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New Vector Tools with a Hygromycin Resistance Marker for Use with Opportunistic Pathogens

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Abstract

The ability of many bacterial strains to tolerate antibiotics can limit the number of molecular tools available for research of these organisms. To help address this problem, we have modified a diverse set of vectors to include a broadly expressed hygromycin resistance (HmR) marker. Hygromycin B is an aminoglycoside antibiotic not used to treat infections in humans and has antimicrobial activity against a wide range of microorganisms. Vectors with four replication origins are represented, with potential applications including general cloning, allelic replacement, and transcriptional analysis. We show that vectors with the broad host range pBBR1-replicon conferred HmR to *Achromobacter xylosoxidans*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Serratia marcescens*, and a pC194-based vector was able to confer HmR to *Francisella tularensis*. We also used a subset of these plasmids to manipulate the genome of *S. marcescens*. Each vector has an origin of transfer for conjugation, and is also able to replicate in *Saccharomyces cerevisiae* to take advantage of the powerful yeast recombineering system.

Keywords

Recombineering; Opportunistic pathogen; Mutation; Vector tools; Plasmid; Cloning

Introduction

The strain of *Serratia marcescens* used in our laboratory tolerates ampicillin, chloramphenicol, erythromycin, and tetracycline at concentrations well above those commonly used to select for resistance cassettes in *Escherichia coli* laboratory strains. We therefore use aminoglycoside resistance markers for gentamicin and kanamycin. These markers, however, are highly limiting in many genetic manipulations when multiple mutations and/or multiple plasmids are desired. Similar limitations are observed for many clinical and environmental isolates from a myriad of species including other opportunistic pathogens, e.g., *Acinetobacter baumannii* and *Pseudomonas* strains. Therefore, there is a need for molecular tools with a wider range of available selectable markers.

A recent report demonstrated the usefulness of a hygromycin (Hm) resistance (HmR) cassette in *Francisella tularensis*, which also confers Hm resistance to *E. coli* [19]. The HmR cassette contains the *hph* gene from *Streptomyces hygrosopicus* [8] driven by the strong *P_{groESL}* promoter from *F. tularensis* [19]. Hygromycin B is not used clinically and the *hph* gene does not confer resistance to other aminoglycosides [8], making it a useful antibiotic for manipulating the genomes of clinically relevant organisms [19]. We generated vector tools that utilize this resistance cassette to increase the number of useful markers available for our research.

This manuscript describes 11 vectors with the HmR cassette made for general cloning, allelic replacement, expression of the I-SceI gene, and transcriptional analysis (Table 1). Four different plasmid replicons were included, so that the resulting vectors can be used for multiple plasmid applications, e.g., the pBBR1-replicon is compatible with the most commonly used replicons, such as p15a and ColE1 [1]. Some of these plasmids were used to make an in-frame deletion in the first gene of the prodigiosin biosynthetic operon, and to complement the pigment defective phenotype of the mutant strain.

Materials and Methods

Microbes and Growth Conditions

All bacteria except *F. tularensis* were cultured using LB medium [2]. *F. tularensis* was cultivated on chocolate II agar plates, or in trypticase soy broth with 0.1% cysteine HCl. *Saccharomyces cerevisiae*, strain InvSc1 (Invitrogen), was cultured with YPD broth (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose), and selections were done using uracil and leucine drop-out medium (SC-ura, SC-leu) [3]. The strains used in this study were: *E. coli* strains S17-1 [21] and EC100D *pir-116* (Epicentre), *S. marcescens* strain PIC-3611 (Presque Isle Cultures), *F. tularensis* subsp. *holarctica* live vaccine strain (LVS) (a gift from Karen Elkins, FDA), *P. aeruginosa* strain UCBPP-PA14 (PA14) [25], and ocular clinical isolates from the Campbell Laboratory of Ophthalmic Microbiology: *A. baumannii* strain K1971 and *Achromobacter xylosoxidans* strain K1893. Plasmids were introduced into microorganisms as previously described using conjugation for bacteria and chemical transformation for *S. cerevisiae* [3, 29, 30]. Hygromycin B was used at 140 µg/ml for *A. xylosoxidans*, *A. baumannii*, *E. coli*, *F. tularensis* and *S. marcescens*, and at 280 µg/ml for *P. aeruginosa*.

