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New Range of Vectors with a Stringent 5-Fluoroorotic Acid-Based Counterselection System for Generating Mutants by Allelic Replacement in *Staphylococcus aureus*

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We have developed a range of vectors for allelic replacements in *Staphylococcus aureus* to facilitate genetic work in this opportunistic pathogen. The central feature of the vector range is a selection/counterselection system that takes advantage of the 5-fluoroorotic acid (FOA) resistance and pyrimidine prototrophy caused by the loss and gain, respectively, of the *pyrF* and *pyrE* genes. This system allows for stringent counterselection of the vectors during the second homologous recombination of a classic allelic replacement. The basic vector pRLY2, which contains the *pyrFE* genes from *Bacillus subtilis*, was combined with chloramphenicol, erythromycin, and tetracycline resistance genes and four different versions of nonreplicative or conditionally replicative origins of replication. The choice between these 12 different pRLY vectors allows for high versatility and ensures that the vectors can be used in virtually any genetic background. Finally, as proof of concept, we present six deletions or modifications of components in the *S. aureus* degradosome as well as the operon containing the *cshB* DEAD box helicase.

Staphylococcus aureus is an important opportunistic pathogen that can cause various diseases from furuncles to life-threatening osteomyelitis and endocarditis (17). Moreover, acquisition of antibiotic resistance and biofilm formation genes resulting in persistent infections are of major concern. An in-depth genetic analysis of *S. aureus* virulence genes is therefore highly desirable.

One of the major problems, especially when examining clinical *S. aureus* strains, is that genetic manipulation is relatively difficult and time-consuming compared to most model organisms. Much work has been carried out using laboratory strains that are easy to work with, such as the mutagenized strains *S. aureus* RN6390 and RN4220 (14, 19, 22). Furthermore, complementation with genes carried on a plasmid can lead to markedly different phenotypes compared to a single chromosomal copy of the gene, and as a consequence, there is increasing interest in performing allelic replacements on the chromosomes of clinical *S. aureus* strains.

Several vectors are currently available for generating mutations in *S. aureus* via double homologous recombination, including pBT2, pMAD, pKOR1, and a range of pCN vectors (1, 2, 5, 7). Common to all of them is that they carry a thermosensitive origin of replication (pE194ts-ORI in pBT2, pMAD, and pKOR1; pT181ts-ORI in pCN39, pCN49, and pCN50), which allows the vectors to replicate in *S. aureus* at 30°C, but not at 42°C. This enables the passage of the vectors through the restriction-defective, but modification-proficient strain RN4220, by maintaining growth at 30°C, before transforming into the final recipient strain (14, 25). Once colonies have been established after transformation, the temperature is shifted to the nonpermissive temperature of 42°C, while antibiotic selection is kept up, thus selecting for cells where the plasmid has recombined into the chromosome via one of the two regions of homology that correspond to the adjacent regions of the desired mutation. The next step is to find colonies where the vector has recombined a second time via the other region of homology, thereby losing the plasmid and leaving only the desired mutation. This is usually done by growing the culture under nonselective conditions at 42°C and by finally screening individual colonies for loss of the plasmid. This screening can be extremely cumbersome, especially if the desired mutation results in a loss of fitness to the cell, thereby allowing wild-type cells to outgrow the mutant cells during the liquid culture passages. Other potential problems include temperature sensitivity of the desired mutant and the risk of acquiring secondary mutations during the passages at elevated temperature. Although pMAD encodes a beta-galactosidase gene, which allows blue/white screening for the loss of the vector on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), in our hands, white colonies could often be shown to retain pMAD, suggesting that the beta-galactosidase is not stably expressed. Moreover, a reoccurring problem with the thermosensitive origins is that their replication is not completely blocked at the nonpermissive temperature, and therefore, if a mutant divides slower than the replication rate of the vector, then it is extremely difficult to isolate plasmid-free cells. The pKOR1 plasmid goes a step further and incorporates a counterselection system based on a tetracycline-inducible transcript that is antisense to the essential *secY* secretion gene (2). However, many clinical and lab strains of *S. aureus*, such as strain COL or our clinical strain S30 (11, 24), carry the pT181 plasmid that carries the gene that encodes a tetracycline efflux pump, which may limit the use of the inducible promoter in pKOR1. A further complication with pKOR1 is the lack of alternatives to the chloramphenicol resistance marker on the backbone vector, which prevents its use in strains that are already resistant to this drug. Additionally, alternative origins would also be desirable, since both pMAD and pKOR1 use the pE194ts origin of replication.

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An efficient counterselection system, based on resistance to the drug 5-fluoroorotic acid (FOA) has been used for many years, primarily in yeasts but also in bacteria and archaea (3, 16, 18, 23). FOA is not toxic in itself, but it is converted to the toxic 5-fluoro-UMP by the two genes encoding orotate phosphoribosyltransferase (*pyrE*) and orotidine 5-phosphate decarboxylase (*pyrF*). Plating cells on rich medium and FOA will allow extremely efficient selection for the inactivation of *pyrE*, *pyrF*, or both. Since *pyrE* and *pyrF* catalyze the last two steps in the pyrimidine biosynthesis pathway, they are essential for growth without pyrimidines in the medium. Thus, revertants to the wild type can easily be selected by plating on a pyrimidine-free medium.

Here we present a range of vectors for allelic replacement in *S. aureus*, incorporating a *pyrFE*/FOA-based counterselection system and using different origins of replication and different antibiotic markers. As proof of principle, the system was used to delete or modify components of the Gram-positive degradosome: four different RNases, the enolase, and *cshA*, an RNA DEAD box helicase. Furthermore, a second DEAD box helicase in the *S. aureus* genome, *cshB*, and its downstream cistron SA1386 in the operon were deleted individually and together.

MATERIALS AND METHODS

Detailed protocols for allelic replacements, using the pRLY vectors, can be found in Protocols S1, S2, and S3 in the supplemental material.

Media and growth of bacteria. *Escherichia coli* was grown in LB medium (Merck, Whitehouse Station, NJ), supplemented as needed with 100 mg/liter ampicillin, 50 mg/liter spectinomycin, and 100 mg/liter X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Fermentas Inc., Glen Burnie, MD).

