Comprehensive Arrayed Transposon Mutant Library of *Klebsiella pneumoniae* Outbreak Strain KPNIH1

Beth Ramage,^{a*} Roxanne Erolin,^{a*} Kiara Held,^a Joe Gasper,^a Eli Weiss,^b
Mitch Brittnacher,^b Larry Gallagher,^a Colin Manoil^a

Departments of Genome Sciences^a and Microbiology,^b University of Washington, Seattle, Washington, USA

ABSTRACT *Klebsiella pneumoniae* and other carbapenem-resistant members of the family *Enterobacteriaceae* are a major cause of hospital-acquired infections, yet the basis of their success as nosocomial pathogens is poorly understood. To help provide a foundation for genetic analysis of *K. pneumoniae*, we created an arrayed, sequence-defined transposon mutant library of an isolate from the 2011 outbreak of infections at the U.S. National Institutes of Health Clinical Center. The library is made up of 12,000 individually arrayed mutants of a carbapenemase deletion parent strain and provides coverage of 85% of the predicted genes. The library includes an average of 2.5 mutants per gene, with most insertion locations identified and confirmed in two independent rounds of Sanger sequencing. On the basis of an independent transposon sequencing assay, about half of the genes lacking representatives in this “two-allele” library are essential for growth on nutrient agar. To validate the use of the library for phenotyping, we screened candidate mutants for increased antibiotic sensitivity by using custom phenotypic microarray plates. This screening identified several mutations increasing sensitivity to β -lactams (in *acrB1*, *mcrB*, *ompR*, *phoP1*, and *slt1*) and found that two-component regulator *cpxAR* mutations increased multiple sensitivities (to an aminoglycoside, a fluoroquinolone, and several β -lactams). Strains making up the two-allele mutant library are available through a web-based request mechanism.

IMPORTANCE *K. pneumoniae* and other carbapenem-resistant members of the family *Enterobacteriaceae* are recognized as a top public health threat by the Centers for Disease Control and Prevention. The analysis of these major nosocomial pathogens has been limited by the experimental resources available for studying them. The work presented here describes a sequence-defined mutant library of a *K. pneumoniae* strain (KPNIH1) that represents an attractive model for studies of this pathogen because it is a recent isolate of the major sequence type that causes infection, the epidemiology of the outbreak it caused is well characterized, and an annotated genome sequence is available. The ready availability of defined mutants deficient in nearly all of the nonessential genes of the model strain should facilitate the genetic dissection of complex traits like pathogenesis and antibiotic resistance.

KEYWORDS Cre, KPC, ST258, *cpxR*, essential gene, phenotypic microarray

Klebsiella pneumoniae and other carbapenem-resistant members of the family *Enterobacteriaceae* are a major source of antibiotic-resistant hospital-acquired infections and are one of three bacterial pathogens thought to represent the greatest current threats to public health by the Centers for Disease Control and Prevention (1). It has been written that “it is possible that no infectious agent since the introduction of HIV has threatened our last line therapies more than these pathogens” (2). The spread of carbapenem-resistant *K. pneumoniae* is largely associated with a single

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Address correspondence to Colin Manoil, manoil@u.washington.edu.

* Present address: Beth Ramage, Department of Biology, University of Washington, Seattle, Washington, USA; Roxanne Erolin, Jefferson College of Population Health, Thomas Jefferson University, Philadelphia, Pennsylvania, USA.

multilocus sequence type (sequence type 258 [ST258]), whose success is not well understood.

Genomic studies of *K. pneumoniae* have characterized relationships among clinical isolates and identified functions required for infection. One study found that ST258 clinical strains isolated worldwide fall into two clades that differ in a 215-kb region that includes capsule biosynthesis genes (3). Another key study used whole-genome sequencing of isolates from an outbreak of carbapenem-resistant *K. pneumoniae* infections at the U.S. National Institutes of Health (NIH) Clinical Center to formulate a scenario for how the infection spread (4).

K. pneumoniae causes a variety of infections, including pulmonary, blood, and urinary tract infections, yet most infecting strains are not particularly virulent in infection models (5, 6). The bacteria usually lack typical virulence factors easily recognizable from genome sequences like type III secretion systems and extracellular toxins, and the most important *Klebsiella* pathogenicity factors identified to date are capsule, lipopolysaccharide, siderophores, and adhesins (6). Strains can encode a remarkable multiplicity of antibiotic resistance functions, e.g., up to six predicted β -lactamases and three aminoglycoside-modifying enzymes. Resistance to biocides, desiccation, and other environmental factors, in addition to antibiotics, probably contributes to the organism's success as a nosocomial pathogen (6).

