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/flrt recombination system for optional marker removal (4). All that is left after FLP/flrt recombination is an 81 bp ‘scar’ containing one flrt site and the cassette primer-binding sequences. The drawback in the REDIRECT system is that FLP/flrt 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 bacteriophage 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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Although an in-frame deletion of part of the \textit{pglY} ORF had been constructed previously (6), we had not been able to construct an in-frame deletion of \textit{pglW} (5). The requirement for \textit{pglW} in the \textit{Pgl} system was inferred using an insertion in \textit{pglW}, which could have also prevented expression of \textit{pglX} due to polarity. Therefore, to complement the \textit{pglX} requirement in the \textit{pglW} insertion mutant, a second copy of \textit{pglX} was introduced ectopically expressed from the \textit{pglW} promoter (5). Although this strain was \textit{Pgl}^− there was still the possibility that the phage sensitivity was due to incomplete complementation of \textit{pglX}. We show here, using Cre-phage for marker removal, that an in-frame deletion of \textit{pglW} is indeed \textit{Pgl}^− and can be complemented by addition of a wild-type allele of \textit{pglW}.

**MATERIALS AND METHODS**

**Bacterial strains**

\textit{E.coli} DH5α was used as a general cloning host (7) and \textit{E.coli} BW25113(pIJ790) was used for the \textit{λ} Red recombination reactions (2). \textit{S.coelicolor} M145 was used as the parent strain for the generation of the \textit{ΔpglW} (SLMW\textsubscript{4,1−4}) and \textit{ΔpglY} (SLMY\textsubscript{4,3} and SLMY\textsubscript{4,4}) mutants. J1929 [\textit{pglY} ; (6)] was used as an indicator strain for \textit{φC31} derivatives KC515 and Cre-pha ge.

**Bacteriophages and plasmids**

A derivative of the \textit{φC31}-based cloning vector KC515 (1) was constructed that contained an allele of \textit{cre} that had been manipulated for expression during phage infection. The \textit{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, SL5 (Table 1), was designed to introduce a 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 \textit{pglX}. The downstream primer was SL5 (Table 1). The modified \textit{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 Scal to form Cre-pha ge. A high titre stock of Cre-pha ge was prepared using \textit{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-pha ge in marker removal via CRE-lossP recombination we constructed a mutant \textit{pglW} using a novel cassette vector, pIJ774, for REDIRECT (2). This plasmid contains \textit{loxP} sites flanking the \textit{aac3(IV)} marker encoding apramycin resistance and the \textit{oriT} site. The apramycin resistance marker \textit{aac3(IV)} and the origin of transfer \textit{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\textplus, 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′-ATTCGGGGATC-CGTGACC. 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′-ATTCGGGGATCGTGACC) 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\textsubscript{774} 5′-TATTCCGGGATC-GTGACC).

**RESULTS AND DISCUSSION**

The \textit{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 \textit{pglW} in SC1F2. Primers were designed using the BMW software provided with the REDIRECT system (13). The initiation codon, GTG, for \textit{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 \textit{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::\textit{ΔpglW}\textsubscript{1} and SC1F2::\textit{ΔpglW}\textsubscript{2}, were introduced into ET12567 (pUZ8002) and transferred to \textit{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 \textit{φC31} and were therefore \textit{Pgl}^−. Two clones, SLMW\textsubscript{4,1} and SLMW\textsubscript{4,2}, derived from each of the two independently mutated cosmids, were chosen for marker removal with Cre recombinase.