S. aureus was grown in Mueller-Hinton broth (Becton Dickinson and Company, Le Pont de Claix, France), supplemented as needed with 10 mg/liter chloramphenicol (MHC), 10 mg/liter erythromycin (MHE), 2 mg/liter tetracycline (MHT), 200 mg/liter 5-fluoroorotate (MHFOA) (US Biological, Swampscott, MA), or 10 mg/liter uracil (MHU). Agar (13 g/liter) was added to make plates. For pyrimidine-free medium, RH medium, a modified RPMI 1640 medium (catalog no. R7388; Sigma), which contains no $NaHCO_3$ but is buffered with 20 mM HEPES (Sigma-Aldrich Chemie, Steinheim, Germany), was used. When used for plates, 500 ml of RH medium was mixed at 55°C with 7 g of agar (Merck, Whitehouse Station, NJ) that had been autoclaved in 100 ml H_2O.

Bacterial strains and plasmids used in this study are listed in Table 1.

Molecular biology methods. All methods using standard molecular biology techniques were performed by the methods of Sambrook et al. (20) or according to the recommendations of the manufacturers. Restriction enzymes from New England BioLabs (Ipswich, MA) were used according to the manufacturer's instructions. PCR products used for cloning were amplified using Phusion high-fidelity DNA polymerase enzyme (New England BioLabs), and screening was carried out using REDTaq ReadyMix (Sigma-Aldrich Chemie) according to the manufacturer's instructions.

The primers for sequencing inserts in the pRLY vectors were pRL-MCS-Seq-F1 (MCS stands for multiple cloning site, Seq stands for sequencing, and F stands for forward) (GCGGCATCAGAGCAGATTG) and pRLY-MCS-Seq-R1 (R stands for reverse) (GGAAACGAAATCCCG AGTC). The primers used for sequencing inserts in the pRLB vectors were pRLB-MCS-Seq-F1 (CTAATACGACTCACTATAGGGC) and pRLB-MCS-Seq-R1 (TGCCCCGTTAGTTGAAGAAGGTT). Primers used for vector construction are shown in Table S1 in the supplemental material.

Strain construction. *S. aureus* strains PR01 and PR02 were constructed using pBS-AB-ERY (ERY stands for erythromycin), which is a pBluescript II KS+ plasmid (Stratagene, Santa Clara, CA) with ~1,000 bp upstream of *pyrFE* cloned between the BamHI and EcoRI sites, ~1,000 bp downstream of the *pyrFE* genes cloned between the EcoRI and XhoI sites, and the *ermC* cassette (see Fig. 2) cloned into the BamHI site. The plasmid was transformed into strains SA564RD (9) and RN4220, respectively, and transformants that grew on RH medium containing erythromycin (ensuring that plasmid integration was downstream of the *pyrFE* genes) were isolated. Then a round of FOA selection was used to select for a second recombination event that removed the plasmid backbone. FOA-resistant colonies were screened for loss of erythromycin resistance and then sequenced to verify the deletion of *pyrFE*.

For strains PR03 and PR04, the pT181ts origin of replication (see Fig. 2) was cloned into the EcoRI site of pBS-AB-ERY, generating pBS-AB-ERY-TREPrev. The procedure for generating the strains was the same as for PR01 and PR02, but it was carried out at 42°C to avoid plasmid replication via the pT181ts origin.

Vector construction. All *in silico* design of vectors and constructs was carried out using pDRAW32 software (www.acaclone.com).

pFL was modified into pRL1 by introducing an XcmI site inside the *lacZ*α gene (generating an unperturbing A53D mutation) and by introducing an NcoI site, an XhoI site, and a second XcmI site. pRL1 was then further modified by introducing an AscI site, an NgoMIV site, and an MluI site near the ClaI site, creating pRL2 (see Fig. 2).

The $Pspac^C$-*pyrFE* cassette was constructed by merging the $Pspac^C$ promoter from pMF35 with the *pyrFE* genes from *Bacillus subtilis*, using fusion PCR. Then the three HindIII sites and a BglII site were removed from the *pyrFE* genes by site-directed mutagenesis to maintain the uniqueness of the corresponding sites.

The resistance cassettes were all prepared with a BamHI site upstream and a BglII site downstream: the chloramphenicol resistance (Cm^r) CAT194 gene, amplified from pMK4 (21); the erythromycin resistance (Ery^r) *ermC* gene, amplified from pMAD (1); and the tetracycline resistance (Tet^r) *tetK* gene from pT181.

The origins of replication were prepared with AscI sites at each end, and the XbaI site in the pT181ts origin was removed by silent mutagenesis.

pRLBC2 was constructed by combining the pSC101-based spectinomycin-resistant vector pGB2 (8) with the multiple cloning site of pBluescript II KS+ and the *S. aureus* plasmid pC194 (Cm^r). In addition, to make plasmid preparation from *E. coli* easier, the copy number of the pSC101 origin was increased by introducing an G-to-A point mutation at position 1026 of the *repA* gene (pSC101 numbering) (26). A basic *E. coli* plasmid, pRLB1, was obtained by removing the pC194 sequence from pRLBC2, while maintaining the MCS. pRLBE2 was generated by introducing a BsrGI site and a BstBI site on each side of the CAT194 gene in pRLBC2, and inserting the *ermC* cassette between the two sites. pRLBER9 was designed with a relatively short, 652-bp region of homology to the *S. aureus* chromosome to prevent too frequent plasmid cross-out (from coordinate 201 in the *pyrF* open reading frame [ORF] to 157 in the *pyrE* ORF).

Nucleotide sequence accession numbers. GenBank accession numbers for the vector constructs can be found in Tables 2 and 3.

RESULTS AND DISCUSSION

One of the major challenges in bacterial genetics is to generate mutations that severely limit growth. Mutation in itself is a rare event, but if the mutation additionally confers severe loss of fitness to the cell, then screening for it can be even more difficult due to faster growth of wild-type cells.

To exploit the stringent counterselection offered by FOA to generate an improved vector system for allelic replacement in *Staphylococcus aureus*, we created a range of plasmids that express *pyrE* and *pyrF*, combined with a range of antibiotic resistance genes and different conditionally replicating staphylococcal origins of replication. Moreover, we facilitated cloning into the vectors by retaining the uniqueness of all the restriction sites in the multiple cloning site of pUC19, as well as blue/white screening by the *lacZ*α complementation system.

