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Efficient Generation of Unmarked Deletions in *Legionella pneumophila*▽†

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Unmarked gene deletions facilitate studies of *Legionella pneumophila* multicomponent processes, such as motility and exonuclease activity. For this purpose, *FRT*-flanked alleles constructed in *Escherichia coli* using λ-Red recombinase were transferred to *L. pneumophila* by natural transformation. Resistance cassettes were then efficiently excised using the Flp site-specific recombinase encoded on a plasmid that is readily lost.

Virulence strategies of *Legionella pneumophila* can be studied by exploiting its genome sequence, natural competence, and growth in artificial media (18, 20). However, research has been complicated by limited selectable markers and the microbe's functional redundancy, including numerous secretion substrates (7, 14, 15). To facilitate construction of strains with multiple unmarked nonpolar deletions, we coupled phage-mediated recombination in *Escherichia coli* with Flp-mediated excision in *L. pneumophila*. By exploiting a λ phage enzyme to mediate homologous recombination between DNA substrates with as few as 35 nucleotides of homology, so-called recombineering offers several advantages over restriction enzyme-based cloning, including increased efficiency (8, 10, 27, 28, 31). The *Saccharomyces cerevisiae* Flp site-specific recombinase excises DNA flanked by directly repeated 34-bp *FRT* sites (9, 12, 13, 19, 21, 23, 24). Here we efficiently generated unmarked deletions in *L. pneumophila* using Flp induced from plasmids, which were then cured from the strain. Compared to traditional methods, this approach generated unmarked deletions at a higher frequency and with greater consistency.

To construct null alleles with *FRT*-flanked antibiotic resistance cassettes, 500 to 1,000 bp flanking each gene to be deleted were amplified by PCR from the chromosome of wild-type *L. pneumophila* and cloned into pGEM-T Easy (Promega) using *E. coli* DH5α as the host (Table 1). The gene of interest was replaced by an *FRT*-flanked *cat* or *kan* cassette from pKD3 or pKD4, respectively (10), using recombineering and *E. coli* DY330 (Table 1) (27, 28). The recombinant allele from pGEM was then transferred to the Lp02 chromosome by natural transformation (25, 26). An *FRT*-flanked gentamicin resistance cassette can be amplified with the same primers used for the *cat* and *kan* genes (Table 1).

***FRT*-flanked cassettes excised using vectors that express Flp.** For construction of a shuttle vector for efficient expression of the Flp recombinase by *L. pneumophila*, *flp* was cloned into a gentamicin-resistant and sucrose-sensitive derivative of the broad-host-range plasmid pMMB206, yielding pMMBFlp (see Table S1 in the supplemental material). Vector pMMBFlp encodes an inducible and functional Flp recombinase (Fig. 1A; see Table S1). However, since this plasmid was difficult to cure from the host strain (Fig. 1B), it is suitable for stable *flp* expression in *L. pneumophila* or for development of tools in other species (see Table S1). For both transient Flp expression and ready segregation from *L. pneumophila*, pBSFlp was constructed by replacing the RSF1010 origin of replication of pMMBFlp with the ColE1 *ori* from pBluescript KS− (Table 1).

Flp-mediated excision of cassettes and removal of vector. pBSFlp was transferred by electroporation (1a, 5, 16) to *L. pneumophila* strains harboring *FRT*-flanked deletion constructs, and transformants were selected on ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid]-buffered charcoal yeast extract (22) supplemented with 100 μg/ml of thymidine (CYET), gentamicin (10 μg/ml), and IPTG (isopropyl-β-D-thiogalactopyranoside) (200 μM). After being incubated for 5 to 6 days, individual colonies from the transformation were patched onto medium without antibiotics and IPTG and incubated overnight. Next, clones were isolated by streaking them onto CYET containing 5% sucrose, and their phenotypes and genotypes were determined. To verify the versatility of the method, deletions were constructed in *lpg1782* (*fliA*), *lpg2217*, *lpg0826* (*xseA*), and *lpg1461* (*recJ*); an *xseA recJ* double mutant was also generated (Table 1).

For every locus examined, the vast majority of clones had excised the resistance cassette and lost the plasmid. When mutants were generated in *fliA*, *lpg2217*, *xseA*, and *recJ*, 100% of the total clones ($n = 96$) lost the desired resistance cassette when plated on medium containing IPTG, whereas 89% ($n = 44$) of the isolates plated on medium without IPTG did so. Loss of vector was also highly efficient; 100% ($n = 96$) of clones lacked the vector after being restreaked on sucrose-containing medium.

