Broad-Host-Range cre-lox System for Antibiotic Marker Recycling in Gram-Negative Bacteria

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ABSTRACT

Complete genome sequences are now available for many bacterial species that lack sophisticated genetic tools. We describe the development of a broad-host-range cre-lox system that allows antibiotic marker recycling in a variety of Gram-negative bacteria. This system consists of an allelic exchange vector bearing a kanamycin cassette flanked by loxP sites and a tetracycline-resistant IncP plasmid that provides expression of the Cre recombinase. We demonstrate this system by generating unmarked deletions of genes in two different bacteria, Methylobacterium extorquens AM1 and Burkholderia fungorum LB400. This new antibiotic marker recycling system offers the possibility of creating unmarked mutants in a wide variety of Gram-negative bacteria. Furthermore, marker recycling allows the generation of strains bearing multiple genetic manipulations in organisms for which few antibiotic markers are currently available.

INTRODUCTION

The availability of complete genome sequences for a wide variety of microbial species has revolutionized microbiology. Although bioinformatics tools permit phylogenetic classification of the majority of the putative gene products into conserved classes (16), the annotation of function based on sequence alone is still, by its very nature, a hypothesis. Experimentation is required to test and refine these hypotheses. For many of these organisms, however, the genetic toolkit available is limited, at best. One key genetic hurdle slowing progress is the lack of facile tools to generate unmarked mutant strains in a wide variety of organisms. The generation of unmarked mutants allows for multiple genetic manipulations of organisms for which few antibiotic markers exist. In recent years, a growing number of systems for antibiotic marker recycling in non-enteric bacteria have been reported that utilize a variety of site-specific recombination systems and antibiotic markers (5). These include the utilization of the RP4 multimer resolution system (8) and both the yeast Flp/FRT (7) and P1 phage cre/lox (2) site-specific recombination systems. This paper describes the development of a simple broad-host-range antibiotic marker recycling system based on cre-lox methodology. Cre recombinase is a site-specific recombinase from the P1 phage that catalyzes in vivo excision of DNA regions flanked by colinear loxP recognition sites (12). The system we describe consists of a mobilizable allelic exchange vector with a loxP-flanked antibiotic resistance cassette, pCM184 or pCM351, and an IncP plasmid that expresses the Cre recombinase, pCM157 or pCM158. We demonstrate the broad utility of this system by generating unmarked mutant strains of two phylogenetically distinct Gram-negative bacteria, Methylobacterium extorquens AM1 (an ß-proteobacterium) and Burkholderia fungorum LB400 (a ß-proteobacterium).

MATERIALS AND METHODS

Media and Growth Conditions

M. extorquens AM1 (11) and B. fungorum LB400 (3) strains were
grown on a minimal salts medium (1) containing carbon sources at the following levels, 0.2% citrate, 0.5% (v/v) methanol, and 0.4% (w/v) succinate. E. coli strains were grown on LB medium (14) (strains and plasmids are described in Table 1). Antibiotics were added at the following final concentrations, unless noted: 50 µg/mL ampicillin, 10 µg/mL chloramphenicol, 50 µg/mL (for E. coli and M. extorquens AM1), or 20 µg/mL (for B. fungorum LB400) kanamycin, 50 µg/mL rifampicin, 35 µg/mL streptomycin, and 10 µg/mL tetracycline. Chemicals were obtained from Sigma (St. Louis, MO, USA). Nutrient agar and Bacto-agar were obtained from Difco (Detroit, MI, USA). Conjugation was performed as described previously (4).

Construction of a Broad-Host-Range cre-lox System for Antibiotic Marker Recycling

Two allelic exchange vectors, pCM184 and pCM351 (Figure 1), were created by inserting loxP-bounded antibiotic resistance cassettes into a variant of the mobilizable suicide plasmid, pAYC61 (4). The 1.3-kb HindII frag-

Figure 1. Plasmid map of the allelic exchange vectors pCM184 and pCM351. Antibiotic resistances are encoded by bla (ampicillin), aacC1 (gentamycin), kan (kanamycin), and tet (tetracycline). The boxes indicate the pair of loxP sites (loxP), the IncP origin of transfer (oriT), and the ColE1 origin of replication (ColE1 ori). The unique restriction sites present in the multiple cloning sites are indicated.