Counterselection vector construction. We used the pFL plas-

TABLE 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Description, relevant genotype/characteristic, or purpose[a]	Source or reference
Bacterial strains		
E. coli DH5α	Standard cloning strain	Invitrogen
S. aureus		
RN4220	Restriction deficient, modification proficient	19
SA564	Clinical isolate	9
SA564RD	SA564 *hsdR* type III mutant (restriction deficient)	9
SA564RD::pRLBER9	SA564RD with the pRLBER9 plasmid recombined into the chromosome, thereby disrupting *pyrE* expression	This work
PR01	SA564RD Δ*pyrFE*	This work
PR02	RN4220 Δ*pyrFE*	This work
PR03	RN4220 Δ*pyrFE*::ORI-pT181ts	This work
PR04	SA564RD Δ*pyrFE*::ORI-pT181ts	This work
PR01-01	PR01 with the RNase J1 gene deleted (between coordinates 1066843 and 1068378) using the pRLYE1 vector	This work
PR01-02	PR01 with the RNase Y gene deleted (between coordinates 1281956 and 1283492) using the pRLYE1 vector	This work
PR01-04	PR01 with the RNase J2 gene deleted (between coordinates 1268765 and 1270394) using the pRLYE1 vector	This work
PR01-05	PR01 with the enolase gene substituted with the *E. coli* enolase gene (between coordinates 837567 and 838878) using the pRLYE1 vector	This work
PR01-06	PR01 with the *cshA* gene substituted with a CAT194 cassette (between coordinates 2136001 and 2137592) using the pRLYE1 vector	This work
PR01-07	PR01 with the PNPase gene deleted (between coordinates 1266484 and 1268492) using the pRLYE1 vector	This work
PR01-08	PR01 with the SA1386 gene deleted (between coordinates 1591115 and 1591987) using the pRLYE1 vector	This work
PR01-09	PR01 with the *cshB* gene deleted (between coordinates 1592017 and 1593367) using the pRLYE1 vector	This work
PR01-10	PR01 with the *cshB* and SA1386 genes substituted by an *ermC* cassette (between coordinates 1591115 and 1593454) using the pRLYC1 vector	This work
PR07	SA564RD with the *spa* gene deleted (between coordinates 122645 and 124007) using the pRLYC1 vector	This work
Plasmids		
pBlueScript II KS+	Standard *E. coli* cloning vector	Stratagene
pFL	pUC19 derivative with added BglII and ClaI sites	4
pGB2	pSC101 origin of replication, spectinomycin resistance marker	8
pMAD		1
pMF35	Carries the Pspac^{C} promoter	10
pMK4		21
pNL9162	Contains the targetron homing intron, adapted for use in *S. aureus*	28
pT181	Naturally occurring plasmid, encoding the *tetK* tetracycline efflux pump	13
pBS-AB-ERY	Used to generate strains PR01 and PR02	This work
pBS-AB-ERY-TREPrev	Used to generate strains PR03 and PR04	This work

[a] Deletions were carried out in strain PR01, which has an *S. aureus* strain SA564 background, but coordinates are given based on the *Staphylococcus aureus* n315 genomic sequence (GenBank accession number BA000018.3).

mid as the basis. pFL is a pUC19 derivative in which a BglII site and a ClaI site have been introduced upstream of the *lacZ*α gene in a region that is nonessential for plasmid functions (4). pFL was modified into pRL2 by introducing several additional restriction sites (Fig. 1).

(i) The *pyrFE* cassette for counterselection. To limit unwanted homologous recombination between the vectors and the *S. aureus* chromosome, endogenous DNA sequence was avoided by fusing the exogenous *pyrFE* genes from *Bacillus subtilis* to the artificial constitutive promoter Pspac^{C} (10), both of which are significantly different from the *S. aureus* sequence. Furthermore, while we were testing the vectors, we discovered that the Pspac promoter is slightly unstable in *E. coli*, due to a 6-bp repeat surrounding the −10 motif, and we therefore introduced a G-to-A mutation that disrupts the repeat but retains promoter activity (Fig. 2A). The resulting construct was introduced into pRL2 between the BglII and NcoI sites, creating pRLY2 (Fig. 1).

Finally, three resistance cassettes, Cm^{r}, Ery^{r}, and Tet^{r} (Fig. 2B), were cloned into the BglII site of pRLY2, creating pRLYC1 (Cm^{r}) (Fig. 1), pRLYE1 (Ery^{r}), and pRLYT1 (Tet^{r}) (see Fig. S1A in the supplemental material).

(ii) Temperature-sensitive origins of replication. While these three constructs are efficient as suicide integrative vectors, it is often necessary to pass a vector through the restriction-deficient *S. aureus* strain RN4220, to get it correctly modified, before introducing the vector into a restriction-proficient *S. aureus* strain. For this to work, the vector will need to replicate in strain RN4220, but not in the wild-type strain.

The pE194ts origin of replication, which is temperature sensitive (12) and the pT181 origin of replication with the cop-634 mutations, which renders it temperature sensitive (6) (Fig. 2C) were therefore cloned into the AscI site of the pRLYx1 vectors (x can stand for C, E, or T), leading to pRLYx8 (pT181ts origin) and pRLYx9 (pE194ts origin) (Fig. 1 and Table 2; also see Fig. S1A in the supplemental material). It should be noted that the *tetK* cassette and the pT181ts origin of replication have an overlapping sequence, which we removed by deleting the small fragment be-

TABLE 2 pRL1-based vectors

Vector	Selection in *S. aureus*[a]	Origin of replication for *S. aureus*	Length (bp)	GenBank accession no.
pRL1	None	None	2,702	JQ410188
pRL2	None	None	2,703	JQ768506
pRLY2	pyr	None	4,414	JQ410189
pRLYC1	Cm, pyr	None	5,417	JQ366074
pRLYC2	Cm, pyr	ORI_{181}[b]	5,627	JQ768507
pRLYC8	Cm, pyr	pT181ts	7,038	JQ768508
pRLYC9	Cm, pyr	pE194ts	6,581	JQ768509
pRLYE1	Ery, pyr	None	5,499	JQ768510
pRLYE2	Ery, pyr	ORI_{181}[b]	5,709	JQ768511
pRLYE8	Ery, pyr	pT181ts	7,120	JQ768512
pRLYE9	Ery, pyr	pE194ts	6,663	JQ768513
pRLYT1	Tet, pyr	None	6,193	JQ768514
pRLYT2	Tet, pyr	ORI_{181}[b]	6,403	JQ768515
pRLYT8	Tet, pyr	pT181ts	7,404[c]	JQ768516
pRLYT9	Tet, pyr	pE194ts	7,357	JQ768517
pRLSAYC9	Cm	pE194ts	8,026	JQ768518
pRLSAYE9	Ery	pE194ts	8,108	JQ768519
pRLSAYT9	Tet	pE194ts	8,802	JQ768520

[a] All pRL1-based vectors carry an ampicillin resistance marker for use in *E. coli*. Abbreviations: pyr, pyrimidine prototrophy; Cm, chloramphenicol; Ery, erythromycin; Tet, tetracycline.
[b] Plasmids with the pT181 origin of replication but missing the *repC* gene, which is provided in *trans* in strains PR03 and PR04.
[c] Deletion between the ClaI sites in the Tet^r cassette and the pT181ts cassette.

tween the ClaI sites in pRLYT8 indicated in Fig. 2, to avoid potential vector instability.