Plasmid Construction

Plasmids were made using yeast homologous recombination as previously described [29, 30]. PCR was performed using Phusion polymerase (New England Biolabs). All plasmids were verified by phenotype (gain of HmR marker and loss of original marker), PCR and sequencing. All vectors made in this report are listed in Fig. 1 and Table 1. Oligonucleotide primers are listed in Table 2.

To generate pMQ297–pMQ300 and pMQ311, previously described vectors pMQ132, pMQ227, and pMQ240 [30] were digested with *Bgl*III. This enzyme linearizes the plasmids roughly in the middle of a gentamicin resistance gene. Primers 1537 and 1538 were used to amplify the HmR cassette (HmR) from pMQ131hyg [11], which was derived from pMP615 [19]. Recombination resulted in completely replacing the gentamicin resistance cassette with HmR in all plasmids as verified by phenotype and sequence.

To generate pMQ352, we amplified the *LEU2* gene from pRS415 [31] with primers 1295 and 1298. Primer 1298 introduces an *Xho*I site, which can be used with another *Xho*I site to remove *S. cerevisiae* replication machinery and *LEU2* gene of pMQ352 if so desired. The HmR cassette was amplified with primers 1300 and 1301. The *sacB* gene was amplified with primers 1303 and 1304. These primers introduce *Mlu*I sites designed to remove the *sacB* gene if desired. All three amplicons were simultaneously recombined into pMQ132 that had been digested with *Sac*II. This recombination removes the *URA3* gene and *aacC1* gene of pMQ132, directing the removal of 2031 bp of pMQ132 on one side of the *Sac*II site and 17 bp on the other side. The resulting plasmid has a *LEU2* selectable marker, HmR cassette with *sacB* under transcriptional control of the *P_{groESL}* promoter. The resulting pMQ352 plasmid was selected for on SC-leu medium and the plasmid conferred HmR and sucrose sensitivity to *E. coli*.

The pMQ225 vector was generated by digesting pMQ352 with *Mlu*I to remove the *sacB* gene, and ligating the vector backbone. The resulting plasmid retains the ability to confer HmR but does not confer sucrose sensitivity.

To generate pMQ235, we amplified oriR6K from pMQ118 [30] with primers 1726 and 1727; pC194 components (replicon, selectable marker, and so forth) were amplified from pSK236 [7] using primers 1729 and F-1730; the *I-Sce*I gene was amplified from pMQ240 [30] with primers 1732 and 1733. The three amplicons were used along with pMQ225 that had been digested with *Msc*I and *Apa*LI to perform yeast recombineering. The four-fragment recombination removed the pBBR1 replicon from pMQ225 and replaced it with the *I-Sce*I gene, flanked by *Mlu*I sites and under control of the *P_{groESL}* promoter, the *pre*, *rep*, and *cat194* genes of pC194, and oriR6K. The resulting pMQ235 plasmid replicates in *E. coli* strains expressing the *pir* gene.

The pMQ351 plasmid was made by digestion of pMQ235 with *Nco*I in the *cat-194* gene and subsequent replacement of *cat-194* by *FGRp-dtomato* that had been amplified from pTC3D [13] using yeast in vivo cloning.

The pMQ353 vector was created by removal of the *I-Sce*I containing *Mlu*I fragment of pMQ235, by restriction digestion and ligation.

The pMQ306 plasmid, designed for allelic replacement schemes, was made by modification of plasmid pMQ227i (to be described elsewhere), digested with *Apa*LI. The HmR cassette was amplified from pMQ299 with primers 2005 and 2006. The ampicillin resistance marker from pMQ227i was completely replaced with the HmR cassette using yeast recombineering.

pMQ354 is a narrow host range vector that is useful as a *lacZ*-fusion promoter probe. This was made by modification of an existing vector with a kanamycin resistance marker, pStvZ3 [16]. The HmR cassette was PCR-amplified from pMQ298 with primers 2001 and 2002. The pStvZ3 vector was digested in the *nptII* and *rpsL* genes with *Bgl*II and *Sma*I, and the HmR cassette completely replaced the *nptII* and *rpsL* genes in a yeast recombination.

