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Genetic Tools To Enhance the Study of Gene Function and Regulation in *Staphylococcus aureus*

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The *bursa aurealis* transposon has been used to create transposon insertion libraries of *Bacillus anthracis* and *Staphylococcus aureus*. To provide a set of genetic tools to enhance the utility of these libraries, we generated an allelic-exchange system that allows for the replacement of the transposon with useful genetic markers and fluorescent reporter genes. These tools were tested in the Nebraska Transposon Mutant Library (NTML), containing defined transposon insertions in 1,952 nonessential *S. aureus* genes. First, we generated a plasmid that allows researchers to replace the genes encoding green fluorescent protein (GFP) and erythromycin resistance in the transposon with a noncoding DNA fragment, leaving a markerless mutation within the chromosome. Second, we produced allelic-exchange plasmids to replace the transposon with alternate antibiotic resistance cassettes encoding tetracycline, kanamycin, and spectinomycin resistance, allowing for the simultaneous selection of multiple chromosomal mutations. Third, we generated a series of fluorescent reporter constructs that, after allelic exchange, generate transcriptional reporters encoding codon-optimized enhanced cyan fluorescent protein (ECFP), enhanced yellow fluorescent protein (EYFP), DsRed.T3(DNT), and eqFP650, as well as superfolder green fluorescent protein (sGFP). Overall, combining the NTML with this allelic-exchange system provides an unparalleled resource for the study of *S. aureus*.

Staphylococcus aureus is a versatile pathogen that is able to cause an array of diseases ranging from simple soft tissue infections to life-threatening illnesses such as toxic shock syndrome, pneumonia, brain abscesses, and endocarditis (1). Recently, strains have appeared that cause outbreaks in otherwise healthy individuals outside of the hospital setting. These so-called community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains are highly virulent and carry genes encoding resistance to multiple antibiotics (2–5). The ability of this pathogen to cause such diverse infections is likely due to the large repertoire of virulence factors it produces, including multiple cell surface proteins that bind to different host matrix components, secreted toxins, hydrolytic enzymes, and immune modulators that are important to establish infection, resist the immune response, and spread to neighboring tissues (6, 7). Although decades of research on individual virulence genes have provided a wealth of information related to the function of specific virulence factors in disease, we are only beginning to realize the role of basic metabolism as the bacteria compete with the host for limited resources. Thus, much work remains to gain a full appreciation for the pathogenic capabilities of this important pathogen and to understand the complex interactions and regulatory processes that underlie disease potential. This work will be essential as we continue the quest for new therapeutic strategies to reduce the impact of staphylococcal infections.

Unfortunately, a critical process in staphylococcal research, the specific disruption of individual genes to study their functions, is time-consuming and expensive, involving the construction of plasmids allowing for homologous recombination into specific sites of the chromosome and resulting in the generation of the allelic replacement of a desired gene. In an era of "omics," where we are inundated with data and the associated hypotheses generated, the need to make specific genetic mutations is even greater. To reduce the burden of this process, we constructed a collection of sequence-defined *bursa aurealis* transposon (Tn) mutants, called the Nebraska Transposon Mutant Library (NTML), in which 1,952 nonessential genes in the *S. aureus* genome have been disrupted (8). This library was generated in a derivative of the strain LAC, a representative of the CA-MRSA USA300 lineage, which accounts for 98% of all CA-MRSA skin and soft tissue infections (9). As a resource for the scientific community, the NTML has been made freely available through the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA; www.narsa.net).

To enhance the functionality of the NTML, as well as other *bursa aurealis* libraries (8, 10, 11), we sought to make an allelic-exchange system that would allow for the simple and universal exchange of transposons inserted in the *S. aureus* chromosome, with a variety of useful selectable markers and reporter genes. For example, one set of plasmids was created to allow for the exchange of the transposon with three different selectable markers, simplifying the generation of multiple defined mutations within the *S. aureus* chromosome. Another set of plasmids allows for the replacement of the transposon with promoterless genes encoding fluorescent reporter proteins, allowing for the creation of single-copy reporter constructs with any gene that is represented in the library. Overall, the combination of these genetic tools with the comprehensive collection of defined transposon mutants in the NTML will greatly enhance the functional analysis of the *S. aureus* genome.