To ensure that the thermosensitivity of the two origins of replication remained intact when introduced into the pRLYx1 vectors, strain PR01 (Fig. 3) carrying either pRLYx8 or pRLYx9 was grown overnight at 30°C in selective medium, whereupon 10 μl was transferred to MH medium at 42°C for 7 h. Dilution series were then spotted on nonselective (MH medium) and selective (MH medium plus antibiotic) plates, which were incubated at 30°C. Counting the colonies in the spots revealed that during the 7 h of nonselective growth at 42°C, about 90% of the cells had lost pRLYx8 or pRLYx9 (see Fig. S2 in the supplemental material), showing that thermosensitivity remains intact in both vectors.

(iii) Strain-specific plasmid replication. Although there are situations where it is desirable to transform with a replicating vector, notably when working with strains for which efficient transformation is not available, the thermosensitive plasmid origins of replication are not always completely inhibited at 42°C. A more stringently conditional system was therefore developed by combining an RN4220 derivative that expresses a plasmid replication protein with vectors that carry the corresponding replication origin without encoding the replication protein.

It has been shown that the replication protein encoded by pT181 (*repC*) can function in *trans* and recognizes an ~168-bp sequence to promote replication (13).

We exploited this relationship by replacing the *pyrFE* genes of *S. aureus* RN4220 with the full pT181ts origin of replication, which encompasses both the *repC* gene and the ~168-bp origin of replication, thus generating strain PR03 (Fig. 3). We then introduced the 168-bp minimal origin of replication (ORI_{181} [Fig. 2C]) into the AscI site of the pRLYx1 vectors, thereby obtaining pRLYx2 vectors that can replicate in strain PR03 at 30°C but that will be nonreplicative in any other *S. aureus* strain (Fig. 1 and Table 2), unless the strain carries another pT181 plasmid. However, it should be noted that the copy number of the pRLYx2 plasmids in strain PR03 is very low, and therefore, we recommend that either the plasmid should be prepared from a large culture volume or phage transduction should be used, when introducing the plasmid into a strain that does not allow plasmid replication.

(iv) Multiple cloning site of the pRLY vectors. The entire multiple cloning site (MCS) of pUC19 has been kept intact by deleting conflicting sites elsewhere in the vectors by silent mutagenesis (Fig. 2). Additionally, the two XcmI sites were designed to each give a single thymidine 3′ overhang, compatible with the single adenosine 3′ overhang created by many DNA polymerases used for PCR, thus allowing ligation to the vector without the need for enzymatic digestion of the PCR product. Thus, the MCS of the pRLY vectors stretch from the NdeI site at position 184 to the XcmI site at position 624 (the NcoI site can also be used in vectors that does not carry the Tet^r cassette), and the *lacZ*α gene is functional for blue/white screening in *E. coli* (Fig. 1).

Construction of new expression vectors to avoid interplasmid recombination. Sometimes it is desirable to maintain an expression vector in a strain while performing an allelic replacement. However, in such situations, it is important that the plasmid

TABLE 3 pRLB-based vectors

Vector	Selection in *S. aureus*[a]	Origin of replication for *S. aureus*	Incompatible vectors	Length (bp)	Description	GenBank accession no.
pRLB1	None	None	None	4,105	Basic *E. coli* vector, with no similarity to pUC-based plasmids	JQ768521
pRLBC2	Cm	pC194	pRLYC vectors	6,325	*E. coli-S. aureus* shuttle vector	JQ768522
pRLBE2	Ery	pC194	pRLYE vectors	6,543	*E. coli-S. aureus* shuttle vector	JQ768526
pRLBC3	Cm	pC194	pRLYC vectors	6,575	pRLBC2 with the Pprot promoter	JQ768523
pRLBE3	Ery	pC194	pRLYE vectors	6,793	pRLBE2 with the Pprot promoter	JQ768527
pRLBER1	Ery	None	pRLYE vectors	5,936	Carries 652 bp of *pyrFE* for temporary disruption of *pyrE*	JQ768528
pRLBCR8	Cm	pT181ts	pRLYC vectors, pRLYx8 vectors	7,468	Carries 652 bp of *pyrFE* for temporary disruption of *pyrE*	JQ768524
pRLBCR9	Cm	pE194ts	pRLYC vectors, pRLYx9 vectors	7,008	Carries 652 bp of *pyrFE* for temporary disruption of *pyrE*	JQ768525
pRLBER8	Ery	pT181ts	pRLYE vectors, pRLYx8 vectors	7,550	Carries 652 bp of *pyrFE* for temporary disruption of *pyrE*	JQ768529
pRLBER9	Ery	pE194ts	pRLYE vectors, pRLYx9 vectors	7,090	Carries 652 bp of *pyrFE* for temporary disruption of *pyrE*	JQ768530

[a] All pRLB vectors carry a spectinomycin resistance marker for selection in *E. coli*. Cm, chloramphenicol; Ery, erythromycin.