The *pigA* deletion construct, pMQ305, was made by amplifying the first 204 bp and the last 249 bp of *pigA* with primers that mediated recombination with the pMQ236 allelic replacement vector [30] and simultaneously direct fusion of the two amplicons. The resulting *pigA*- Δ gene was designed to code for a mutated PigA protein with a 235 amino acid deletion. The amplicons were generated with primers 2081 and 2086, and 2082 and 2085, respectively.

The full-length *pigA* complementation plasmid was made by amplifying the *pigA* gene from *S. marcescens* strain PIC-3611 using primers 2081 and 2082. The amplicon was then recombined with pMQ300 using yeast recombineering.

Allelic Replacement

The pMQ305 plasmid, with the *pigA*- Δ allele and an I-SceI site in its vector backbone, was introduced into *S. marcescens* by conjugal transfer, with selection for kanamycin resistance. The pMQ299 plasmid, with I-SceI expressed from the *E. coli* *P_{lac}* promoter, was then introduced into the *S. marcescens* with pMQ305 merodiploid strain by conjugal transfer, with selection for HmR. Colonies were streaked to singles on LB plates supplemented with hygromycin. Single colonies were screened for loss of kanamycin resistance, indicating that pMQ305 had recombined out of the chromosome as catalyzed by digestion of the I-SceI site on pMQ305, by the I-SceI nuclease expressed from pMQ299. Approximately equal numbers of pigmented and colorless colonies were found among the kanamycin-sensitive colonies. Chromosomal DNA was extracted from several of the resulting colonies (Quick Extract, Epicentre), and the *pigA* locus was analyzed by PCR using primers 2081 and 2082 described above.

Prodigiosin Production Assay

Five single colonies per strain were inoculated into five separate test tubes containing 5 ml of LB medium supplemented with hygromycin (140 μ g/ml) and were incubated for 16–18 h on a rotary shaker (TC-7, New Brunswick). Ten microliters of each culture was spotted on the surface of LB plates supplemented with hygromycin (140 μ g/ml) and incubated for 20 h at 30°C. Bacteria were scraped from the agar plate and suspended in 1-ml PBS (phosphate buffered saline, pH 8). The cell density (A600 nm) was taken with one half of the bacterial suspension, and the remaining 500 μ l of bacteria in PBS was pelleted by centrifugation. Prodigiosin was extracted from centrifuged cells with acidified ethanol as Slater et al. [32], and measured spectrophotometrically at 534 nm (Beckman DU-70 spectrophotometer) using a 1 cm² cuvette. The A534/A600 ratio was determined as an expression of prodigiosin levels. The experiment was repeated twice on different days, and combined data was analyzed by one-way ANOVA with a Tukey post-test with significance set at $P < 0.05$, using Graphpad Prism software.

Results

pSC101-Based Vectors

A temperature-sensitive version of the pSC101 replicon is useful for numerous applications including general cloning and expression of genes in enteric bacteria such as *E. coli*, *Salmonella* species, and *Serratia* species [4, 15]. It is also useful for delivery of transposons

or conditional expression of genes, such as recombinases [5, 28]. We added the HmR cassette to previously published plasmids [30] with a temperature-sensitive pSC101 replicon [10]. One resulting plasmid, pMQ297 has the *E. coli* P_{lac} promoter driving the *lacZ α* gene containing several restriction sites for general cloning use. This and subsequent vectors are depicted and listed in Fig. 1 and Table 1. This plasmid is a low-copy cloning vector that can be removed from cells or forced to integrate by shifting to high temperature (42°C).