MATERIALS AND METHODS

Strains and media. *Escherichia coli* DH5α was utilized for cloning and was grown in Luria-Bertani medium with ampicillin (100 μg ml^{-1}) as needed for selection. *S. aureus* strains used in the present study are listed in Table 1 and were grown in tryptic soy broth (TSB) unless otherwise noted.

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Bacterial strains		
RN4220	Highly transformable *S. aureus*	14
JE2	USA300 CA-MRSA strain LAC without plasmids	8
NE1260	JE2 *pckA*::ϕNΣ	8
NE1260C	JE2 *pckA*::*ecfp*	This study
NE1260F	JE2 *pckA*::*fp650*	This study
NE1260G	JE2 *pckA*::*sgfp*	This study
NE1260R	JE2 *pckA*::*rfp*	This study
NE1260RR	JE2 *pckA*::*rfp* with *rfp* in opposite direction	This study
NE1260Y	JE2 *pckA*::*eyfp*	This study
NE1354	JE2 *hla*::ϕNΣ	8
NE1354K	NE1354 with *aphA-3* (Kan^r) replacement	This study
NE1354T	NE1354 with *tet*(M) (Tet^r) replacement	This study
NE1354TnT	NE1354 with truncated Tn	This study
NE1354S	NE1354 with *aad9* (Spc^r) replacement	This study
Plasmids		
pBFP-F	pTnT with *ecfp* in forward orientation	This study
pBFP-R	pTnT with *ecfp* in reverse orientation	This study
pCL10	pET194ts oriV, Chl^r Amp^r, pBR322 oriV	22
pCN34	Source of *apha-3*	21
pCN36	Source of *tet*(M)	21
pCN55	Source of *aad9*	21
pFP650-F	pTnT with *fp650* in forward orientation	This study
pFP650-R	pTnT with *fp650* in reverse orientation	This study
pGFP-F	pTnT with *sgfp* in forward orientation	This study
pGFP-R	pTnT with *sgfp* in reverse orientation	This study
pJB33	pCL10 with $P_{xyl/tetO}$-*secY570*	This study
pJB38	pJB33 with XhoI site removed	This study
pJB68	Source of *sgfp*	Lab stock
pKAN	pTnT with *aphA-3*	This study
KOR1	Source of $P_{xyl/tetO}$-*secY570*	23
pRFP-F	pTnT with *rfp* in forward orientation	This study
pRFP-R	pTnT with *rfp* in reverse orientation	This study
pSPC	pTnT with *aad9*	This study
pTET	pTnT with *tet*(M)	This study
pTnT	pJB38 with homologous DNA to *bursa aurealis*	This study
pYFP-F	pTnT with *eyfp* in forward orientation	This study
pYFP-R	pTnT with *eyfp* in reverse orientation	This study

[a] Antibiotic resistance abbreviations: Amp^r, ampicillin resistance (*bla*); Chl^r, chloramphenicol resistance (*cat*); Kan^r, kanamycin resistance (*aphA-3*); Tet^r, tetracycline resistance [*tet*(M)]; Spc^r, spectinomycin resistance (*aad9*). *rfp*, *ecfp*, *eyfp*, *sgfp*, and *fp650* encode DsRed.T3(DNT), ECFP, EYFP, superfolder GFP, and eqFP650, respectively.