Large-scale mutant screens have been carried out to identify *K. pneumoniae* pathogenesis determinants. For example, signature-tagged mutagenesis with different strains and infection models has identified a number of candidate virulence functions, although there has been limited follow-up validation (6–8). A recent transposon sequencing (Tn-seq) screen also identified hundreds of potential factors needed for lung infection in a mouse model (9). That study validated six of the virulence genes with constructed deletion mutants and identified potential reasons for their virulence defects with additional phenotypic tests.

One factor that has limited genome-scale genetic studies of *K. pneumoniae* has been the need to construct individual mutants to test genotype-phenotype associations suggested by experiment or genome sequence analysis. To help address this limitation, we have constructed an arrayed library of transposon mutants with multiple insertions in most of the nonessential genes represented. The mutants are derivatives of strain KPNIH1, an early isolate from the NIH Clinical Center outbreak that belongs to ST258 (clade 2) and carries three plasmids (pKPN-498 [244 kb], pKpQIL [114 kb], and pAAC154 [15 kb]) (4). Strains making up the arrayed library are available to the research community and should be useful both for mutant hunts and for testing of genotype-phenotype associations suggested by other approaches.

RESULTS AND DISCUSSION

Overall approach. The primary goal of this work was to create an arrayed, sequence-defined transposon mutant library of *K. pneumoniae* KPNIH1 with near-saturation coverage of nonessential genes. We wanted it to include multiple mutants for each gene to allow immediate confirmation of genotype-phenotype associations in screens and to minimize missed associations due to noninactivating mutations or strain cross-contamination. We created the library in two stages. First, a large primary collection of mutants generated by random insertion mutagenesis was arrayed and transposon junction fragments were individually sequenced. This collection contained more than seven unique mutants per coding gene. Second, individual mutants from this primary collection corresponding to two or three unique insertions per gene were colony purified, rearrayed, and resequenced. This smaller, resequenced library is called the “two-allele library.”

Construction of a carbapenemase gene deletion strain (MKP103). As a parent strain for transposon mutagenesis, we created a KPNIH1 derivative with the KPC-3 carbapenemase-encoding gene deleted. The strain was constructed in order to limit inadvertent spread of the resistance determinant, which is carried on a conjugal plasmid (pKpQIL). We created the mutation by recombination by transforming KPNIH1

FIG 1 Transposon T30. (A) Overall structure. The transposon is a 1,271-bp Tn5 derivative carrying mosaic ends (ME) and *loxP* recombination sites flanking the chloramphenicol resistance determinant (*cat*). The resistance marker can be eliminated by Cre recombination at the *loxP* sites, leaving a 219-bp insertion “scar” carrying both mosaic ends and one *loxP* site. (B) Whole transposon sequence. (C) Insertion scar sequence. The 219-bp sequence remaining after *loxP* × *loxP* recombination is shown. The sequence encodes stop codons in all except the +2 reading frame.

with a PCR fragment carrying the chloramphenicol resistance gene (*cat*) in place of the KPC-3-encoding gene, followed by elimination of the *cat* gene by FLP-mediated recombination at *FRT* sites flanking the gene (see Materials and Methods). As expected, MKP103 was imipenem sensitive and carried a deletion of the predicted size (see Materials and Methods).

Transposon T30. For mutagenesis of MKP103, we created transposon T30, a mini-Tn5 derivative encoding chloramphenicol resistance (Fig. 1). T30 carries mosaic end sequences to support the formation of transposon-transposase complexes *in vitro*. The transposon also carries *loxP* recombination sites near its ends, making it possible to eliminate the *cat* gene by Cre recombination (Fig. 1). It should be possible to create double mutants from library strains by deleting the resistance marker from one mutant and introducing a second insertion by transformation of genomic DNA from a different mutant (10).

Primary mutant collection. To create the primary transposon mutant collection, MKP103 was mutagenized by transformation of transposon T30-transposase complexes, followed by selection for chloramphenicol-resistant colonies (11). Colonies were then arrayed robotically in a 384-well format (see Materials and Methods). To identify transposon insertion locations, arrayed cells were grown overnight and PCR fragments corresponding to genome-transposon junction regions were amplified and individually sequenced (see Materials and Methods). A total of 41,769 unique insertions were identified, corresponding to 86% of the strain’s predicted coding genes (Table 1).