The pMQ297 was modified by addition of the I-SceI coding gene, cloned under transcriptional control of the *E. coli* P_{lac} promoter, to generate pMQ299. The I-SceI protein is an endonuclease used in allelic replacement strategies and it initiates a double stranded break at I-SceI restriction site [22, 24, 33].

pBBR1-Based Vectors

The pBBR1 amplicon is capable of replication in Gram-negative bacterial species [1, 18]. We modified a previously described vector, pMQ132 [30], by addition of the HmR cassette to generate pMQ300. This vector is a medium copy cloning vector useful for complementation purposes. This plasmid was sufficient to confer HmR to *A. baumannii*, *S. marcescens*, and *P. aeruginosa* (Fig. 2), and is to our knowledge the first example of using hygromycin as a selectable marker in these organisms. Without the HmR cassette, *A. baumannii* and *S. marcescens* exhibited weak growth at 70 $\mu\text{g/ml}$ but not at 140 $\mu\text{g/ml}$, whereas *P. aeruginosa* exhibited growth up to 140 $\mu\text{g/ml}$ but not 280 $\mu\text{g/ml}$. With the pMQ300 vector *A. baumannii* grew well on LB plates with hygromycin up to 420 $\mu\text{g/ml}$ and with reduced colony size from 560 to 840 $\mu\text{g/ml}$. *S. marcescens* and *P. aeruginosa* bearing the pMQ300 vector grew with the maximum tested hygromycin concentration of 980 $\mu\text{g/ml}$. The HmR cassette on the pMQ300 plasmid was also sufficient to confer HmR to *A. xylosoxidans*, but insufficient to confer HmR to *S. cerevisiae* (data not shown).

The pMQ132 vector was modified to generate pMQ352 for use in *Francisella* species. This was done using yeast recombineering by the simultaneous addition of the *S. cerevisiae* selectable marker *LEU2*, the HmR cassette, and *sacB*, which was placed under control of the P_{groESL} promoter that also drives transcription of the *hph* gene. The *sacB* gene confers sucrose sensitivity to many Gram-negative organisms and is often used in allelic replacement vectors [27]. The pBBR1 replicon is not stable in *F. tularensis* and this plasmid can be used for allelic replacement in this organism [12]. pMQ352 can also be used for general cloning and complementation purposes and is expected to replicate in a broad range of Gram-negative bacteria because of the pBBR1 replicon.

The pMQ352 vector was designed with MluI sites flanking the *sacB* gene, and these were cut to remove the *sacB* gene and the remaining backbone was self-ligated to generate pMQ225. The pMQ225 vector is similar to pMQ300 except that it contains the *LEU2* marker, rather than the *URA3* marker, and it has an MluI site that can be used with recombineering and restriction enzyme-based cloning strategies.

oriR6K-Based Vectors

The oriR6K- γ replicon has a narrow host range, but is a versatile replicon in that it can be maintained in commercially available *E. coli* strains at either low copy, high copy, or used as a suicide plasmid, depending upon whether the strain has a wild-type *pir* gene or the *pir-116* allele or no *pir* gene, respectively [17, 20]. This replicon is widely used for allelic replacement and integration purposes [21]. The pBBR1 replicon of pMQ225 was replaced by the oriR6K- γ replicon along with the replication and chloramphenicol resistance determinants of the pC194 plasmid, and the I-SceI gene in a single step, to generate pMQ235. The pC194 plasmid is a small R-plasmid originally isolated from *S. aureus* coding

for the chloramphenicol resistance gene, *cat194* [14]. pC194 replicates in several Gram-positive and Gram-negative organisms, including *Staphylococcus* species and *F. tularensis*. The I-SceI gene codes for an endonuclease from *S. cerevisiae* mitochondrial DNA [22], and is useful in some allelic replacement strategies [12, 24]. On pMQ235, the I-SceI gene was placed under transcriptional control of the *P_{groESL}* promoter that drives the *hph* gene to express I-SceI in a wide range of hosts.

Because chloramphenicol resistance markers are prohibited for use in virulent *Francisella* species in the United States [6], we replaced the *cat-194* gene of pMQ235 with *dtomato*, a gene that codes for a fluorescent red protein to generate pMQ351. In *E. coli*, pMQ351 confers a pink color to the EC100D *pir-116* strain, such that one can visually determine which colonies have the plasmid. In *F. tularensis* and other organisms, where the pC194 replicon can replicate, this vector can be used to deliver the I-SceI for allelic replacement strategies. After allelic replacement, loss of the I-SceI delivery plasmid is desired, so an easy strategy to identify bacteria that have lost this plasmid would be to grow cultures without selection and then separate single colonies on agar plates without selection. Subsequently, one could scan replicas with a fluorescent microscope for non-fluorescent clones that have lost the plasmid, followed by testing for hygromycin susceptibility. We could easily observe red fluorescence among *F. tularensis* strain LVS colonies transformed by pMQ351 (data not shown).