TABLE 2 Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)[a]	Source
JBHLA1	CGAAAGGTACCATTGCTGGTCAATATAGAG	This study
JBHLA2	CTATCCATAGTAATAACTGTAGCGAAGTC TGGTG	This study
JBPCKA1	CTTGGTGCTGTTAATGCAAGTACTGGAA AATATAC	This study
JBPCKA2	GCATGCTCATAATATCTTGCATCGGTAAGAG	This study
JBTN3	ccgaattcTCTCTAGACACATAGATGGCGTC	This study
JBTN15	ccgctagcTTAGCCTGCCATGATGTATACATTG TGTGAG	This study
JBTN16	ccgctagcGCCATACCACAGATGTTCCAG	This study
JBTN17	ccgtcgacGTCTCTAGACGCGTATAACTATATAG GAAC	This study
JBTN18	gcctaggTGATTAACTTTATAAGGAGGAAA AACATATGGA	This study
JBTN19	cgcctaggTTATAAAAACAAATGATGACGAC CTTCTGTAC	This study
JBTN20	gcctaggTGATTAACTTTATAAGGAGGAAA AACATATGGG	This study
JBTN21	cgcctaggTTAACTATGACCTAATTTTGATGGT AAATC	This study
JBTN22	ggcctaggTGATTAACTTTATAAGGAGGAAA AACATATGG	This study
JBTN23	ggcctaggTTATTTGTATAATTCATCC ATACCTAATGTAA	This study
JBTN26	ggcctaggTTATTTATATAATTCAT CCATACCTAATGTAA	This study
JBTN29	gaCCTAGGGGTTTCAAAATCGGCTC	This study
JBTN30	ggcCTAGGTACTAAAACAATTCATCCAGTAAA	This study
JBTN31	gaCCTAGGCAAATATGCTCTTACGTGC	This study
JBTN32	ggcctagGCACTAAGTTATTTTATTG AACATATATCTTAC	This study
JBTN33	gaCCTAGGATCGAATCCCTTCGTGAGCGTC	This study
JBTN34	ggcctaggCTAATTGAGAGAAGTTTCTA TAGAATTTTTC	This study
JBTN37	gcctaggTGATTAACTTTATAAGGAGGAA AAACATATGAG	This study
JBTN38	cgcctaggTTATTTGTAGAGCTCATCC ATGCCATGTG	This study
JBSECY1	GCCGACTGCGCAAAAGACATAATCGATTC	This study
JBSECY2	GGGTGATCTAATGATTCAATGATTCAAACC	This study

[a] Lowercase indicates nonhomologous sequences added for cloning purposes.

When necessary for *S. aureus*, chloramphenicol (10 μg ml^{-1}), tetracycline (5 or 0.625 μg ml^{-1}), erythromycin (5 μg ml^{-1}), spectinomycin (1,000 μg ml^{-1}), or kanamycin (250 or 75 μg ml^{-1}) was added for selection. Reagents were purchased from Sigma-Aldrich (St. Louis, MO), EMD Chemical, Inc. (Gibbstown, NJ), or Becton Dickinson (Sparks, MD).

Molecular genetic techniques. Plasmids were purified using a Wizard Plus SV Minipreps DNA purification system (Promega Corp., Madison, WI). Oligonucleotides (Table 2) were synthesized by Integrated DNA Technologies (Coralville, IA). DNA ligase and restriction enzymes were obtained from New England BioLabs (Beverly, MA). DNA fragments were recovered using the DNA Clean and Concentrator-5 Kit (Zymo Research, Orange, CA). PCR was performed with an Applied Biosystems GeneAmp PCR System 9700 (Life Technologies Corp., Carlsbad, CA), using KOD DNA polymerase (Novagen, Madison, WI). PCR products were sequenced at the University of Nebraska Medical Center DNA Sequencing Core to ensure that there were no unintended changes during the generation of the plasmids described in the present study. Sequences were analyzed using Vector NTI (Invitrogen, Carlsbad, CA).

