Sequence analysis revealed a single base pair deletion of a 'G' within the primer sequence, P1, of pIJ774 so that the sequence is 5′-ATTCCGGGATC-CGTCGACC. In the REDIRECT procedure P1 and P2 are used to prime amplification of the cassettes encoding the markers/*oriT*. Despite the sequence change in pIJ774, the original P1 sequence (5′-ATTCCGGGGATCCGTCGACC) was used for the primers, LJM5 and LJM6, in this work. Ultimately the deletion in the pIJ774 P1 sequence had no adverse effect on formation of the scar sequence, i.e. no unwanted frameshifts were introduced (see below). Our current experiments employ a modified P1 sequence ($P1_{774}$ 5′-TATTCCGGGATCC-GTCGACC).

RESULTS AND DISCUSSION

The *pglW* gene is located on cosmid SC1F2 (11,12). pIJ774 was used as a template in a PCR with primers LJM5 and LJM6 to generate the recombination substrate for replacement of *pglW* in SC1F2. Primers were designed using the BMW software provided with the REDIRECT system (13). The initiation codon, GTG, for *pglX* was replaced with the more efficient ATG to compensate for the removal of a putative ribosome-binding site during deletion. An alternative strategy for primer design would have been to move the deletion end point 21 bp upstream thus leaving in place the putative translational signals for optimal expression of *pglX*. The PCR products were digested with DpnI overnight (37°C) and then introduced by electroporation into BW25113(pIJ790) containing SC1F2 (2). About 70% of the apramycin resistant colonies obtained contained mutant cosmids. Two independently mutated cosmids, SC1F2::Δ$pglW_4$1 and SC1F2::Δ$pglW_4$2, were introduced into ET12567 (pUZ8002) and transferred to *S.coelicolor* M145 by conjugation, selecting for apramycin resistance (1). Apramycin-resistant, kanamycin-sensitive clones were subcultured and tested for phage resistance. All of the apramycin-resistant, kanamycin-sensitive clones tested supported plaque formation with ϕC31 and were therefore Pgl^-. Two clones, $SLMW_4$1 and $SLMW_4$2, derived from each of the two independently mutated cosmids, were chosen for marker removal with Cre recombinase.

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               >>>>>>>>>>>>>>>>>>>  *********************
GGGAACAGGCATGATTCCGGGGATCCGTCGACCCATAACTTCGTATAGCATACATTA
          M  I  P  G  I  R  R  P  I  T  S  Y  S  I  H
          ↳ ΔpglW

*********** <<<<<<<<<<<<<<<<<<<<
TACGAAGTTATCGAAGCAGCTCCAGCCTACATGATCGACCGCAA
Y  T  K  L  S  K  Q  L  Q  P  T  -
                              M  I  D  R
                              ↳ pglX
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Figure 1. Deletion of *pglW* in M145. (**A**) Schematic representation showing (i) the use of the pIJ774 REDIRECT disruption cassette in the generation of a Δ*pglW* gene replacement, (ii) the organization of the SC1F2::Δ*pglW* gene replacement in the mutated cosmids, SC1F2::Δ*pglW*$_4$1 and SC1F2::Δ*pglW*$_4$2, and in the ex-conjugants SLMW$_4$1 and SLMW$_4$2 and (iii) the remaining *loxP* site after removal of *aac3(IV)*/*oriT* using Cre-phage. The black arrowheads represent the *loxP* sites and are in direct repeat; the arrows P1 and P2 show the locations of the primer-binding sites used to amplify the REDIRECT cassette from pIJ774 with the custom primers. (**B**) PCR amplification of the *pglW* region from the various strains of *S.coelicolor* generated using the REDIRECT/Cre-phage method. SLMW$_4$1 and SLMW$_4$2 are two independently isolated ex-conjugants from ET12567(1 SC1F2::Δ*pglW*$_4$1) and ET12567(1 SC1F2::Δ*pglW*$_4$2) conjugation with M145. SLMW$_4$3 clones a-d and SLMW$_4$4 clones a-d are derivatives from SLMW$_4$1 and SLMW$_4$2, respectively, that have survived Cre-phage infection and have lost the apramycin resistance marker. The expected PCR products of the *pglW* locus amplified using the primers LJM11 and LJM12 are 5099 bp when M145 is the template, 1794 bp with SLMW$_4$1 and SLMW$_4$2 as templates and 507 bp with SLMW$_4$3 clones a-d and SLMW$_4$4 clones a-d as templates. The markers are the 100 bp ladder from new England Biolabs. Arrows indicate the expected positions of the 1794 and 507 bp fragments. The predicted 5099 bp product from M145 was not observed, probably because the conditions for PCR precluded synthesis of such a large product. (**C**) DNA sequence of the scar sequence obtained from SLMW$_4$3 clone a and SLMW$_4$4 clone b. The initiation codon for *pglW* initiates the synthesis of a 26 amino acid peptide (sequence shown below the DNA sequence) ending in a termination codon, TGA, that overlaps with the initiation codon, ATG for *pglX*. The arrowheads and asterisks above the DNA sequence show the cassette primer sequences, P1 and P2, and the *loxP* site, respectively.