The ability of Flp recombinase to excise two cassettes in one step was also examined. A double *recJ*::*FRT xseA*::*FRT* unmarked deletion mutant was constructed in three steps: one step for each cassette insertion and then Flp-mediated excision of both cassettes in a single final step (Table 1). In contrast, traditional allelic exchange methods utilizing counter selection would require four selection steps, costing the researcher more time while increasing

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TABLE 1. Abbreviated list of bacterial strains and plasmids used in this study[a]

Bacterium and strain	Genotype	Source or reference
E. coli strains		
DH5α	*supE44* Δ*lacU169* (80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
DY330	W3110 Δ*lacU169 gal490* λ*cI857* Δ(*cro-bioA*)	31
	pGEM-T Easy	Promega
	pKD3 (*FRT-cat-FRT* allele)	10
	pKD4 (*FRT-kan-FRT* allele)	10
MB838	DH5αλ*pir* pR6Kγ*FRT-gent-FRT*	This work
MB790	DH5α pMMBFlp	This work
MB791	DH5α pBSFlp	This work
MB750	DH5α pGEM*fliA*	This work
MB751	DH5α pGEMΔ*fliA*::*FRT-cat-FRT*	This work
L. pneumophila strains		
MB110	Lp02 wild type; *thyA hsdR rpsL* (Str^r)	1
MB811	Lp02 Δ*fliA*::*FRT-cat-FRT*	This work
MB818	Lp02 Δ*fliA*::*FRT*	This work
MB758	Lp02 *recJ*::*FRT-cat-FRT*	1a
MB819	Lp02 Δ*recJ*::*FRT*	This work
MB759	Lp02 *xseA*::*FRT-kan-FRT*	1a
MB820	Lp02 Δ*xseA*::*FRT*	This work
MB760	Lp02 *recJ*::*FRT-cat-FRT xseA*::*FRT-kan-FRT*	1a
MB821	Lp02 *recJ*::*FRT xseA*::*FRT*	This work

[a] See Table S1 for a complete list of strains.

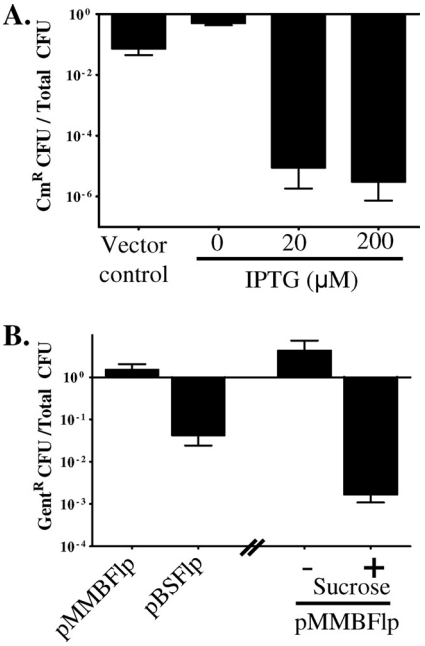

FIG. 1. After antibiotic resistance cassettes are efficiently cured from the *L. pneumophila* chromosome, plasmids expressing the Flp recombinase can be segregated from the host strain. (A) Flp-mediated excision of a *cat* cassette was examined in a *letA*::*FRT-cat-FRT* strain using pMMBFlp and IPTG induction in broth and then plating on medium with and without chloramphenicol (see Table S1 in the supplemental material). Data represent means ± standard errors of the means (SEMs) of 3 experiments. (B) Loss of Flp-encoding plasmids with either an RSF1010 (pMMBFlp) or ColE1 (pBSFlp) origin of replication. For cultures that were selected for plasmid loss with sucrose, overnight cultures were first grown in the absence of sucrose and then exponential-phase cultures were normalized, resuspended, and grown overnight in ACES-buffered yeast extract broth with thymidine plus 5% sucrose without antibiotics. After cultures were plated on media with and without gentamicin, the presence of the resistance cassette carried on the vector was scored. Data represent means ± SEMs of ≥3 experiments.

the chances of second-site mutations. Minimizing strain passage was particularly important for the nuclease mutants, whose DNA repair is predicted to be defective.

Mutations in multiple genes with redundant functions reveal phenotypes. To examine whether functionally redundant genes can be studied by constructing multiple unmarked deletions in a single strain, we investigated the individual and combined effects of the RecJ and ExoVII (XseA) nucleases. *E. coli* has four canonical single-stranded nucleases (ssExos) with a great deal of redundancy (2, 11, 29, 30), whereas only two of these enzymes are annotated in the *L. pneumophila* genome (1a, 6). In *E. coli*, increased sensitivity to the base analog 2-aminopurine (2-AP) is not revealed until three or four of its ssExos are mutated (29). Nuclease mutants also exhibit increased sensitivity to nalidixic acid (Fig. 2) (4, 29). To test genetically if the *L. pneumophila* RecJ and ExoVII nucleases are redundant and together provide the majority of ssExo activity, we examined the susceptibilities of single and double mutants to 2-AP and nalidixic acid. When the mutants were treated with either DNA-damaging agent, the plating efficiency of the double mutant was less than that of either single mutant, suggesting overlapping function (Fig. 2). It is notable that the *L. pneumophila* double mutant was more susceptible to 2-AP than the corresponding *E. coli* double mutant, yet it was more resistant than the *E. coli* quadruple mutant (2). Since in *E. coli* nucleases are necessary for methyl-directed mismatch repair (MDMR), *L. pneumophila* may have a less responsive MDMR system than *E. coli* or recruit other mechanisms to repair base mismatches; alternatively, this pathogen may have unrecognized ssExos. However, the extreme sensitivity of the double mutant to nalidixic acid suggests that RecJ and ExoVII are *L. pneumophila*'s main ssExos.