Generation of pTnT. The antisense *secY* expression cassette from pKOR1 was amplified by PCR using primers JBSECY1 and JBSECY2. The resulting fragment was digested with ClaI and cloned into pCL10 digested with SnaBI and ClaI to generate pJB33. To facilitate further cloning, the XhoI site was removed from pJB33 by first digesting with XhoI, followed by treating with Klenow to create blunt ends, and then self-ligating the resulting fragment. The ligation product was digested with XhoI to remove any remaining pJB33 prior to transformation, resulting in pJB38. To make the *bursa aurealis* markerless exchange plasmid pTnT, ~525 bp of the 5′ and 3′ ends of the transposon were amplified using primer pairs JBTN3/JBTN15 and JBTN16/JBTN17, respectively. The 5′ end PCR product was digested with EcoRI-HF and NheI, while the 3′ end PCR product was digested with NheI and SalI-HF. After digestion, the PCR products were simultaneously ligated into pJB38 digested with EcoRI-HF and SalI-HF to generate pTnT (Fig. 1).

Construction of reporters. Fluorescent reporter genes were codon optimized for *S. aureus* and synthesized by Invitrogen. The synthesized genes were amplified by PCR from the provided plasmids using the primer sets JBTN18/JBTN19, JBTN20/JBTN21, JBTN22/JBTN23, and JBTN22/JBTN26 for DsRed.T3(DNT), eqFP650, enhanced cyan fluorescent protein (ECFP), and enhanced yellow fluorescent protein (EYFP), respectively. The gene encoding superfolder green fluorescent protein (sGFP) was amplified by PCR from pJB68 using primers JBTN37/JBTN38. All forward primers included the TIR sequence (12, 13) to provide enhanced translation of these genes. PCR products were digested with AvrII and ligated into NheI-digested pTnT. The ligation products were digested with NheI to remove any self-ligated pTnT and transformed into *E. coli* DH5α. Orientation of insert was determined by PCR using primer JBTN22, which binds the TIR sequence of all inserts, and JBTN17, which is found downstream of the insertion site in pTnT. Plasmids with the reporter gene in the same orientation as *bursa aurealis ermB* were given the designation "F"; those in the opposite orientation were labeled "R" (Fig. 1).

Construction of antibiotic resistance cassettes. Genes encoding antibiotic resistance were cloned with their native promoters into pTnT. The *aphA-3* gene was amplified by PCR from pCN34, using the primers JBTN29 and JBTN30. Subsequently, the resulting product was digested