TABLE 1 *K. pneumoniae* transposon mutant library^a

Parameter	Result in:	
	Primary set	Two-allele library
No. of arrayed strains	54,528	12,000
No. of sequence-mapped insertions	42,951	11,985
No. of insertion locations confirmed by resequencing		10,259
No. of unique insertions	41,769	11,750
No. of unique insertions within open reading frames	34,697	11,564
No. of genes hit (of 5,411)	4,666	4,583
% of genes hit	86	85
No. of mutants/gene hit	7.4	2.5

^aThe two-allele library was constructed from the primary set of mutants by colony purification and rearraying and resequencing two or three mutants per gene represented.

Two-allele library. A smaller library with two or three unique insertion mutants per gene was constructed from the primary mutant collection (Table 1). Mutants with insertion sites in the centers of genes were chosen for inclusion where possible, and strains were colony purified and resequenced to verify genome insertion locations. The identities of mutants in this two-allele library and additional information are provided in Data Set S1 in the supplemental material and in a genome browser (http://tools.uwgenomics.org/tn_mutants/index.php) (Fig. 2).

KPNIH1 essential genes. Mutants deficient in 828 KPNIH1 genes were absent from the two-allele library (Table 1; Data Set S2). We assume that these genes represent both functions essential for growth under the mutant selection conditions used and non-essential genes missed by chance. To help distinguish the essential subset, we gener-

FIG 2 Mutant browser screenshot. The locations of transposon insertions in the two-allele library are shown for one of two *lac* regions in the KPNIH1 genome. The searchable browser (https://tools.uwgenomics.org/tn_mutants/index.php) provides linked information about each insertion and can be used to create lists of mutants for request.

FIG 3 Essential genes unrepresented in the two-allele library. (Top) Overlap between *K. pneumoniae* genes unrepresented in the two-allele library and identified as essential by Tn-seq. (Bottom) Overlap of *E. coli* essential genes with *K. pneumoniae* genes.

ated a pool of ~100,000 transposon T30 mutants and characterized it by Tn-seq (12) (see Materials and Methods). That analysis identified 642 putative essential genes, 373 of which were also unrepresented in the two-allele library. (Fig. 3, top; Data Set S2). Genes scored as essential by Tn-seq thus account for nearly half (373/828) of those absent from the KPNIH1 two-allele library.

To help identify any KPNIH1 essential genes represented in the two-allele library by nonnull mutations, we examined orthologues of the highly validated *Escherichia coli* K-12 essential gene set (13). Since *K. pneumoniae* and *E. coli* are closely related, most *K. pneumoniae* orthologues of *E. coli* essential genes should also be essential. Of the 289 KPNIH1 orthologues we identified, more than two-thirds (205) fell into the high-confidence set of 373 *E. coli* essential genes (Fig. 3, bottom; Data Set S2) (13). However, another 46 orthologues were scored as essential by Tn-seq but had mutants in the two-allele library (Fig. 3, bottom; Data Set S2). Most of these 46 genes were underrepresented in the arrayed primary mutant collection (data not shown). We therefore assume that most or all of the 46 genes are essential in KPNIH1 and that insertions mapping to them in the two-allele library are not inactivating. Five orthologues were absent from the two-allele library but scored as nonessential by Tn-seq, and 33 orthologues were nonessential by both criteria (Fig. 3; Data Set S2). Mutants deficient in many of these additional 38 genes were underrepresented in the Tn-seq pool, suggesting that they may grow slowly (Data Set S2). Overall, this analysis bolsters the conclusion that about half of the genes without mutants in the two-allele library are missing because they are essential and identifies mutants in the library that are unlikely to have fully inactivating mutations because they affect essential genes. A consensus set of 424 KPNIH1 essential genes based on our studies is presented in Data Set S2.