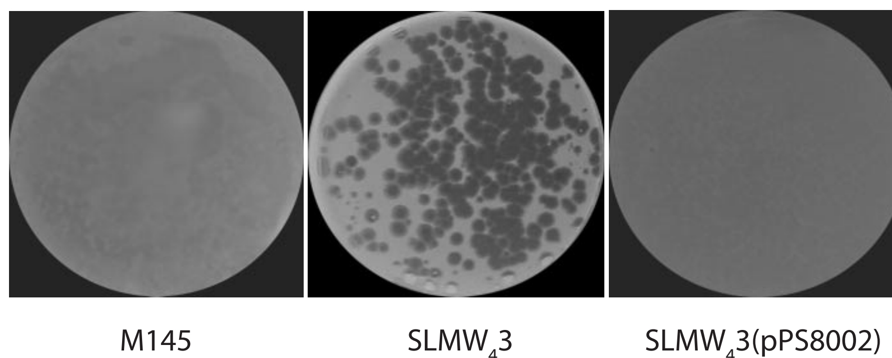

Figure 2. Phage sensitivity of the SLMW$_4$3 Δ*pglW* strain. Difco nutrient agar plates containing 10 mM $MgSO_4$ and 8 mM $Ca(NO_3)_2$ were inoculated with ~300 p.f.u. of φC31*c*Δ*25* (22) and overlaid with soft nutrient agar containing M145, SLMW$_4$3 or SLMW$_4$3(pPS8002) spores. pPS8002, constructed previously, integrates into the *attB* site for φC31 and encodes a functional *pglW* allele expressed from the *ptipA* promoter (5).

We chose as a delivery vector for Cre, the cloning vector KC515, a derivative of the phage φC31 (1). KC515 has a functional phage repressor and a defective *int* gene, encoding the integrase, and no *attP* site. This should result in some phage infections that enter the lysogenic pathway but fail to persist as stable lysogens. It is not clear in φC31 infections at what point the decision is made to enter lysogeny. The developmental cycle begins with transcription of the early lytic genes from early promoters (9,14,15). It is likely that at some point during early lytic growth a decision between lysis and lysogeny is made and the phage repressor shuts down lytic transcription resulting in cessation of DNA replication (16). As KC515 (and Cre-phage) has no efficient means of integration into the host chromosome, the phage DNA will virtually always be lost by dilution or nucleolytic activity. In addition, a proportion of cells within φC31 plaques survive infection through some ill-defined phenotypic resistance. Thus there are at least two routes by which cells infected by Cre-phage might survive yet have experienced a burst of Cre synthesis. Approximately 10^7 spores of SLMW$_4$1 and SLMW$_4$2 were plated on R2YE plates and 5×10^6 p.f.u. (in 100 μl) of Cre-phage were inoculated onto the centre of each plate. After overnight incubation the areas of slightly reduced growth, indicative of phage infection, were marked on the base of the plates. Incubation was continued until a whitish layer of spores appeared within the infected areas. The spores were harvested in sterile water, and 10-fold dilutions were plated on mannitol soya (MS) medium. Replica-plating of plates containing between 15 and 60 colonies onto NA plates with or without apramycin was used to identify apramycin-sensitive colonies. The frequency of apramycin-sensitive clones varied from ~30 to ~60%. Eight apramycin sensitive colonies, SLMW$_4$3 clones a–d and SLMW$_4$4 clones a-d, derived from Cre-phage-infected SLMW$_4$1 and SLMW$_4$2, respectively, were subcultured and genomic DNA samples were prepared. PCR, using primers LJM11 and LJM12, generated an expected product of 507 bp from SLMW$_4$3 clones a-d and SLMW$_4$4 clones a-d and 1794 bp from SLMW$_4$1 and SLMW$_4$2 genomic DNA templates (Figure 1B). The 507 bp product was sequenced from four isolates and found to contain the expected scar sequence of 81 bp (Figure 1B). The supernatants from spore preparations of SLMW$_4$3 clones a-d and SLMW$_4$4 clones a-d were tested for any remaining phage by plating with indicator spores, but no plaques were obtained, indicating that Cre-phage failed to persist through subculture. When this assay is performed with a spore preparation of a φC31 lysogen, the titre is usually between 10^3–10^4 pfu/ml (M.C.M. Smith, unpublished data). SLMW$_4$3 clones a-d and SLMW$_4$4 clones a-d were able to plaque φC31, and could be complemented to phage resistance by integration of pPS8002 encoding PglW fused to 6× His tag and expressed from *ptipA* (5) (Figure 2). This experiment provided proof that *pglW* is indeed required for Pgl. We have also used this technique to generate knockout mutations of the complete *pglY* ORF (SLMY$_4$3 and SLMY$_4$4).