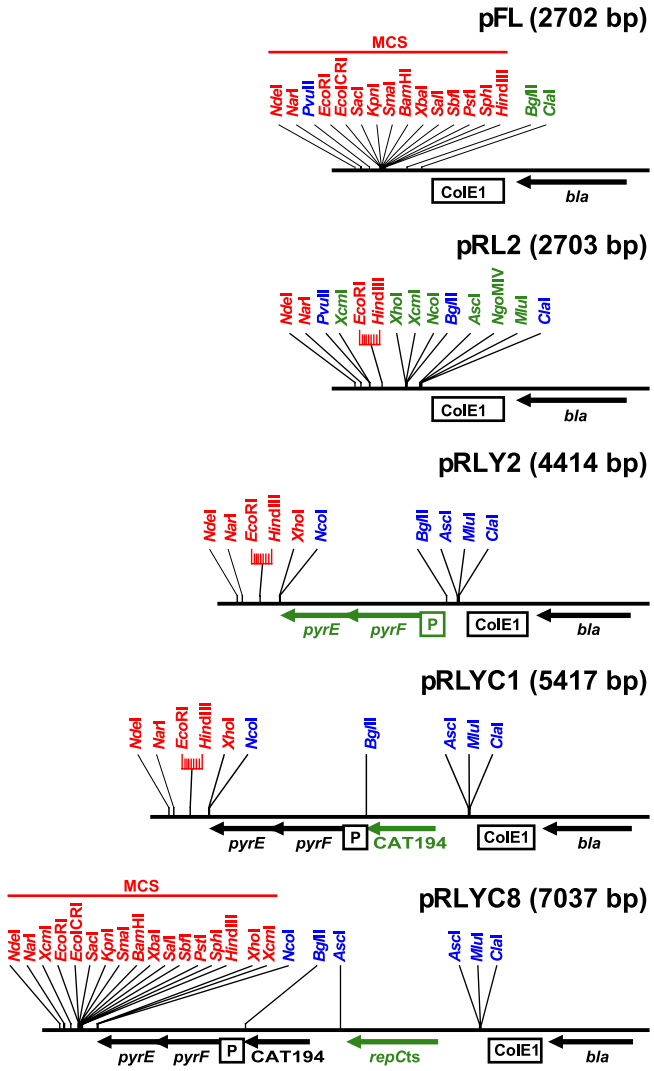

FIG 1 Different steps in vector construction, exemplified by the pRLYC8 vector. Features that are introduced at each step are shown in green. Unique restriction sites in the multiple cloning site (MCS) are shown in red. Other restriction sites of interest are shown in blue. *bla* is the beta-lactamase gene. ColE1 is the origin of replication for *E. coli*. *pyrF* and *pyrE* are genes taken from *B. subtilis* and fused to the Pspac^{C} promoter (which is shown as a boxed P). CAT194 is the *S. aureus* chloramphenicol resistance gene. *repCts* is the pT181ts replication gene. The full MCS is shown only in pFL and pRLYC8, but the region between the EcoRI and HindIII sites remains unchanged in all vectors.

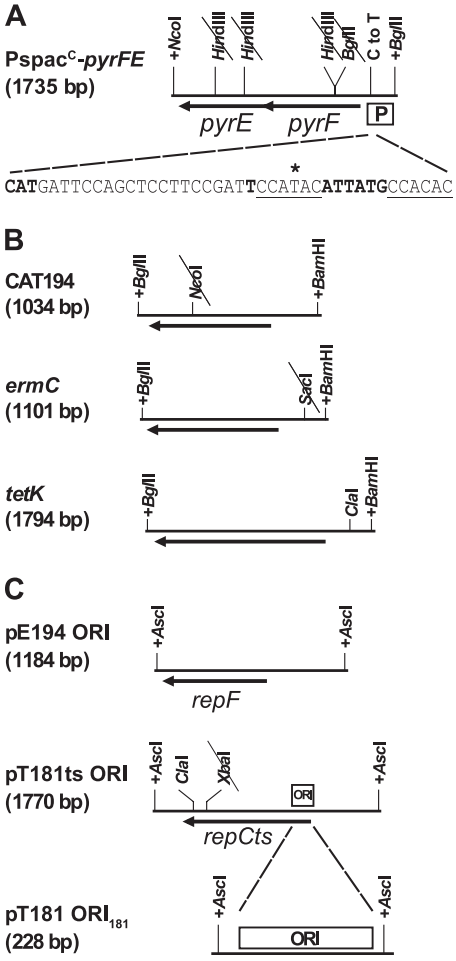

FIG 2 Cassettes used in vector construction. Added restriction sites are indicated by a plus sign before the restriction site, whereas a slash through the restriction site indicates that the restriction site was removed by a silent point mutation. The two ClaI sites used for the deletion in pRLYT8 are indicated. (A) The Pspac^{C}-*pyrFE* cassette, with *pyrF* and *pyrE* genes from *B. subtilis*. The C-to-T point mutation used to avoid instability in *E. coli* is indicated. The −10 box, the transcription start site, and the start codon are indicated in bold type. The 6-bp repeats are underlined, and the asterisk indicates where we introduced the C-to-T point mutation. The three HindIII sites and a BglII site were removed from the *pyrFE* genes by site-directed mutagenesis to maintain the uniqueness of the corresponding sites. (B) Antibiotic resistance cassettes used for construction of the pRLYx1 vectors. An NcoI site in CAT194 and a SacI site in *ermC* were removed by site-directed mutagenesis via silent mutations. (C) Origins of replication used for construction of the pRLYx2, pRLYx8, and pRLYx9 vectors. ORI$_{181}$ (ORI) is indicated, and broken lines indicate the section of the full-length pT181ts origin of replication, which comprises the ORI$_{181}$, used for the pRLYx2 vectors. The XbaI site was mutated in the pT181ts origin.

used for expression of proteins has no similarity to the allelic replacement vector (such as the pRLY vectors) to prevent homologous recombination between the two. We therefore designed a series of *E. coli-S. aureus* shuttle vectors based on the *E. coli* vector pGB2, which has no homology to pUC19 (8), and the *S. aureus* pC194 origin of replication, which has no homology to the pE194 or pT181 origins of replication. Two versions of the vector were made: pRLBC2 with chloramphenicol resistance and pRLBE2 with erythromycin resistance (Table 3 and Fig. 4). Furthermore, to allow continuous strong expression of genes and to allow expression to be uncoupled from native promoters, we introduced the constitutive Pprot promoter from a streptococcal plasmid (15) upstream of the KpnI site in the MCS of pRLBC2 and pRLBE2, generating pRLBC3 and pRLBE3, respectively (Table 3; see also Fig. S3 in the supplemental material).

Generating *pyrFE* mutants and revertants in *S. aureus*. To determine the optimal concentration of FOA for restricting growth of *pyrFE*$^{+}$ strains while allowing growth of *pyrFE* mutant strains, we spotted dilution series of a wild-type and a *pyrFE* mutant on MH plates with 50, 100, 150, and 200 mg/liter FOA. The 50- and 100-mg/liter concentrations of FOA allow slight growth of wild-type cells, whereas the 150- and 200-mg/liter concentrations abolish growth (not shown). The *pyrFE* mutant grew on all plates,

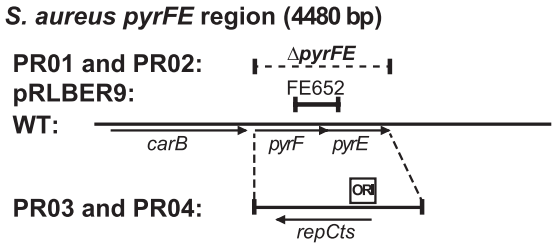

FIG 3 Various modifications to the *pyrFE* locus in *S. aureus*. The broken horizontal line indicates the chromosomal region of strains SA564RD and RN4220 that was deleted to generate strains PR01 and PR02, respectively. FE652 is the region of the *pyrFE* locus that allows pRLBER9 to recombine into the chromosome and disrupt *pyrE* expression. Strains PR03 and PR04 were generated by substituting the *pyrFE* genes with the pT181ts-ORI cassette from Fig. 2 in strains RN4220 and SA564RD, respectively. The ORI_{181} region (ORI) is indicated.

which shows that the FOA concentrations tested are nontoxic in cells without *pyrFE*, and also indicates that MH medium contains sufficient pyrimidines to support pyrimidine auxotrophic *S. aureus* without adding additional uracil, although we find that adding 10 mg/liter uracil is preferable to obtain faster growth and denser cultures. Note that the optimal concentration of FOA might be dependent on the strain.