Genotype-phenotype validation tests using custom PM plates. We evaluated the use of the two-allele mutant library to identify genotype-phenotype associations for seven clinically relevant antibiotic resistance traits. Candidate resistance genes corresponded to two-component regulators, efflux pumps, and additional genes orthologous to established *E. coli* resistance functions (14, 15). We wished to screen candidate mutants by using phenotype microarray (PM) assays (Biolog) (16, 17), but the levels of many antibiotics in the commercially available plates were too low to support effective screening. Thus, we designed custom PM plates tailored to the resistance profile of the MKP103 parent strain. The plates provided coverage of three classes of antibiotics used to treat *K. pneumoniae* infections: an aminoglycoside (amikacin), five different β -lactams, and a fluoroquinolone (ciprofloxacin) (Fig. S1).

We assayed antibiotic sensitivity in triplicate in a total of 76 mutants corresponding to insertions in 46 genes (Data Set S3). For genes represented by multiple mutant

TABLE 2 Antibiotic sensitivities of candidate mutants^a

Mutant			No. of alleles tested	Sensitivity (MIC μ g/ml)					<i>E. coli</i> phenotype(s) ^c
Locus tag	Gene	Function ^b		Amikacin	Aztreonam	Cefazolin	Imipenem	Levofloxacin	
Parent strain (MKP103)				≥ 11	0.5	195	≥ 0.3	150	
KPNIH1_00370	<i>cpxA</i>	TCR	1	2.2	0.22	58	0.06	44	1, 2
KPNIH1_00375	<i>cpxR</i>	TCR	2	3.3	0.18	58	0.04	55	1, 2
KPNIH1_03580	<i>slt</i>	PG	2	7.5	0.22	87	≥ 0.3	150	1, 2
KPNIH1_04480	<i>mcrB</i>	PG	1	≥ 11	0.22	87	0.06	≥ 225	2
KPNIH1_05950	<i>acrB</i>	RND	2	≥ 11	0.18	162	≥ 0.3	150	2, 3
KPNIH1_10025	<i>phoQ</i>	TCR	2	7.5	0.36	87	0.13	100	2, 3
KPNIH1_10030	<i>phoP1</i>	TCR	2	5	0.27	103	0.2	100	2, 3
KPNIH1_24525	<i>envZ</i>	TCR	2	≥ 11	0.22	78	0.09	125	2, 3
KPNIH1_24530	<i>ompR</i>	TCR	2	≥ 11	0.22	72	0.17	150	

^aSensitivities to five of seven antibiotics tested (the aminoglycoside amikacin; the β -lactams aztreonam, cefazolin, and imipenem; and the fluoroquinolone ciprofloxacin) are shown for genes with significant mutant phenotypes (Fig. S1 and Data Set S2). Assays were carried out in triplicate, and average values for two mutant alleles are provided for all genes except *cpxA* and *mcrB*. *E. coli* phenotypes are based on lists of aminoglycoside (amikacin, gentamicin, streptomycin, and tobramycin), β -lactam (aztreonam, ceftoxime, cefsulodin, amdinocillin, and oxacillin), and quinolone (ciprofloxacin, nalidixic acid, and norfloxacin) sensitivity phenotypes (15).

^bRND, resistance-nodulation-division family efflux; PG, peptidoglycan metabolism; TCR, two-component regulation.

^c1, aminoglycoside sensitive; 2, β -lactam sensitive; 3, quinolone sensitive.

alleles, different alleles generally led to congruent phenotypes. Mutants deficient in nine genes exhibited significant increased-sensitivity phenotypes (Table 2). Mutations inactivating the two-component regulator *cpxAR* strongly increased sensitivities to all three classes of antibiotics tested, whereas the other mutations caused less dramatic changes. The phenotype caused by *cpxAR* is similar to that seen in *E. coli* (increased sensitivity to aminoglycosides and β -lactams) (18, 19), although the *K. pneumoniae* mutant phenotypes were stronger. Other *K. pneumoniae* phenotypes also largely agree with those seen in *E. coli*, including those caused by five genes in which mutations increased sensitivity to multiple β -lactams (*acrB1*, *mcrB*, *ompR*, *phoP1*, and *slt1*) (Table 2) (15). Overall, the results confirm the utility of the KPNIH1 mutant library for identifying genotype-phenotype associations and also suggest that known *E. coli* associations provide a valuable source of *K. pneumoniae* candidate genes for traits of interest.