Unmarked *fliA* deletion alleles are not polar on *motAB*. Unmarked mutations pose a lower risk of polar effects on downstream genes than insertion mutations. For example, a transposon mutation in the gene encoding the flagellar sigma

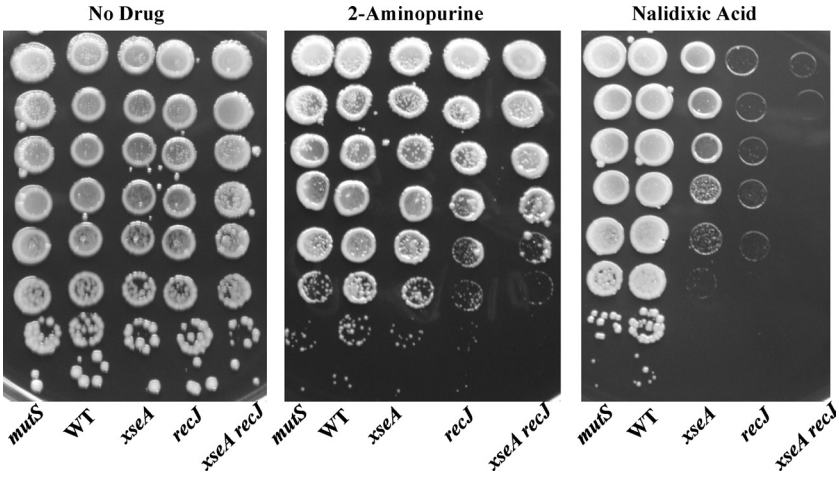

FIG. 2. Exonuclease double mutants are sensitive to 2-aminopurine and nalidixic acid. The indicated strains were grown to the postexponential phase, as assessed by motility, cultures were normalized to an OD_{600} of 1.0, and 10-fold serial dilutions were plated on CYET without a drug or on CYET supplemented with 350 μg/ml of 2-AP or 2 μg/ml of nalidixic acid. A *mutS*::*cat* strain (1a) is shown as a specificity control.

factor, *fliA*, completely eliminates flagella and motility, and these defects are only partially complemented (17). Positioned 9 bp 3′ of *fliA* is the *motAB* locus, which encodes ion channels critical for flagellar motion, as <5% of *motAB* mutant *L. pneumophila* exhibit motility (17).

To test the polarities of *fliA* mutations generated with Flp, we compared the resulting phenotypes to those of the *fliA* transposon mutant (17). Motility was scored microscopically, since soft-agar assays are not applicable for the nonchemotactic *L. pneumophila* (3). Wet mounts were observed through an inverted-phase microscope at least three times as the broth cultures achieved optical densities at 600 nm (OD_{600}s) of 3.70 to 4.70. Plasmid-borne *fliA* restored full motility only to the *fliA* unmarked-mutant strain (Table 2). Since the partial motility of both the *fliA* transposon and the *FRT-cat-FRT* mutant strains carrying *fliA* in *trans* resembled that of the *motAB* mutant, both of the insertion mutations are likely polar.

Conclusions. A powerful approach to study fundamental bacterial processes is the construction and analysis of unmarked nonpolar mutations. Certain experimental questions, including whether factors are functionally redundant, require that multiple mutations be constructed in a single strain. To facilitate the genetic manipulation, we developed an efficient tandem approach in which recombinant alleles are first constructed in *E. coli* using the λ-Red recombinase system and then Flp-mediated excision is induced in *L. pneumophila.*

TABLE 2. Motility phenotypes

Strain	Chromosome	Plasmid	% motile[a]
Lp02	Wild-type postexponential phase	None	>95
Lp02	Wild-type exponential phase	None	0
MB560	*motAB*::*gent*	None	<5
MB808	*fliA*::*kan* transposon insertion	pMMBGent-empty	0
MB510	*fliA*::*kan* transposon insertion	pMMBGent-FliA	<5
MB814	Δ*fliA*::*FRT-cat-FRT*	pMMBGent-FliA	<5
MB816	Δ*fliA*::*FRT*	pMMBGent-empty	0
MB817	Δ*fliA*::*FRT*	pMMBGent-FliA	>95

[a] Motility of $>10^4$ cells scored as a population in two independent experiments, as observed periodically from OD_{600}s of 3.70 to 4.70, except for the wild-type exponential phase, which was observed at OD_{600}s of 0.90 to 1.00.

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