This simple and reliable technique for marker removal greatly facilitates the construction of in-frame, unmarked mutants in *Streptomyces*. As φC31 has a fairly broad host range within the genus *Streptomyces* [(17,18) and D. Cowlishaw and M. C. M. Smith, unpublished data], Cre-phage should be applicable to many species. Although we have used the technique for marker removal in a Pgl^- strain that can support plaque formation by φC31 and derivatives, it can also be used in Pgl^+ strains. This is because φC31 (and therefore Cre-phage) prepared from a Pgl^- strain can undergo a full lytic cycle in a Pgl^+ strain releasing progeny phage. However multiplication of this progeny phage in further infectious cycles is severely attenuated resulting in an inability to form plaques (19). To demonstrate the use of Cre-phage in the Pgl^+ strain *S.coelicolor* M145, SCO6073, the putative cyclase, *cyc2*, previously shown to be required for biosynthesis of geosmin (2), was deleted. Using the same primers as described previously [(2) Sc9B1.20forw and Sc9B1.20rev] and pIJ774 as a template, SCO6073 was replaced in cosmid SC9B1 with apramycin/*oriT* flanked by the two *loxP* sites. Apramycin-resistant, kanamycin-sensitive exconjugants were treated with Cre-phage and an infected zone could still be discerned after overnight incubation. Spores from the infected area were streaked on MS for single colonies. Replica plating onto DNA plates with and without apramycin revealed the loss of the resistance marker in ~90% of colonies. DNA of three apramycin-sensitive mutants was analysed by PCR using primers 9B1.20T1 and 9B1.20T2 (2) resulting in a PCR product of 1353 bp (data not shown). This fragment was sequenced and the *loxP*-scar was identical to the scar sequence in Figure 1. Cre-phage has also been used by others for marker removal in a derivative of M145 (F. Barona-Gomez and G. Challis, personal communication).

Marker deletion via Cre-phage in *Streptomyces* leaves a 'scar' containing a *loxP* site, and this could potentially be a target for undesirable rearrangements, if one should need to generate multiple mutants. Whilst the transient nature of Cre expression from Cre-phage will help to minimize recombination between distant *loxP* sites, a more effective deterrent to these unwanted rearrangements would be for the genomic interval to contain an essential gene. Another way to solve this problem would be to incorporate variant *loxP* sites into the REDIRECT cassettes that can only recombine with each other, e.g. *lox2722* recombines efficiently with another *lox2722* but not with a *loxP* site (20,21). Furthermore, there may be occasions when one might wish to make two knockouts in the same cosmid in *E.coli*. One way of doing this may be to combine the use of the FLP/*frt* and Cre/*loxP*. The first mutation could be made using the FLP/*frt* sites on the REDIRECT cassette, pIJ773, and the second mutation made using the pIJ774 cassette. The doubly mutated cosmid would then be transferred to *Streptomyces* by conjugation selecting for apramycin resistance, and Cre-phage used to remove the marker. Overall the use of Cre-phage in marker removal represents an improvement to the current use of the FRT/*flp* and adds further versatility to the REDIRECT system.

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