Figure 2. Plasmid map of the cre expression plasmids pCM157 and pCM158. The E. coli lacZp drives expression of Cre recombinase. These two plasmids differ only in the antibiotic resistance genes present, tetAR (tetracycline) or kan (kanamycin). The boxes indicate the IncP origin of replication (oriV), the ColE1 origin of replication (ColE1 ori), and the IncP origin of transfer (oriT).
ment bearing the kanamycin resistance cassette from pUC4K (17) was inserted into pLox1 (12), which had been cut with XbaI and blunt-ended, to create pCM161. To introduce convenient multiple cloning sites, the loxP-bounded kanamycin resistance cassette of pCM161 was amplified with following primer pairs, CM-mrkMCS, 5'-TGACGTCTAGATCCATGGTACATGTTAGCATATGGCGGCCGCA-3'; and CM-frkMCS, 5'-GACTAGTGGACTCACGGTTAACACGCGTACGATAGGCCCCTCGGTATCGATAAGCTGGATCC-3'. The resulting 1.4-kb PCR product was purified and cloned into pCR2.1 (Invitrogen, Carlsbad, CA, USA) to create pCM183. To preserve useful cloning sites, pAY661 was cut with EcoRI and Smal, blunted using T4 DNA polymerase, and self-ligated to produce pCM182. Finally, the 1.4-kb AatI-SpeI fragment from pCM183 containing the loxP-flanked kanamycin resistance cassette was ligated between the AatI and XbaI sites of pCM182 to create pCM184 (GenBank® accession no. AY093429). A gentamicin-resistance conferring version, pCM351, was also generated. The loxP-flanked gentamyce resistance cassette (encoded by aacC1) was amplified from pLoxGen4 (12), using CM-urtkMCS and CM-dfrMCS, and cloned into pCR2.1 (Invitrogen) to produce pCM350. The 1-kb AatI/SacI fragment from pCM350 was cloned between the AatI and SacI sites of pCM184 to generate pCM351 (GenBank accession no. AY093430).

Two broad-host-range cre expression vectors, pCM157 and pCM158 (Figure 2), were created based on a pair of small, mobilizable IncP plasmids (10). The 1.1-kb XbaI-EcoRI fragment from pJW168 (19) was cloned between the XbaI and EcoRI sites of pCM62 (10) to generate the tetracycline-resistance conferring cre expression plasmid pCM157. A kanamycin-resistant version, pCM158, was generated by cloning the same XbaI-EcoRI fragment from pJW168 between the XbaI and EcoRI sites of pCM66 (10). These plasmids contain cre behind the E. coli lac promoter. In M. extorquens AM1, this promoter provides only low constitutive activity (10). Despite this low expression, the majority of cells obtained from the first passage onto plates lacking kanamycin are already kanamycin sensitive (data not shown).

**Generation of a Δfar Mutant of M. extorquens AM1**

M. extorquens AM1 mutants defective for far (encodes formaldehyde-activating enzyme) (18) were generated using pCM184 (Figure 2). The regions immediately flanking far were amplified by PCR using the following primer pairs: CM-Dfae1, 5'-CGGTTGTTCGTGACCTGTTC-3', and CM-Dfae2, 5'-GTTATGCGGCCCACTCTGATGGAAGCCCATTTGTTC-3'; and CM-Dfae3, 5'-GGTTATGCGGCCCACTCTGATGGAAGCCCATTTGTTC-3', and CM-Dfae4, 5'-CGGTTGTTCGTGACCTGTTC-3'. The purified PCR products for far-upstream and far-downstream were cloned into pCR2.1 to produce pCM195 and pCM196, respectively. The 0.6-kb EcoRI-NotI fragment from pCM195 was introduced between the EcoRI and NotI sites of pCM184 to produce pCM197. Subsequently, the 0.6-kb ApaI-SacI fragment from pCM196 was ligated between the ApaI and SacI sites of pCM197 to produce pCM198.

A Δfar::kan mutant of M. extorquens AM1 was generated by introducing pCM196 by conjugation from E. coli S17-1 (15). Kanamycin-resistant transconjugants obtained on succinate medium containing rifamycin were screened for tetracycline sensitivity to identify potential null mutants. To date, our laboratory has generated more than 30 different null mutant strains utilizing this system, and the frequency of double-crossover events has varied from 5% to 80% (unpublished data). One such Δfar::kan mutant, CM198K.1, was chosen for further study. The plasmid pCM157 was introduced by conjugation into CM198K.1 using the helper plasmid pRK2073 (6). Tetracycline-resistant strains were streaked for purity until the resulting strain produced only kanamycin-sensitive colonies (generally only two transfers). Subsequently, pCM157 was cured from the strain by two successive transfers on medium lacking tetracycline to produce the Δfar strain CM198.1. Analytical PCR was

![Figure 3. Strategy for antibiotic marker recycling. DNA flanks upstream and downstream of the target gene are amplified by PCR and cloned into pCM184. Allelic exchange leads to a kan insertion mutant, which can then be unmarked through the introduction of the cre expression plasmid pCM157. The process can then be repeated with a second target gene to generate a strain bearing multiple genetic manipulations.](image-url)