### Table 1. Oligonucleotides

| Oligonucleotide | Sequence                                      |
|-----------------|-----------------------------------------------|
| SL6             | 5′-GAAGCCCGGTTGACCCGGGTTTGCGACTCCCTATCGGTCTCCGAGCACTACGAGAAGGGAGTCGATATGATAACCAAACAAAATTTGG TTTCTCGACCTACCAACAAATTTGG |
| SL5             | 5′-TTACGGGTATACGGCTAATCCGGCTACATCCAGCGCGGAGCC |
| 774FLOXP        | 5′-ATTCCGGGGATCCTGGACCCCATATACTTCGTATAGCATACTTATACGAAGTTATGTAAGATGTTCGGGACCCTCGC |
| 774RLOXP        | 5′-GCTGCTTACGGTCTTACAAAGACGAGCCTTGGCCTATCAGTAGTGGCTTGAAGCAGTCTTTC |
| LJM5            | 5′-GGCTCTTTGAGAACACGAGGACGGGAGGGAACAGGCATGATTTCCGGGGATCCGTCGACC |
| LJM6            | 5′-GCTGCTTACGGTCTTACAAAGACGAGCCTTGGCCTATCAGTAGTGGCTTGAAGCAGTCTTTC |
| LJM11           | 5′-CCGAGGCTATACCCGGTCCACC |
| LJM12           | 5′-ACCTCTATCGAGGCGCTTCAC |

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Figure 1. Deletion of \( \text{pglW} \) in M145. (A) Schematic representation showing (i) the use of the plJ774 REDIRECT disruption cassette in the generation of a \( \Delta \text{pglW} \) gene replacement, (ii) the organization of the SC1F2::\( \Delta \text{pglW} \) gene replacement in the mutated cosmids, SC1F2::\( \Delta \text{pglW} \), and in the ex-conjugants SLMW\(_1\) and SLMW\(_2\), and (iii) the remaining \( \text{loxP} \) site after removal of \( \text{aac3(IV)}/\text{oriT} \) using Cre-phage. The black arrowheads represent the \( \text{loxP} \) 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 plJ774 with the custom primers.

(B) PCR amplification of the \( \text{pglW} \) region from the various strains of \( S.\text{coelicolor} \) generated using the REDIRECT/Cre-phage method. SLMW\(_1\) and SLMW\(_2\) are two independently isolated ex-conjugants from ET12567(1\ SC1F2::\( \Delta \text{pglW} \)) and ET12567(1\ SC1F2::\( \Delta \text{pglW} \)) conjugation with M145. SLMW\(_3\) clones a-d and SLMW\(_4\) clones a-d are derivatives from SLMW\(_1\) and SLMW\(_2\), respectively, that have survived Cre-phage infection and have lost the apramycin resistance marker. The expected PCR products of the \( \text{pglW} \) locus amplified using the primers LJM11 and LJM12 are 5099 bp when M145 is the template, 1794 bp with SLMW\(_1\) and SLMW\(_2\) as templates and 507 bp with SLMW\(_3\) clones a-d and SLMW\(_4\) 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\(_3\) clone a and SLMW\(_4\) clone b. The initiation codon for \( \text{pglW} \) 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 \( \text{pglX} \). The arrowheads and asterisks above the DNA sequence show the cassette primer sequences, P1 and P2, and the \( \text{loxP} \) site, respectively.
We chose as a delivery vector for Cre, the cloning vector KC515, a derivative of the phage φ31 (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 if φ31 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 φ31 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 φC31 activity. Approximately 10^7 spores of SLMW41 and SLMW42 derivatives, it can also be used in PglW strain. Difco nutrient agar plates containing 10 mM MgSO4 and 8 mM Ca(NO3)2 were inoculated with ~300 p.f.u. of φC31ΔC25 (22) and overlaid with soft nutrient agar containing M145, SLMW3, or SLMW4(pPS8002) spores. pPS8002, constructed previously, integrates into the attB site for φC31 and encodes a functional pglW allele expressed from the pglA promoter (5).

This fragment was sequenced and the loxP-scar was identical to the scar sequence in Figure 1. Cre-phage failed to persist through subculture. When this assay is performed with a spore preparation of φC31 lysogen, the titre is usually between 10^3–10^4 pfu/ml (M.C.M. Smith, unpublished data). SLMW3 clones a-d and SLMW4 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 pglA (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 (SLMY3 and SLMY4).

This simple and reliable technique for marker removal greatly facilitates the construction of in-frame, unmarked mutants in Streptomyces. As φ31 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 PglW 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 PglW 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 PglW 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 pU774 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 on 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 9B1.20 forw and 9B1.20 rev 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/flp and Cre/loxP. The first mutation could be made using the FLP/flp 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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**Conflict of interest statement.** None declared.

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