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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 co-directional *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 α-proteobacterium) and *Burkholderia fungorum* LB400 (a β-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 rifamycin, 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 anti-

biotic resistance cassettes into a variant of the mobilizable suicide plasmid, pAYC61 (4). The 1.3-kb *Hin*cII fragment bearing the kanamycin resistance cassette from pUC4K (17) was inserted into pLox1 (12), which had been cut with *Xba*I and blunted, to create pCM161. To introduce convenient multiple cloning sites, the *loxP*-bounded kanamycin cassette of pCM161 was

amplified with following primer pair, CM-ufkMCS, 5′-TGACGTCTAGATC-TGAATTCAGCTGTACAATTGGTA-CCATGGATGCATATGGCGGCCG-CA-3′, and CM-dfkMCS, 5′-GACTA-GTGAGCTCACCGGTTAACACGC-GTACGTAGGGCCCGCGGTATCGA-TAAGCTGGATCC-3′. The resulting 1.4-kb PCR product was purified and cloned into pCR2.1 (Invitrogen, Carls-

bad, CA, USA) to create pCM183. To preserve useful cloning sites, pAYC61 was cut with *Eco*RI and *Sma*I, blunted using T4 DNA polymerase, and self-ligated to produce pCM182. Finally, the 1.4-kb *Aat*II-*Spe*I fragment from pCM183 containing the *loxP*-flanked kanamycin cassette was ligated between the *Aat*II and *Xba*I sites of pCM182 to create pCM184 (Gen-
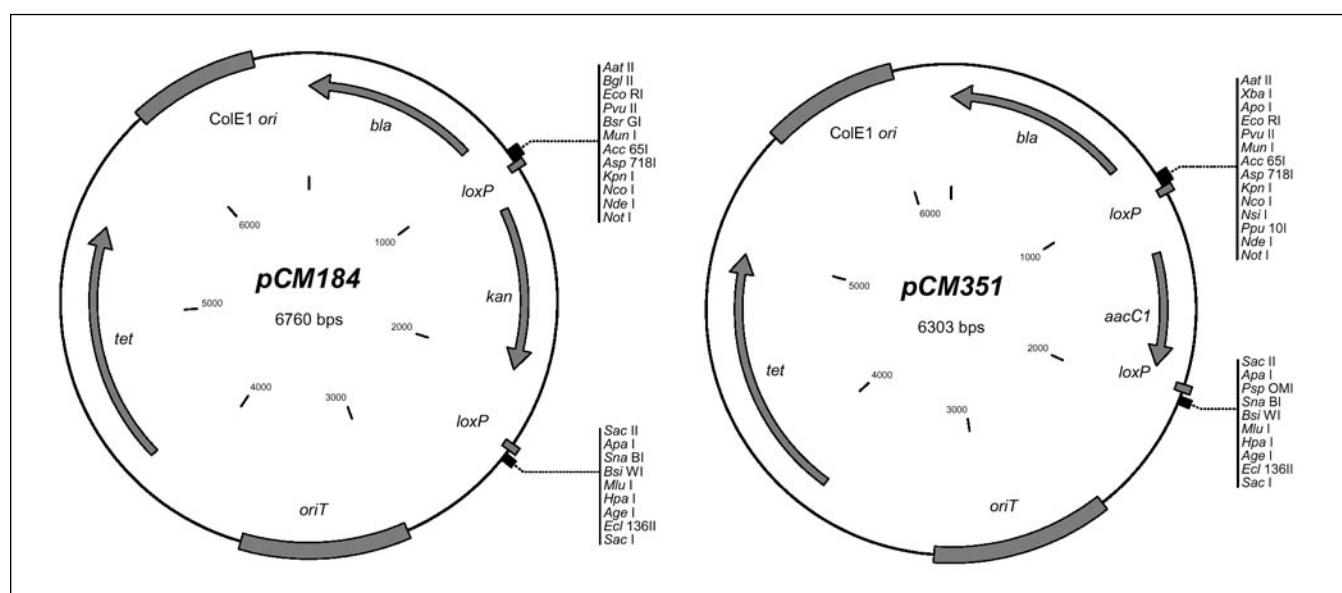