To use this counterselection system efficiently in almost any *S. aureus* strain, we developed several strategies to easily generate *pyrFE* mutations taking in consideration the possibility to revert to a wild-type PyrFE background after having the mutants constructed.

Strains with spontaneous mutations in either *pyrE* or *pyrF* can be obtained by plating on MHFOA medium. Single colonies are restreaked on MHFOA medium and then verified by replica plating on MH, MHFOA, and RH media. Strains growing on MHFOA medium but not on RH medium are strong candidates for *pyrFE* mutants. Revertants with functional *pyrFE* genes can be obtained by plating on RH medium. However, if it is important to ensure a perfect reconstitution of the *pyrFE* genes, then it is preferable to use either phage transduction from a wild-type strain or classical allelic replacement of the *pyrFE* genes, using pyrimidine prototrophy as a marker. For the latter, we have constructed the pRLSAYC9, pRLSAYE9, and pRLSAYT9 vectors (Table 2), where the *pyrFE* genes from strain SA564, with an additional ~1 kb of chromosomal sequence on each side, have been cloned between the XbaI and BglII sites of pRLYC9, pRLYE9, and pRLYT9, respectively. These vectors allow complete reconstruction of the *pyrFE* locus, regardless of mutation type (see Protocol S2 in the supplemental material for details).

(i) A system for temporary disruptions of *pyrFE*. While it is easy to select on FOA to isolate naturally occurring *pyrFE* mutants, it is practical to be able to disrupt the *pyrFE* genes in a predictable and completely reversible manner. To accomplish this, the pRLBER9 plasmid was designed to recombine into the *pyrFE* genes, via a single region of homology (FE652 in Fig. 3 and 4), thereby disrupting expression of the downstream *pyrE* gene. This disruption can be maintained by selecting on FOA, allowing the pRLY counterselection system to be used for one or more allelic replacements. Once the genetic manipulations have been accomplished, then the *pyrFE* locus can be reverted back to the wild type by using pyrimidine-free medium to select for cells in which pRLBER9 is removed (see details in Protocol S3 in the supplemental material).

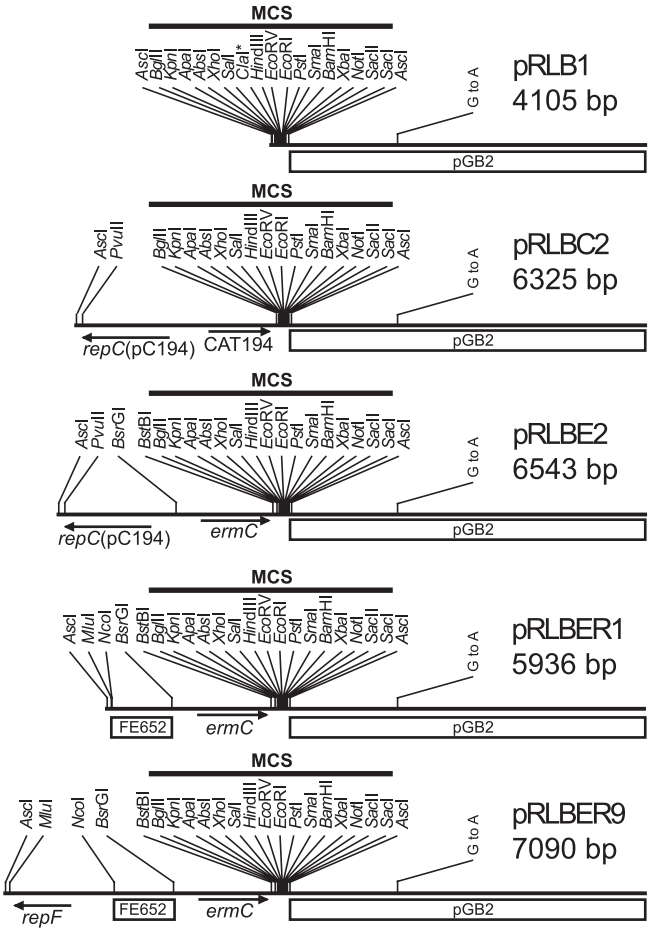

FIG 4 Layout of selected pRLB vectors. Important restriction sites and genes are indicated. The multiple cloning site (MCS) is indicated. The asterisk on ClaI indicates a ClaI site that is unique in pRLB1 but not in the rest of the vectors. The G-to-A mutation (G to A) that was introduced to increase plasmid copy number in *E. coli* is shown. pGB2 is the *E. coli* backbone vector. *repC*(pC194) is the replication gene from pC194. CAT194 is the chloramphenicol resistance marker. *ermC* is the erythromycin resistance marker. *repF* is the pE194ts replication gene. FE652 is the 652-bp sequence from the *pyrFE* locus in *S. aureus*.

In order to create pRLBER9, the pRLBE2 vector was modified by substituting the pC194 origin of replication with the pE194ts origin, and a 652-bp section of *pyrFE* was introduced between the origin and the *ermC* cassette (Fig. 4). pRLBER9 can therefore not be used with pRLY vectors that carry *ermC* cassettes and/or pE194ts origins, and to remedy this deficiency, versions of pRLBER9 were generated with pT181ts origins and CAT194 cassettes in various combinations (Table 3) to ensure compatibility with any of the pRLY vectors (or any other *E. coli-S. aureus* shuttle vectors).

In addition to disrupting *pyrE*, pRLBER9 and its related plasmids can also be used to express genes from a single copy per chromosome by cloning into the MCS and introducing the vector into the *pyrFE* locus on the chromosome.

Proof of principle. (i) Allelic replacement procedure. A protocol for allelic replacement, exemplified by using pRLYE1 to exchange your favored gene, *yfg*, with a kanamycin resistance marker, is briefly summarized here (see Protocol S1 in the supplemental material for details) (Fig. 5 shows a schematic flowchart).