The I-SceI gene from pMQ235 was designed with flanking MluI sites. These were digested and the vector backbone self-ligated to generate pMQ353. This plasmid can be used as a general cloning vector or suicide vector as dictated by the host's ability to support replication of pC194 or oriR6K- γ -based vectors.

The pMQ310 plasmid is an oriR6K- γ -based vector, modified from pMQ236 with the HmR cassette. This plasmid contains the I-SceI restriction site that can be used to introduce double-stranded breaks into chromosomal DNA to drive homologous recombination. It is therefore useful for allelic replacement strategies in conjunction with a plasmid expressing the I-SceI gene.

The pMQ354 vector is designed as an integrative promoter probe. This vector has a full-length *lacZ* gene from pYC2-lacZ-CT (Invitrogen), the oriR6K- γ replicon, and HmR. A piece of targeting DNA can be moved into pMQ354 upstream of the *lacZ* gene, to make either a transcriptional or translational fusion. When this is integrated into a strain of bacteria without the *pir* gene, it can integrate into the chromosome as directed by the targeting DNA. The strength of proximal promoters can then be measured using β -galactosidase assays.

Mutation of *pigA*

To demonstrate the utility of some of the presented vectors, we chose to perform an allelic replacement experiment to mutate a gene on the chromosome of *S. marcescens*. The *pigA* gene codes for a putative short chain acyl-CoA dehydrogenase, and is the first gene in the prodigiosin pigment operon required for the red coloration of many *S. marcescens* strains [9]. An in-frame deletion in the *pigA* gene was designed such that the resulting gene should code for a protein with a 235 amino acid deletion out of the original 385 residues. This new *pigA* allele, *pigA*- Δ , was introduced into *S. marcescens* (PIC-3611) using allelic replacement vector pMQ236 [30], which is similar to pMQ310 except that it has a kanamycin resistance marker. The resulting merodiploid strain has pMQ236 + *pigA*- Δ integrated at the *pigA* locus, and the pMQ236 plasmid has an I-SceI restriction site to facilitate allelic replacement. Plasmid pMQ299 (Table 1) was moved into the merodiploid strain by conjugation to express I-SceI and initiate recombination. Candidates were grown at

42°C on medium without hygromycin to enrich for cells that have lost pMQ299, and were patched on separate agar plates with kanamycin, hygromycin, or no antibiotic. Approximately half of the kanamycin-susceptible single colonies lacked the red color of the parent strain and the merodiploid strain. Several kanamycin-susceptible strains were screened by PCR for deletion of *pigA*. We found that red isolates maintained the full-length *pigA* gene, whereas all the tested white colonies exhibited the expected 705-bp deletion (Fig. 3a).

The full-length *pigA* gene was cloned from a laboratory strain of *S. marcescens* (PIC-3611) and introduced into pMQ300 for complementation purposes. The pMQ300 + *pigA* plasmid (p*pigA*) was able to restore pigment production to *pigA*-Δ mutants (Fig. 3b). Together, these data show that these HmR-resistant vectors can be used for genetic manipulation of the *S. marcescens* genome.

Discussion

The toolkit of novel vectors presented here, each vector with a broadly expressed HmR cassette and a replicon useful for genetic manipulations, can expand studies with bacterial pathogens of medical importance. This report shows that the *P_{groESL}-hph* HmR cassette can be used with diverse species of opportunistic pathogens, *A. xylosoxidans*, *A. baumannii*, *F. tularensis*, *P. aeruginosa*, and *S. marcescens*. The vectors are by no means limited to the organisms used in this study, as several can be used as suicide-vectors in other species and the pBBR1-replicon has an extremely broad host range. Additionally, all these vectors contain *S. cerevisiae* replication machinery and selectable markers so that researchers can use the native recombination ability of yeast to modify genes in vivo [23, 26, 29, 30].