Figure 1. Plasmid map of the allelic exchange vectors pCM184 and pCM351. Antibiotic resistances are encoded by *bla* (ampicillin), *aaaC1* (gentamycin), *kan* (kanamycin), and *tet* (tetracycline). The boxes indicate the pair of *loxP* sites (*loxP*), the IncP origin of tranfer (*oriT*), and the ColE1 origin of replication (ColE1 *ori*). The unique restriction sites present in the multiple cloning sites are indicated.
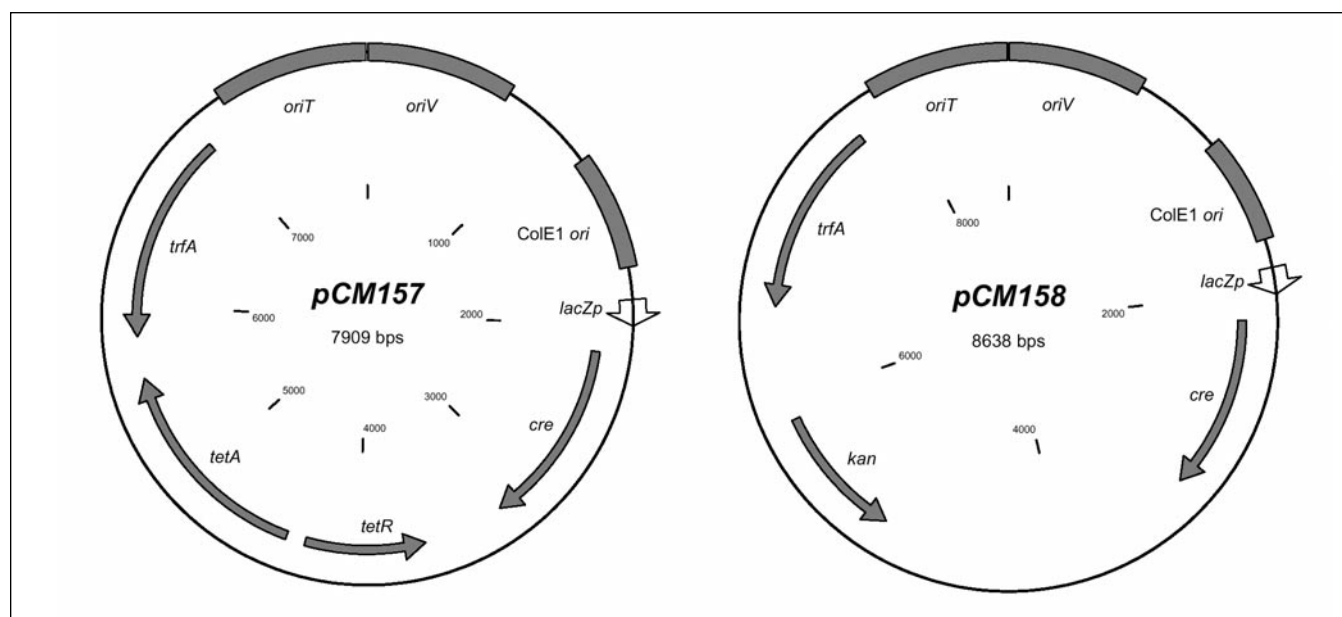

Figure 2. Plasmid map of the *cre* expression plasmids pCM157 and pCM158. The *E. coli lacZp* drives expression of Cre recombinase. These two plasmids differ only in the antibiotic resistance genes present, *tetAR* (tetracycline) or *kan* (kanamycin). The boxes indicate the IncP origin of replication (*oriV*), the ColE1 origin of replication (ColE1 *ori*), and the IncP origin of tranfer (*oriT*).

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Bank® accession no. AY093429). A gentamycin-resistance conferring version, pCM351, was also generated. The *loxP*-flanked gentamycin-resistance cassette (encoded by *aaaC1*) was amplified from pLoxGen4 (12), using CM-ufkMCS and CM-dfkMCS, and cloned into pCR2.1 (Invitrogen) to produce pCM350. The 1-kb *Aat*II/*Sac*I fragment from pCM350 was cloned between the *Aat*II and *Sac*I 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 *Xba*I-*Eco*RI fragment from pJW168 (19) was cloned between the *Xba*I and *Eco*RI 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 *Xba*I-*Eco*RI fragment from pJW168 between the *Xba*I and *Eco*RI 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 Δ*fae* Mutant of *M. extorquens* AM1