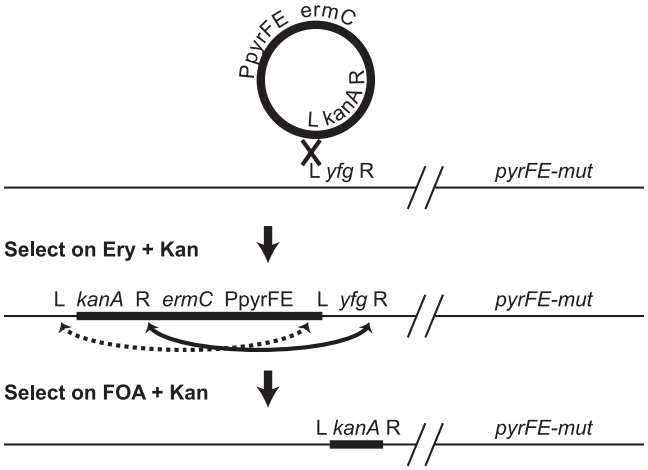

FIG 5 Allelic replacement flowchart for replacing the *yfg* allele with a kanamycin resistance cassette (*kanA*). A nonreplicative vector is forced to recombine (thick X) with the chromosome by selection on erythromycin (Ery) and kanamycin (Kan). The recombination can occur only via L or R, which are homologous to the regions adjacent to *yfg* in the chromosome (recombination with L is shown). Once the vector has been integrated into the chromosome, then FOA is used to counterselect the presence of the backbone vector, while kanamycin is used to select for the recombination that results in a substitution of *yfg* by *kanA* (solid black double-headed arrow). The dotted double-headed arrow indicates an alternative recombination which can happen if there is no selection with kanamycin and which will result in a wild-type strain. Vector DNA (thick black lines) and chromosomal DNA (thin black lines) are indicated. *yfg* is the gene that is substituted by *kanA*. *kanA* is the kanamycin resistance marker. L and R are ~1 kb of sequence to the left and right of *yfg*, respectively. *ermC* is the erythromycin resistance marker. PpyrFE is *pyrF* and *pyrE* from *B. subtilis* with a Pspac constitutive promoter. The chromosomal copies of the *pyrF* and/or *pyrE* genes that have been inactivated (*pyrFE*-mut) (see the text for further information) are indicated.

An *S. aureus pyrFE* mutant strain is transformed with 1 μg plasmid DNA and plated on MHE. A liquid culture of each colony to be tested is spotted on plates containing MH, MHE, MHFOA, and RH media (pyrimidine-free defined medium, see Materials and Methods). Strains growing on MHFOA are discarded (the remaining strains can be checked by PCR to verify vector insertion), and one or two strains are chosen for growth in MHU containing kanamycin for 6 h, whereupon they are streaked on MHFOA plus kanamycin. The colonies are picked and restreaked on MHFOA plus kanamycin, then transferred to liquid medium, and spotted onto plates containing MH, MHE, MHFOA, and RH media. Candidates that grow on MHE and/or RH medium are discarded, and remaining candidates are checked by PCR and subsequent sequencing of the genomic region of *yfg*.

(ii) Degradosome mutants generated. The counterselection system worked well in our hands when using spontaneously obtained FOA-resistant (FOA^r) mutants. However, for testing purposes, it was decided to use strain PR01, a Δ*pyrFE* mutant of the clinical strain SA564, because this strain is also mutated in the two restriction systems (9, 27), allowing direct transformation with plasmids isolated from *E. coli* DH5α.

The *S. aureus* degradosome consists of five RNases (RNase J1, RNase J2, RNase Y, polynucleotide phosphorylase [PNPase], and RnpA), two metabolic enzymes (enolase and phosphofructokinase), and a DEAD box RNA helicase (CshA) (27).

To test our counterselection system, deletions of SA0940 (RNase J1), SA1118 (RNase J2), SA1117 (PNPase), and SA1129 (RNase Y), and SA1885 (*cshA*) were performed, generating strains PR01-01, PR01-04, PR01-02, PR01-07, and PR01-06, respectively (Fig. 6). The mutations were checked by sequencing the entire region from ~1,300 bp downstream to ~1,300 bp upstream of the deletion. Moreover, since enolase is an essential enzyme in the glyconeogenetic and glycolysis pathways, the *S. aureus* enolase gene (*eno* [SA0731]) was substituted with the enolase gene from *E. coli*, which encodes an enolase that should be sufficiently different to prevent normal assembly of the degradosome, but which still provides the essential enzymatic activity for glycolysis and glyconeogenesis (strain PR01-05) (Fig. 6).

(iii) Generating a *cshB* SA1386 double mutant. To further test the strength of the counterselection, we decided to delete the two genes SA1386 and SA1387 (*cshB*), coding for a putative DNA endonuclease and an RNA helicase, respectively. First, *cshB* and SA1386 were deleted individually, giving rise to strains PR01-09 and PR01-08, respectively. PR01-09 is cold sensitive, whereas PR01-08 is able to grow at the same temperatures as the parent strain.

We had attempted several times, unsuccessfully, to obtain an SA1386 *cshB* double mutant in our laboratory, both via allelic replacement using pMAD and by disruption with a mobile intron, using the pNL9162 targetron system (28), and our continued failure suggested that the double mutation might be synthetically lethal. However, using the pRLYC1 vector, we encountered no problem replacing *cshB* and SA1386 with an *ermC* cassette (strain PR01-10 [Fig. 6]), testing three colonies where one had the deletion, thus showing not only that the double mutant is viable (although slowly growing and cold sensitive), but also that the FOA counterselection can be used to obtain mutants that could not be obtained by other means. We speculate that the fitness loss in the double mutant (which grows about 25% slower and has a long lag phase when inoculated) prevented a sufficiently high rate of plasmid loss (using plasmid pMAD or pNL9162) to allow detection of plasmid-free cells via screening, and thus, *pyrFE* counterselection was needed to overcome this barrier.

(iv) Attempting to delete the essential *eno* gene. Finally, we attempted to delete the essential enolase gene (*eno*) in order to force the appearance of false positives. A construct with pRLYE1 as the backbone and with a Cm^r marker situated between the L and R regions (Fig. 6) was recombined into the chromosome. After brief growth in MHC supplemented with uracil, the culture was plated on MHC containing FOA. All six colonies that appeared were restreaked and then tested for erythromycin sensitivity (which would indicate a loss of the backbone vector) and chloramphenicol resistance (which would indicate substitution of the enolase). The six clones were all erythromycin resistant, indicating that the FOA resistance had arisen from a mutation in the *pyrFE* genes of the integrated vector. No FOA^r Ery^s clones were observed, and we conclude that double mutations, destroying both the vector backbone resistance marker (*ermC* in this case) and the *pyrFE* genes, are rare. Furthermore, when performing a parallel experiment where the MHC-plus-uracil culture was plated on MHFOA, many more colonies appeared, and 30 tested colonies had lost both the Cm^r marker and the Ery^r marker, indicating homologous recombination via the same region as the initial integration into the chromosome (exemplified by the dotted double-headed arrow in Fig. 5).