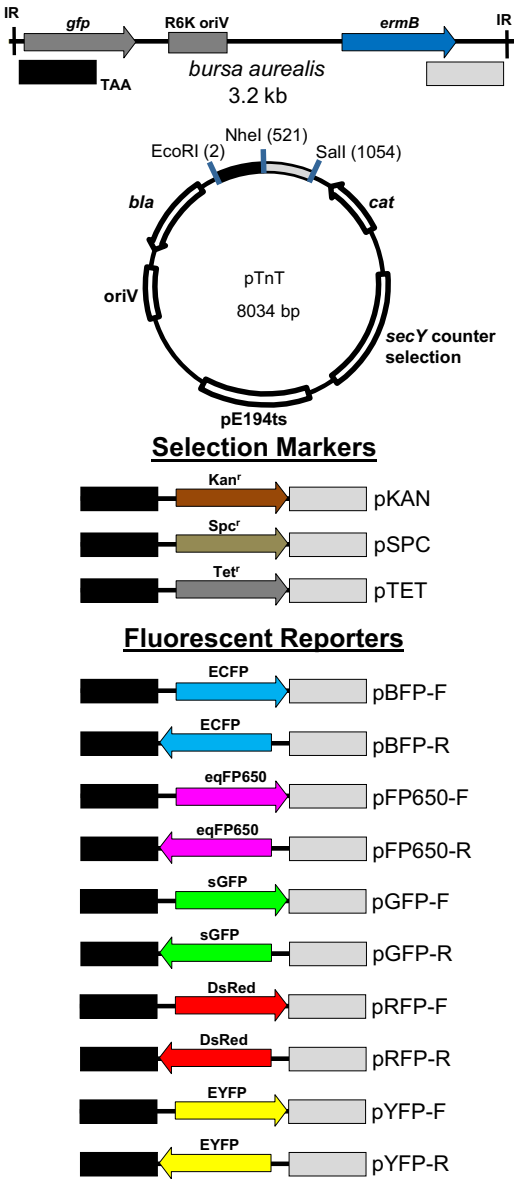

FIG 1 *bursa aurealis* replacement system. A schematic and the names of plasmids generated during the present study are shown. All reporters are codon optimized for *S. aureus* and include the TIR for enhanced expression. Antibiotic selective markers have their own promoters and encode resistance to kanamycin (Kan^r), tetracycline (Tet^r), and spectinomycin (Spc^r). For pTnT, *bla* encodes ampicillin resistance in *E. coli* and contains *cat* for chloramphenicol resistance in *S. aureus*.

with AvrII and ligated into NheI-digested pTnT. The ligation product was digested with NheI and transformed into *E. coli*. Similarly, *tet*(M) and *aad9* were amplified using primers JBTN31/JBTN32 and JBTN33/JBTN34, respectively. The products were digested with AvrII and ligated into pTnT as described for *aphA-3*. The orientation of the insert was determined using the 5′ primer for the resistance cassette and JBTN17, which binds downstream of the insertion site. The resulting plasmids were named based on the antibiotic selection conferred by them (Fig. 1).

Allelic exchange. Chromosomal changes in *S. aureus* were engineered by allelic exchange. Plasmids were generated in *E. coli* and transferred to the highly transformable, restriction-deficient *S. aureus* strain RN4220 (14). Transduction into JE2 derivatives was performed using ϕ11 propagated on plasmid-containing RN4220. Transductants were maintained at the replication-permissive temperature of 30°C for plasmid maintenance and confirmation. To initiate recombination, freezer stocks of strains containing the mutagenesis plasmid were struck onto tryptic soy agar (TSA) supplemented with 10 μg of chloramphenicol ml^{-1} and incubated at 44°C overnight. Large colonies were considered to be those that had undergone a single recombination event and were struck onto TSA with chloramphenicol for isolated colonies a second time and then incubated at 44°C overnight. These single recombinants were used to inoculate 3 ml of TSB and incubated at 30°C without selection to promote a second round of recombination and plasmid loss. After several days of serial dilutions, cultures were plated on TSA with 100 ng of anhydrotetracycline ml^{-1}, and the resulting colonies were replica patched onto TSA alone, TSA with erythromycin, and TSA with chloramphenicol to identify cells that had undergone a second recombination event and lost the plasmid. Erythromycin-sensitive colonies were screened by PCR to verify the presence of the appropriate allele, i.e., primers JBHLA1 and JBHLA2 for *hla* insertions and JBPCKA1 and JBPCKA2 for *pckA* mutants (Table 2). At times, erythromycin-sensitive and chloramphenicol-resistant colonies were identified, presumably because recombination had occurred but with some plasmid maintenance. These colonies were struck for isolated colonies on TSA and rescreened to identify those that had lost the plasmid.

Hemolysis activity. Cultures grown overnight in TSB were diluted in TSB to an optical density at 600 nm of 1.0. One microliter of cell suspension was spotted onto TSA containing 5% defibrinated rabbit's blood (Becton Dickinson). The plates were incubated at 37°C for 24 h and imaged using an AlphaImager (Proteinsimple, Santa Clara, CA).

***pckA* expression analysis.** *pckA* expression in response to glucose was performed as previously described with modification (15). Strains were struck for isolated colonies on either TSA without glucose or TSA containing 50 mM glucose and incubated overnight at 37°C. The resulting colonies were resuspended in phosphate-buffered saline (PBS) and fluorescence visualized by confocal microscopy at the UNMC Confocal Laser Scanning Microscope Core Facility using a Zeiss LSM 710 microscope. Images were prepared using Zen 2011.