M. extorquens AM1 mutants defective for *fae* (encodes formaldehyde-activating enzyme) (18) were generated using pCM184 (Figure 2). The regions immediately flanking *fae* were amplified by PCR using the following primer pairs: CM-Dfae1, 5′-CGGGTTTCGT-GACCTGTTC-3′, and CM-Dfae2, 5′-GTTATGCGGCCGCCATCTGCATG-GAAGCCATCCTTGTTTGC-3′; and CM-Dfae3, 5′-GCTTATCGATACCG-TCGACCTCGAGGCAGTCCTGGG-CAGA-3′, and CM-Dfae4, 5′-CGGGC-ATCGAGCGTTTCAC-3′. The purified PCR products for *fae*-upstream and *fae*-downstream were cloned into pCR2.1 to produce pCM195 and
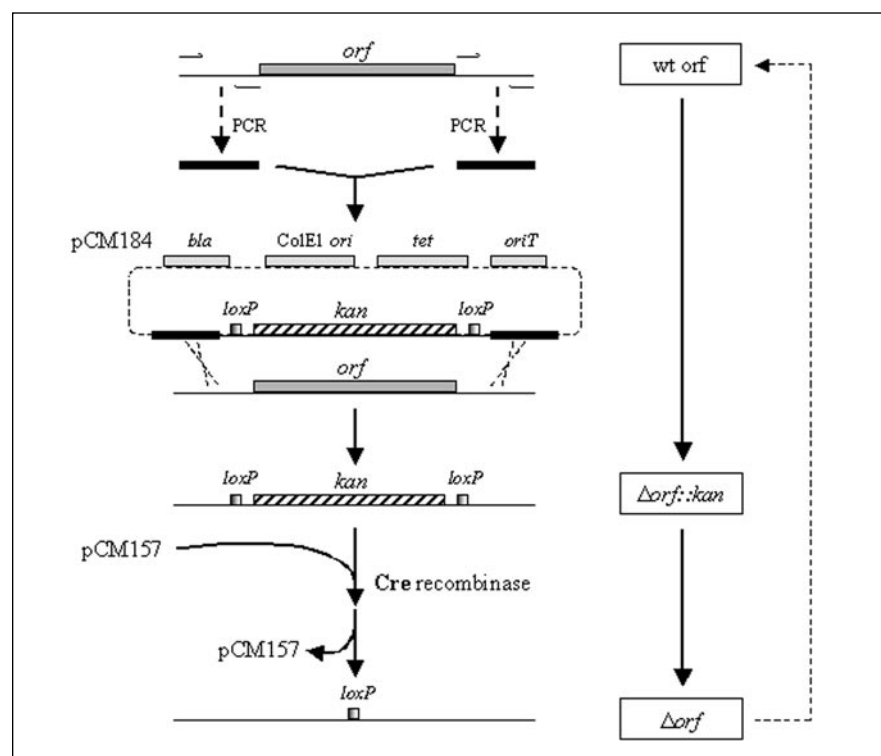

Figure 3. Strategy for antibiotic marker recycling. DNA flanks upstream and downstream of the target gene are amplified by PCR and cloned into pCM184. Allelic exchange leads to a *kan* insertion mutant, which can then be unmarked through the introduction of the *cre* expression plasmid pCM157. The process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