(v) Generating an *spa* deletion, using pRLBER9 to temporarily disrupt *pyrE*. In order to test the functionality of pRLBER9, the plasmid was transformed into strain SA564RD, the parent strain

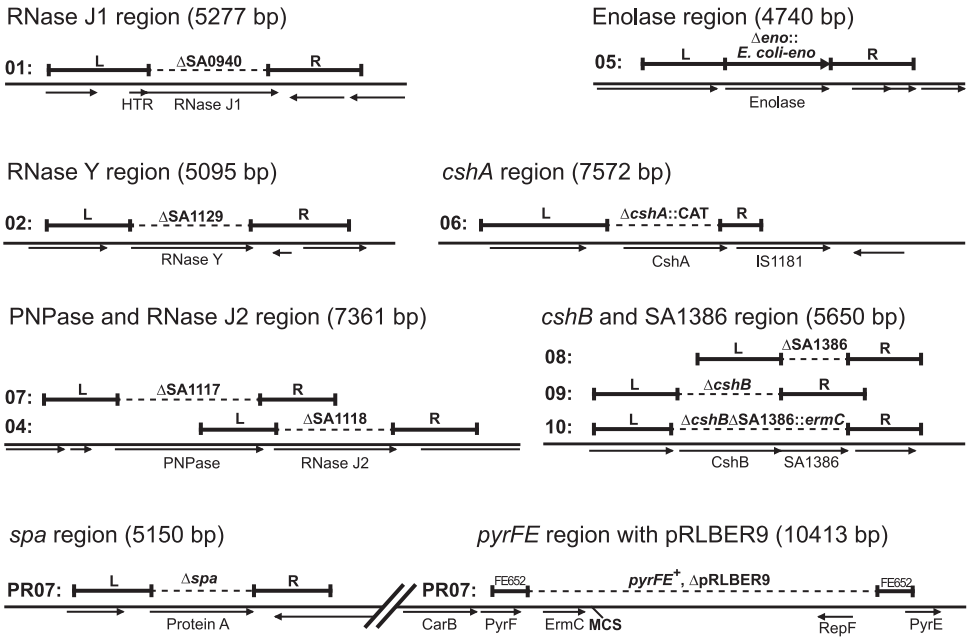

FIG 6 Overview of deletions and replacements generated in order to test the FOA counterselection system. The deleted regions (broken lines) are indicated. The two regions used for homologous recombination, the regions left and right of the deletion, are indicated by the thick lines with the letter L and R, respectively. Horizontal arrows indicate significant ORFs in the vicinity of the deletions. HTR indicates a hypothetical transcriptional regulator gene, which is 1 bp upstream of the RNase J1 gene. The numbers 01 to 10 indicate the strain name, PR01-01 to PR01-10, respectively. The bottom row shows strain PR07, where a *spa* deletion (bottom left) was carried out in strain SA564RD that was temporarily a *pyrFE* mutant, due to a pRLBER9 insertion. Subsequently, pRLBER9 was removed, leaving an intact wild-type *pyrFE* region (bottom right). FE652 indicates the two 652-bp regions which recombine to cross-out pRLBER9. The multiple cloning site in the chromosomally integrated pRLBER9 (MCS) is indicated.

of PR01, which has an intact and functional *pyrFE* locus. Plasmid replication was inhibited by shifting the temperature to 42°C, and erythromycin selection was used to select for cells where the plasmid had recombined into the *pyrFE* locus. It was verified that the resulting strain, SA564RD::pRLBER9, was FOA resistant, and then competent cells were prepared. A pRLYC1-based construct, which was designed to delete the *spa* gene, encoding protein A, was then transformed into SA564RD::pRLBER9. Transformants were selected on chloramphenicol, and the continued presence of pRLBER9 was confirmed by erythromycin resistance. Following the procedure described above (and in protocol S1 in the supplemental material), we obtained a *spa* deletion mutant, strain RD::pRLBER9-Δ*spa*. To return to a wild-type *pyrFE* locus and eliminate pRLBER9, a selection on pyrimidine-free medium was performed (to select for plasmid cross-out) at 42°C (to inhibit plasmid replication). After one restreaking, a screen for loss of erythromycin resistance showed that all 16 colonies had lost pRLBER9. One colony was chosen (PR07; Fig. 6), and the *spa* and *pyrFE* loci were PCR amplified and sequenced to confirm the Δ*spa pyrFE*$^+$ genotype (protocol S3 provides details for using pRLBER9).

Conclusion. We have developed a new and highly efficient system for generating mutants by allelic replacement in *S. aureus*. The strong FOA counterselection allows for quick and clean selection of mutants, which means that the expensive and time-consuming screening has been reduced to a verification step. While any system for obtaining mutants, including the one presented here, carries the probability of acquiring secondary spontaneous mutations in the genome, a shorter workflow should lessen that risk. Following the detailed protocol presented in supplemental material, we routinely obtain mutants in 8 days after transforming into *S. aureus*, about 2 to 3 days less than existing systems. Moreover, we have combined our counterselection system with a range of antibiotic resistance genes, and a range of conditional replication origins, which allow the choice of an optimal combination for a given project (Table 2). Therefore, our range of counterselection vectors solves several of the problems inherent in previous vectors, especially for obtaining mutants with significant reduction in fitness.

Furthermore, we have created a range of auxiliary vectors without sequence similarity to the pRLY vectors. These pRLB vectors can be used not only for gene expression during the allelic replacement procedure but can also be used for generating completely reversible *pyrFE* mutants.

Finally, we have tested our vectors by generating a variety of allelic replacements, including metabolic genes, RNases, and DNA repair enzymes. We have been able to delete or substitute all the alleles we have attempted, with the special exception of a deletion of the essential enolase gene, thus demonstrating the effectiveness and general application of the vector system.

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ADDENDUM IN PROOF

While this paper was in proof, a report by Monk and coworkers [mBio **3**(2)**:**e00277-11, 2012], describing direct transformation into *S. aureus* wild-type strains with DNA prepared from their newly developed *E. coli* strain (DC10B), was published. This should greatly facilitate genetic manipulations of *S. aureus* in the future, especially in combination with the work presented here.

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