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Fig. 1.

Vector maps. Maps of HmR vectors that have been linearized at a consistent but arbitrary origin. *Arrows* indicate open reading frames and *lines* indicate other regions of interest. *T1* and *T2* are transcriptional terminators; *CENARS* is the *CEN6* and *ARSH4* replication elements for *S. cerevisiae*; *palA*, *pre* and *palB* are components of pC194; *lacZa* is *lacZa*; the *lacZ* open reading frame from pMQ354 codes for a C-terminal V5 epitope and poly-histidine tag. Other markers are defined in the text. Sequence information is available at GenBank-accession numbers GU339044–GU339053, HQ418395

Fig. 2.

Plasmid conferred HmR phenotype to opportunistic pathogens. Use of vectors with hygromycin markers in opportunistic pathogens. *P. aeruginosa* strain PA14 (Pa), *S. marcescens* strain PIC-3611 (Sm), *A. baumannii* strain K1971 (Ab)

Fig. 3.

pigA mutagenesis and complementation. **a** PCR analysis of colonies from an allelic replacement strategy using pMQ299 to make a 705-bp deletion in the *pigA* gene. The amplicon from the pigmented WT *pigA* gene is 1229 bp. Colonies from pigmented colonies and the parental strain (WT) exhibited the full-length amplicon, whereas pigment-deficient colonies exhibited the 524 bp amplicon expected for the *pigA*-Δ mutation. **b** Prodigiosin production by *S. marcescens* *pigA* mutant. Prodigiosin levels were severely reduced in the *pigA*-Δ mutant made with pMQ299, and complemented by the wild-type *pigA* gene expressed from the pMQ300 vector (*ppigA*). Error bars depict one standard deviation, and the asterisk indicates a significant difference ($P < 0.05$) from the WT strain with empty vector control (pMQ300)

Table 1

Vectors with HmR markers

Plasmid name ^a	Resistance marker ^b	Replicon ^c	Promoter/feature	Counter selection ^d	oriT (RP4) ^e	Host range ^f	Yeast marker	Possible use ^g
pMQ297	<i>hph</i>	pSC101 ^{ts}	<i>P_{lac}-lacZα</i>	No	+	NHR, N	<i>URA3</i>	A, C, I
pMQ299	<i>hph</i>	pSC101 ^{ts}	<i>P_{lac}-I-SceI</i>	No	+	NHR, N	<i>URA3</i>	A
pMQ300	<i>hph</i>	pBBR1	<i>P_{lac}-lacZα</i>	No	+	BHR, N	<i>URA3</i>	C
pMQ311	<i>hph</i>	pBBR1	<i>P_{lac}-I-SceI</i>	No	+	BHR, N	<i>URA3</i>	A
pMQ352	<i>hph</i>	pBBR1	<i>P_{lac}-lacZα</i>	sacB	+	NHR, N	<i>LEU2</i>	C
pMQ225	<i>hph</i>	pBBR1	<i>P_{lac}-lacZα</i>	No	+	BHR, N	<i>LEU2</i>	C
pMQ235	<i>hph, cat194</i>	R6K-γ/ pC194	<i>P_{lac}-lacZα, I-SceI^h</i>	No	+	NHR, N, P	<i>LEU2</i>	A, C
pMQ351 ⁱ	<i>hph</i>	R6K-γ/ pC194	<i>P_{lac}-lacZα, I-SceI^h</i>	No	+	NHR, N, P	<i>LEU2</i>	A, C
pMQ353	<i>hph, cat194</i>	R6K-γ/ pC194	<i>P_{lac}-lacZα</i>	No	+	NHR, N, P	<i>LEU2</i>	C, I
pMQ310	<i>hph</i>	R6K-γ	<i>P_{lac}-lacZα</i>	<i>I-SceI</i> site	+	NHR, N	<i>URA3</i>	A, I
pMQ354	<i>hph</i>	R6K-γ	<i>lacZ-6his</i>	<i>rpsL</i>	+	NHR, N	<i>URA3</i>	A, I, P

^aPlasmid sequences are deposited at GenBank, accession numbers GU339044–GU339053, HQ418395