Table 1. Bacterial Strains and Plasmids Used in This Study

Strain/Plasmid	Relevant Properties	Reference
Strains		
B. fungorum LB400	Polychlorinated biphenyls-degrading isolate	3
CM363K.1	Δ*flhA::kan B. fungorum* LB400	This study
CM363.1	Δ*flhA B. fungorum* LB400	This study
E. coli S17-1	C600::RP-4 2-(Tc::Mu) (Kn::Tn*7*) *thi pro hsdR hsdM⁺ recA*	15
M. extorquens AM1	Rif^r derivative	11
CM198K.1	Δ*fae::kan M. extorquens* AM1	This study
CM198.1	Δ*fae M. extorquens* AM1	This study
Plasmids		
pAYC61	Ap^r, Tc^r; mobilizable allelic exchange vector	4
pCM62	Tc^r; broad-host-range cloning vector	10
pCM66	Kn^r; broad-host-range cloning vector	10
pCM157	Tc^r; pCM62 with *cre* from pJW168; *cre* expression vector	This study
pCM158	Kn^r; pCM66 with *cre* from pJW168; *cre* expression vector	This study
pCM161	Ap^r, Kn^r; pLox1 with *kan* cassette from pUC4K	This study
pCM182	Ap^r, Tc^r; pAYC61 cut with *Eco*RI and *Sma*I, blunted, and self-ligated	This study
pCM183	Ap^r, Kn^r; pCR2.1 with *kan* cassette amplified from pCM161	This study
pCM184	Ap^r, Kn^r, Tc^r; pCM182 with *kan* from pCM183; allelic exchange vector	This study
pCM195	Ap^r, Kn^r; pCR2.1 with *fae* upstream flank	This study
pCM196	Ap^r, Kn^r; pCR2.1 with *fae* downstream flank	This study
pCM197	Ap^r, Kn^r, Tc^r; pCM184 with *fae* upstream flank	This study
pCM198	Ap^r, Kn^r, Tc^r; pCM197 with *fae* downstream flank; donor for Δ*fae::kan*	This study
pCM350	Ap^r, Gm^r, Kn^r; pCR2.1 with *aaaC1* cassette amplified from pLoxGen4	This study
pCM351	Ap^r, Gm^r, Tc^r; pCM184 with *aaaC1* from pCM350; allelic exchange vector	This study
pCM360	Ap^r, Kn^r; pCR2.1 with *flhA* upstream flank	This study
pCM361	Ap^r, Kn^r; pCR2.1 with *flhA* downstream flank	This study
pCM362	Ap^r, Kn^r, Tc^r; pCM184 with *flhA* upstream flank	This study
pCM363	Ap^r, Kn^r, Tc^r; pCM362 with *flhA* downstream flank; donor for Δ*flhA::kan*	This study
pCR2.1	Ap^r, Km^r; PCR cloning vector	Invitrogen
pJW168	Ap^r; *cre* expression plasmid	19
pLox1	Ap^r; mobilizable suicide vector with *loxP* sites	12
pLoxGen4	Ap^r, Gm^r; pLox1 with *aaaC1* cloned between loxP sites	12
pRK2073	Sm^r; helper plasmid supplying IncP *tra* functions	6
pUC4K	Ap^r, Km^r; vector with *kan* cassette	17

Antibiotic resistances are indicated as follows, Ap (ampicillin), Gm (gentamycin), Kn (kanamycin), Rif (rifamycin), Sm (streptomycin), and Tc (tetracycline).

pCM196, respectively. The 0.6-kb *Eco*RI-*Not*I fragment from pCM195 was introduced between the *Eco*RI and *Not*I sites of pCM184 to produce pCM197. Subsequently, the 0.6-kb *Apa*I-*Sac*I fragment from pCM196 was ligated between the *Apa*I and *Sac*I sites of pCM197 to produce pCM198.

A Δ*fae::kan* mutant of *M. extorquens* AM1 was generated by introducing pCM198 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 Δ*fae::kan* mutant, CM198K.1, was chosen for further study. The plasmid pCM157 was introduced by conju-

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gation 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 Δ*fae* strain CM198.1. Analytical PCR was performed with wild-type *M. extorquens* AM1, CM198K.1, and CM198.1 for the confirmation of allelic exchange and the subsequent deletion of the kanamycin cassette (data not shown). Where examined, the sequence of the analytical PCR product indicated faithful recombination between the *loxP* sites (data not shown).

Generation of a Δ*flhA* Mutant of *B. fungorum* LB400

B. fungorum LB400 mutants defective for *flhA* (predicted to encode a NAD- and glutathione-dependent formaldehyde dehydrogenase) (13) were generated using pCM184, as described earlier with *M. extorquens* AM1. The regions flanking *flhA* were amplified by PCR using the following primer pairs: CM-BfflhAuf, 5′-GGTGACGG-CATTGAAGCTG-3′, and CM-BfflhAur, 5′-CATGCATCTTTGGTCTTC-ATCGTGAATG-3′; and CM-BfflhAdf, 5′-ACCGCGGTCGTGCTGTACTAAT-CC-3′, and CM-BfflhAur, 5′-AGAGC-TCGATACCGACCGATAGATCTC-3′. The *flhA* upstream and downstream PCR products were cloned into pCR2.1 (Invitrogen) to produce pCM360 and pCM361, respectively. The 0.6-kb *Sac*II-*Sac*I downstream fragment from pCM361 was introduced between the *Sac*II and *Sac*I sites of pCM184 to produce pCM362. Subsequently, the 0.5-kb *Eco*RI-*Nsi*I upstream fragment from pCM360 was ligated between the *Eco*RI and *Nsi*I sites of pCM362 to produce pCM363.