Resource sharing. One goal of this project was to make a simple, easy-to-use system available to the scientific community. All of the plasmids generated in the present study are available through NARSA. In addition, protocols, plasmid maps, and plasmid sequence data are available at http://app1.unmc.edu/fgx/.

Nucleotide sequence accession numbers. The TIR and gene sequences for the synthesized codon-optimized fluorescent alleles have been deposited in GenBank and assigned accession numbers JX486120 [DsRED.T3(DNT)], JX486121 (ECFP), JX486122 (EYFP), and JX402762 (eqFP650).

RESULTS

Generation of a markerless exchange plasmid. The NTML is a collection of 1,952 strains in which most of the nonessential genes in the *S. aureus* chromosome have been disrupted by the insertion of a transposon encoding erythromycin resistance (8). Given the common use of this antibiotic resistance marker, we sought to generate a plasmid that could be used to remove the erythromycin resistance gene in any of the mutants of the NTML, thus easing subsequent genetic manipulation of these mutant strains. Ideally, use of this plasmid would truncate the transposon and leave a markerless "scar" that continued to inactivate the gene originally disrupted by the inserted transposon and would be useful for additional rounds of mutagenesis on the chromosome. To accomplish this, we generated the plasmid pTnT (Fig. 1), which includes adjacent (~500 bp) DNA sequences derived from the ends of the *bursa aurealis* transposon, a temperature-sensitive origin, chloramphenicol resistance, and anhydrotetracycline-inducible counterselection. In addition, we included an NheI restriction endonu-

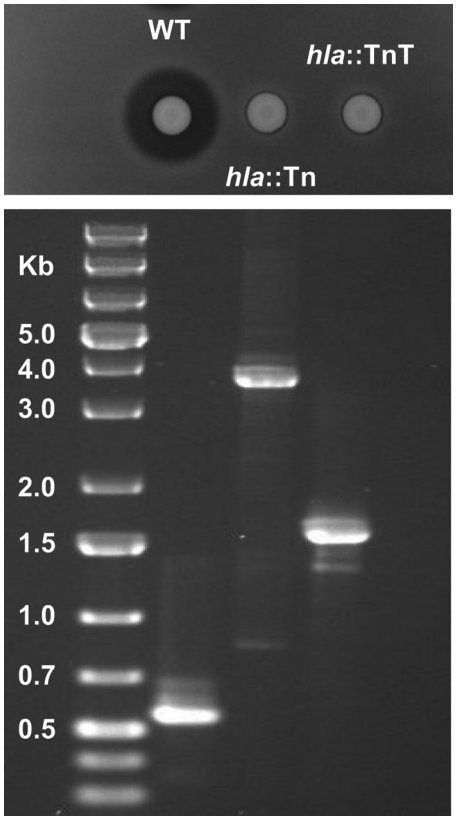

FIG 2 *bursa aurealis* size reduction in *hla*::Tn mutant. Findings for the hemolytic activity (top) and a PCR of chromosomal alleles (bottom) of JE2 (0.54 kb), *hla*::ϕNΣ mutant NE1354 (3.7 kb), and Tn replacement strain NE1354TnT (1.6 kb) are presented.

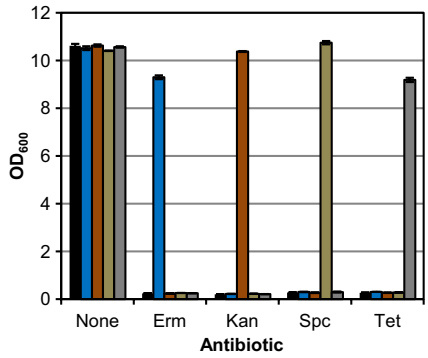

FIG 3 Growth of strains with alternate antibiotic markers. The optical density at 600 nm of JE2 (black), *hla*::ϕNΣ mutant NE1354 (blue), and allelic-exchange mutants NE1354K (brown), NE1354S (tan), and NE1354T (gray) grown in 3 ml of TSB in test tubes with the indicated antibiotics for 15 h at 37°C with shaking (250 rpm) was determined. The antibiotics were used at 5.0, 75, 1,000, and 0.625 μg ml^{-1} for erythromycin (Erm), kanamycin (Kan), spectinomycin (Spc), and tetracycline (Tet), respectively. Values represent the average ($n = 2$) with the standard error.