Transfer of mutations between strains. Transposon insertion alleles could be transferred between KPNIH1 strains by homologous recombination after electroporation of PCR fragments carrying insertions and flanking genome sequences (see Materials and Methods). For example, mutations in *cpxR* (KPNIH1_00375) and a capsule gene (KPNIH1_17405) were transferred to carbapenemase-positive KPNIH1, with successful transfer confirmed by PCR and by phenotype analysis (not shown). The transfer was not highly efficient (see Materials and Methods), and it may be possible to increase its efficiency by the expression of foreign recombinases in recipient cells (20).

Mutant availability. Individual strains making up the two-allele library, as well as copies of the entire library, are available through a web-based request mechanism from a University of Washington cost recovery center (http://www.gs.washington.edu/labs/manoil/kpneumoniae_library.htm).

Concluding remarks. Comprehensive mutant libraries such as that described here have proved to be a valuable resource for genetic studies for two reasons. First, the libraries can be screened directly for relatively complete identification of nonessential genes contributing to a given trait. Second, the libraries are a source of strains that can be used to test the validity of genotype-phenotype associations suggested by genome-scale experimental methods like Tn-seq and transcriptome sequencing. Single-mutant verification of candidate genes identified by such approaches is commonly modest in scale because of the effort required to construct mutants yet is critical for confirming the authenticity of associations they indicate. Having defined mutants already available should improve the extent of such verification that is feasible in studies of *K. pneumoniae*.

MATERIALS AND METHODS

Strains. *K. pneumoniae* KPNIH1 was generously provided by Karen Frank (National Institutes of Health Clinical Center) (4). MKP103, a carbapenemase (KPC-3) deletion derivative of KPNIH1, was generated in two steps. First, KPNIH1 was transformed by electroporation with a 3,059-bp PCR fragment (~100 ng) carrying the chloramphenicol resistance (*cat*) gene and adjacent *FRT* recombination sites derived from pKD3 (21) bracketed by ~1-kb regions corresponding to sequences on each side of the KPC-3-encoding gene on plasmid pKpQIL (22). A chloramphenicol-resistant transformant (MKP102) selected on LB agar plus 175 μ g/ml chloramphenicol exhibited greater imipenem sensitivity than KPNIH1 and was confirmed by PCR to carry the *cat* gene in place of the KPC-3-encoding gene. Second, the *cat* gene in MKP102 was eliminated by FLP recombination. MKP102 was electroporated with a plasmid carrying the FLP recombinase-encoding gene (pFLP3) (21) with selection for tetracycline-resistant transformants on LB agar plus 50 μ g/ml tetracycline. Transformants were then grown in the absence of tetracycline to allow spontaneous loss of pFLP3, and a cured derivative that had lost the *cat* gene was identified by its chloramphenicol sensitivity. That strain (MKP103) carries a deletion of codons 15 to 291 of the 294-codon KPC-3-encoding gene, with an *FRT* "scar" in its place. MKP103 showed an ~100-fold lower imipenem minimal growth inhibitory concentration and was confirmed by PCR to carry a KPC-3-encoding gene deletion of the expected size.

Construction of transposon T30. Transposon T30 was constructed from transposon T26 in plasmid pLG123 (23) by replacement of a *Not*I-*Asc*I restriction fragment carrying the tetracycline resistance gene and adjacent promoter with a PCR fragment carrying *cat* and its promoter amplified from plasmid pACYC184.

Transposon mutagenesis. T30 mutagenesis was carried out by transformation of transposon-transposase complexes (11). The transposon was amplified from plasmid pT30 by using primers and procedures described previously for transposon T26 (23). Transposon-transposase complexes were generated by mixing transposon DNA with EZ-Tn5 transposase (Epicentre) and introduced by electroporation into MKP103, which had been grown in LB to an optical density at 600 nm of 0.4 to 0.6, washed three times in distilled water, and resuspended in 1/133 volume of 10% glycerol (23). Electroporation was carried out for mixtures of 50 μ l of cells and ~0.1 μ l of the transposon-transposase complex in 1-mm cuvettes by pulsing at 200 Ω , 25 μ F, and 1.75 kV with a Bio-Rad Gene Pulser. Electroporated cells were added to ~0.5 ml of Super Optimal broth with catabolite repression (SOC), grown for 1 h at 37°C, and then plated on LB agar plus chloramphenicol (175 μ g/ml). A typical mutagenesis yielded ~20,000 colonies per electroporation.