A Δ*flhA::kan* mutant of *B. fungorum* LB400 was generated by introducing pCM363 by conjugation. Kanamycin-resistant transconjugants were obtained on citrate medium containing chloramphenicol (wild-type *B. fungorum* LB-400 was found to be naturally resistant below 10–20 μg/mL). One tetracycline-sensitive strain representing a Δ*flhA::*

kan mutant, CM363K.1, was chosen for further study. The plasmid pCM157 was used as described earlier to produce the Δ*flhA* strain CM363.1. Analytical PCR was performed with wild-type *B. fungorum* LB400, CM363K.1, and CM363.1 for confirmation (data not shown).

The minimal inhibitory concentration of formaldehyde was determined by comparing the rate and extent of colony formation of wild-type *B. fungorum* LB400 to that of the *flhA* mutants CM363K.1 and CM363.1 on solid medium containing succinate as a growth substrate with various concentrations of formaldehyde. Formaldehyde was added to the plates immediately before the addition of the molten agar. Because an undetermined fraction of the formaldehyde will volatilize, the reported minimal inhibitory concentration of formaldehyde is a maximum value.

RESULTS AND DISCUSSION

To test the broad-host-range *cre-lox* antibiotic marker recycling system, unmarked mutants were generated in *M. extorquens* AM1 (an α-proteobacterium) and *B. fungorum* LB400 (a β-proteobacterium). Analytical PCR confirmed replacement of each deleted gene with *kan* and the subsequent excision of *kan* to produce the unmarked deletion (data not shown). The Δ*fae* mutant of *M. extorquens* AM1 grew like the wild-type on succinate but failed to grow on methanol or medium containing succinate and methanol. This mutant phenotype is in agreement with previous observations with a *fae::kan* mutant (18). The CM198.1 Δ*fae* strain can serve as a convenient host for structure-function studies that require the expression of variant Fae proteins.

As a second demonstration of this broad-host-range antibiotic marker recycling system, a Δ*flhA* mutant of *B. fungorum* LB400 was generated. In other bacteria, the *flhA* gene encodes a glutathione-dependent formaldehyde dehydrogenase (13). This enzyme is involved in formaldehyde detoxification in *E. coli* (9) and *Paracoccus denitrificans* (13) and is required for methylotrophic growth by the latter. The Δ*flhA* strain CM363.1 was found to be

somewhat more sensitive to the presence of formaldehyde during growth on citrate than wild-type *B. fungorum* LB400, with a minimal inhibitory concentration of 0.1 mM compared to 0.2 mM for the wild-type. This finding demonstrates that the glutathione-dependent pathway is involved in formaldehyde detoxification across multiple branches of the proteobacteria.

In conclusion, this new broad-host-range *cre-lox* antibiotic marker recycling system offers the possibility to create unmarked mutants in a wide variety of Gram-negative bacteria. Utilization of allelic exchange with counter-selection against integrants and an inherently unstable minimal IncP Cre expression plasmid obviates the need for successful negative selection in the target organism, a feature of some previously developed marker recycling systems (7). The use of PCR to generate flanks for gene replacement allows for the facile generation of precise deletion mutants (Figure 3), as well as truncations through the introduction of start or stop codons in the primers, as needed. Variants of this system can be readily developed to allow the construction of chromosomal transcriptional or translational fusions (T. Strovas, C.J. Marx, and M.E. Lidstrom, unpublished data). Marker recycling systems such as ours described here offer a substantial advantage over standard allelic exchange methods because it can be used iteratively to enable the generation of unmarked strains bearing multiple genetic modifications. Our laboratory has already used this system to generate an *M. extorquens* AM1 strain bearing four separate mutations (C.J. Marx, L. Chistoserdova, and M.E. Lidstrom, unpublished data). Finally, engineered strains generated with these tools are more acceptable for environmental release, owing to the absence of introduced antibiotic resistance markers.

ACKNOWLEDGMENTS

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