clease recognition site between the *bursa aurealis*-derived regions. NheI was chosen because overhangs generated by this enzyme are compatible with those following digestion with AvrII, XbaI, and SpeI. This versatility facilitates insertion of desired reporters or selection markers, since it is likely that one or more of these enzymes would be absent in products to be cloned into pTnT. After allelic exchange, this system reduces the size of *bursa aurealis* by ~2 kb and removes 72% of *ermB*, as well as 35% of *gfp*, leaving the resulting strain green fluorescent protein (GFP) negative as well as erythromycin sensitive.

To test this system, we used pTnT to truncate the transposon inserted in the alpha-hemolysin-encoding *hla* gene in the NTML mutant NE1354. Specifically, pTnT was transduced into NE1354, and allelic exchange was performed as described in Materials and Methods. After a second round of genetic recombination, mutants were screened for sensitivity to both chloramphenicol (pTnT) and erythromycin (*bursa aurealis*). The resulting strain, NE1354TnT, was confirmed by PCR of the chromosome allele using primers flanking the inserted transposon. As shown in Fig. 2, while the wild-type strain produced a band that was 0.54 kb in size, the *bursa aurealis* mutant NE1354 produced a 3.7-kb band due to the insertion of the 3.2-kb transposon. In contrast, PCR analysis of the truncated mutant NE1354TnT generated a band that was only 1.6 kb. As expected, this mutant was hemolysis deficient on agar containing rabbit blood (Fig. 2). These results demonstrate that pTnT is a useful tool for removing the transposon-encoded erythromycin resistance determinant, while still leaving a gene-inactivating insertion mutation within the target gene.

Exchange of antibiotic selection markers. Although a markerless mutation is useful for various purposes, it is often advantageous to have the ability to associate a mutation with alternate selectable markers, for example, for studies comparing the relative virulences of mutants in coinfection models of infection. Thus, we generated exchange plasmids that would incorporate alternate antibiotic resistance markers while removing the erythromycin resistance cassette. This would also allow for the transduction of other transposon insertions into the newly generated replacement strain to generate multiple selectable mutations in a single background. We chose to clone *aphA-3*, *tet*(M), and *aad9*, for resistance to kanamycin, tetracycline, and spectinomycin, respectively, into pTnT, generating the plasmids pKAN, pTET, and pSPC (Fig. 1). These plasmids are stable in *S. aureus* when grown at 30°C and conferred resistance to kanamycin (250 μg ml^{-1}), tetracycline (5 μg ml^{-1}), or spectinomycin (1,000 μg ml^{-1}) on solid media in both RN4220 and JE2 derivatives.

To demonstrate the utility of these plasmids and to test the level of resistance they conferred once integrated into the chromosome, we replaced the *bursa aurealis* transposon in the *hla* mutant NE1354 with each of these antibiotic resistance cassettes and analyzed the resistance of the resulting strains to antibiotics (Fig. 3). After allelic exchange, the strains generated were named NE1354S, NE1354K, and NE1354T, based on the replacement of the transposon with the spectinomycin, kanamycin, and tetracycline resistance cassettes, respectively. NE1354S was found to grow on TSA media, as well as in TSB broth, at 1,000 μg of spectinomycin ml^{-1}, as with the replicating plasmid. NE1354K and NE1354T were unable to grow well at antibiotic concentrations as high as those used for plasmid-based selection. However, NE1354K was found to grow well in TSB supplemented with 75 μg of kanamycin ml^{-1}, while the parent strain NE1354 and JE2 could not. Likewise, 0.625 μg of tetracycline ml^{-1} in TSB was selective, allowing only NE1354T to grow. All of the replacement strains were confirmed to be susceptible to 5 μg of erythromycin ml^{-1}, whereas NE1354 was resistant, demonstrating that the use of these