Construction of the primary mutant collection. To generate the primary mutant collection, T30 insertion mutant colonies were robotically arrayed into 142 384-well plates containing LB with 5% dimethyl sulfoxide (DMSO) and 100 μ g/ml chloramphenicol by a QPix2 colony-picking robot (Genetix). Plates were incubated for 24 h at 37°C and then stored at -80°C. Insertion sites were identified by two-stage semidegenerate PCR amplification and Sanger sequencing of transposon-genome junctions (24, 25). The sequences of the primers used for PCR 1 were TATCAACAGGGACACAGGATTTA and a mixture of GGCCACGCGTCGACTAGTACNNNNNNNNNCTGAG, GGCCACGCGTCGACTAGTACNNNNNNNNN NNAGTGC, and GGCCACGCGTCGACTAGTACNNNNNNNNNNTGCT; those of the primers used for PCR 2 were CTGCGAAGTGATCTTCGTCAC and GGCCACGCGTCGACTAGTAC; and that of the primer used for sequencing was CGGCCGCATAACTTCGTATAATGT. Insertion sites were mapped to the KPNIH1 genome with an overall success rate of 89.6%.

Construction of the two-allele library. To create the two-allele library from the primary mutant collection, custom scripts and manual curation were used to choose up to three unique mutants per gene whose insertions had been mapped by using high-quality sequence data and that were located at distributed sites between 5 and 90% of the coding sequences. Strains were cherry picked from the primary library, colony purified, rearrayed, and resequenced to confirm the initial assignments. Mutants were stored in a 96-well format at -80°C in LB with 5% DMSO and 100 μ g/ml chloramphenicol.

Tn-seq identification of KPNIH1 essential genes. Genes essential for growth on nutrient medium were identified by Tn-seq analysis of a pool of ~10⁵ transposon T30 mutants selected on LB agar containing 175 μ g/ml chloramphenicol (23). For each gene, the number of transposon sequence reads per kilobase was calculated for insertions within 5 to 90% of the coding sequence. Hit and read density was calculated by dividing reads (5 to 90%) by locus length (5 to 90%), and the distributions of reads per gene were calculated. Genes with zero values were considered essential. The remaining assignments (reads per gene) were log₂ transformed, and data were fitted to a normal distribution. Loci with values falling below a *P* value cutoff of 0.01 were added to the zero-value set for the final set of candidate essential loci (12).

Custom PM analysis. Custom PM plates were formulated for six antibiotics (amikacin, aztreonam, cefazolin, ceftriaxone, imipenem, levofloxacin, and piperacillin-tazobactam [8:1, wt/wt]) with six concentrations per antibiotic and 1.5-fold concentration steps. The highest concentrations of each of the antibiotics in the custom plates were chosen in preliminary tests to give at least partial inhibition of MKP103. The PM assays were carried out in a 96-well format in triplicate for each strain in accordance with the procedures recommended by the supplier (Biolog). Growth in each well was monitored as the rate of tetrazolium reduction with an OmniLog reader (16, 17).

Transfer of transposon insertions between strains. Insertion mutations were transferred into new strains by electroporation of PCR products carrying the insertions and flanking regions of homology. PCR products with 2- to 2.5-kb sequences flanking T30 insertions were purified, dialyzed against water, and electroporated into cells grown and prepared as described above for transposon

mutagenesis. Electroporated cells were grown in SOC and plated on LB agar supplemented with chloramphenicol (175 μ g/ml). The efficiency of transformation was approximately 10 transformants/ μ g of PCR DNA.

Deletion of transposon T30 sequences by Cre/lox recombination. Transposon T30 sequences between directly repeated *loxP* sequences near the transposon ends were eliminated by recombination through transient introduction of a nonreplicating plasmid (pCre2) expressing Cre recombinase (26). Transposon insertion mutants were conjugated with SM10 λ pir/pCre2 by mixing cells at a donor-to-recipient ratio of 10:1, spotting them on 0.45- μ m-thick nitrocellulose membranes (Millipore), and incubating them for 4 h at 37° on LB agar. Cells were then resuspended in 1 ml of LB and streaked onto LB agar. Resulting colonies were screened to identify those that had become chloramphenicol sensitive (~50% of the total). Such sensitive strains (in three cases examined) were found by PCR and DNA sequence analysis to carry the expected 219-bp recombination product “scar.”

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00352-17>.

SUPPLEMENTAL FILE 1, XLSX file, 1.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 4, PDF file, 0.3 MB.

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