^bThe HmR gene, *hph*, is under control of the *P_{groESL}* promoter from *F. tularensis* and confers hygromycin resistance to resistance to tested Gram-positive and Gram-negative organisms. The *cat194* gene from pC194 confers chloramphenicol resistance in Gram-positive and tested Gram-negative organisms when used at high-copy

^cReplicon information is listed in text

^dThe I-SceI site is used in conjunction with an I-SceI expressing plasmid; *sacB* confers sucrose sensitivity, *rpsL* confers streptomycin sensitivity in certain *rpsL* mutant strains

^eThe oriT can be used for conjugal transfer of plasmids

^f*NHR* narrow host range, *BHR* broad host range, *N* Gram-negative, *P* Gram-positive. These host ranges are expected based upon the replicon

^gA allelic replacement, *C* complementation or gene expression, *I* insertion, *P* promoter probe

^hThis vector also expresses the I-SceI gene from the *P_{groESL}* promoter

ⁱThis vector also expressed dtomato from the *FGRp* promoter

Table 2Oligonucleotide primers used in this study Name Sequence^a

Name	Sequence ^a
1537	tccgcggccgggaagccgatctcgcttgaacgaattgTCAGGCGCCGGGGGCGGT
1538	cttcaataattgaaaaaggaagagtatgagTTGAAAATTTTTTTTGGACTCAATATC
1295	ttttatagcacgtgatgaaaaggacccatcgataagctATCGACGGTCGAGGAGAACTTC
1298	ctagatattgagtcacaaaaaaatttcaactcgcgCCTCCTTTTTCTCCTTCTTGAT
1300	atcaagaaggagaaaaaggaggctcgcgTTGAAAATTTTTTTTGGACTCAATATCTAG
1301	ctttttgatgttcacgttcctcctcgcgTCAGGCGCCGGGGGCGGTGTCC
1303	ggacaccgccccggcgctgaacgcgTAGGAGACATGAACGATGAACATCAAAAAG
1304	gtgcaccataatcgcatcttttttgcgtttacgcgTGAGACGGTCACAGCTTGCTCTG
1726	tgtcgtgccactgcattaatgcgcctacggccttgcCAAAGTGAACAACACTCAACC
1727	catatagttttatgcctaaaaacctacagCTGTGAGCCGTTAAGTGTTC
1729	ggaacacttaacggctgacagCTGTAGGTTTTAGGCATAAACTATATG
1730	cgaacttctctgaataataaggacgcgGCTTTTAAAAAGCAAATATGAGCCA
1732	tggctcatattgttttttaaaagcacgcgtTTATTATTTCAGGAAAGTTTCG
1733	ctgggggcccggacaccgccccggcgctgaacgcgTAGGAGAACGCATATGCATCA
1887	aaaccgactgtaaaaagtacagtcgcattatctcataGAATTCCTTACTTGTACAGCTCG
1888	taattcacttttagataaaaatttaggagcatatcaaTATCATCCTGAGTCACAACCTAC
2005	aacggctgacaggacgaaaaggcctcgtgatacgcGAGTTGAAAATTTTTTTTGGACTC
2006	atctaaagtatatagtaaaacttggtctgacagtaCGTTCAGGCGCCGGGGGCGGTG
2001	tcttgccgcaaggatctgatgcgcaggggatcCGAGTTGAAAATTTTTTTTGGACTC
2002	agttaaacgtttggccttactaacggagaaccaCGTTCAGGCGCCGGGGGCGGTGTC
2081	acgccagggttttccagtcacgacgttgtaaaacgaTCATGCGCTCCCCGCAGACCCA
2086	ggattggcatgaatgcgctgatGCCTGTTGAAACACGATCG
2082	tgtgagcggataacaatttcacaggaacagctATGGATTTTAACTTATCAAGTGAGC
2085	cgatcgtgtttccaacagcATCGAGCGCATTCATGCCCAATCC

^a *Upper-case letters* in these primer sequences direct amplification of the desired DNA, whereas the *lower-case letters* target homologous recombination with the cut plasmid. *Underlined* sequences are added restriction sites, as noted in the text