allelic-exchange plasmids inactivate erythromycin resistance while providing resistance to alternate antibiotics. All mutants generated remained defective in the production of alpha-hemolysin, indicating that the *hla* gene was disrupted in each strain. Based on these findings, the concentrations described here should be appropriate for selection during growth in liquid media for these antibiotic resistance cassettes when placed on the chromosome.

Generation of fluorescent reporter system. The third goal of this project was to construct a series of plasmids that would allow for the replacement of the GFP reporter in *bursa aurealis* with other fluorescent reporter options. To accomplish this, we cloned genes encoding DsRed.T3(DNT) (16), eqFP650 (17), ECFP, sGFP, and EYFP into the NheI site of pTnT. To maximize expression, and therefore the detection of the fluorescent proteins, we included an optimized translation initiation region (TIR). This TIR contains a consensus Shine-Dalgarno sequence and the phage T7 enhancer region to minimize secondary structure (12, 13). Since the *bursa aurealis* transposon can integrate into the chromosome in two orientations relative to the gene in which it has inserted (e.g., "forward" or "reverse" relative to the direction of the interrupted gene), we made two constructs for each fluorescent reporter so that selection of the appropriate allelic-exchange plasmid will allow for the placement of the reporter downstream of the promoter of the target gene. For simplicity, the plasmid names were based on the reporter as well as the orientation in which it will integrate the fluorescent reporter gene into the *bursa aurealis* transposon. Thus, each fluorescent reporter plasmid has two versions (Fig. 1 and Table 1), one designated "R" and the other "F." As an example, transposon insertions that are in the reverse orientation relative to the disrupted gene (indicated in the NTML website [http://app1.unmc.edu/fgx/]) would require the use of the "R" version of the plasmid to generate a transcriptional fusion to the fluorescent reporter gene.

As an illustration of the functionality of these reporter plasmids, we replaced the transposon insertion in the *pckA* gene within the NTML mutant NE1260 with our genes encoding the different fluorescent reporters. *pckA* encodes phosphoenolpyruvate carboxykinase, and the expression of this enzyme is repressed in the presence of glucose by the regulator CcpA (15, 18, 19). Based on these previous studies, we expected to observe fluorescence when the mutants were grown in the absence of glucose, with no expression in the presence of glucose. To test this, we first replaced *bursa aurealis* in the *pckA*::Tn mutant NE1260 with the DsRed.T3(DNT) reporter using pRFP-F. As seen in Fig. 4, we observed glucose-dependent fluorescence of DsRed.T3(DNT) in the replacement strain NE1260R. However, when the gene encoding DsRed.T3(DNT) was placed in the opposite orientation (NE1260RR) using pRFP-R, no fluorescence was observed. Likewise, we were able to demonstrate this same glucose-dependent expression when the sGFP, eqFP650, ECFP, and EYFP reporters were used to replace the transposon inserted within *pckA*. The ECFP reporter was the most difficult to detect induction, likely due to the lower fluorescence yield of this protein than of the other fluorescent proteins used. These data demonstrate that these allelic-replacement plasmids can be effectively used to construct chromosomally encoded fluorescent reporters of multiple colors.

DISCUSSION

The *bursa aurealis* transposon has recently been used to generate a sequence-defined transposon mutant library (designated NTML) with the CA-MRSA USA300 strain LAC (8) and is freely available for use by the research community. The objectives of the present study were to provide an allelic-replacement system (Fig. 1) that would complement and enhance the power of the NTML. Specifically, our goals were 3-fold and are all based on the generation of plasmids that would replace the inserted transposon via homologous recombination in any of the mutants of the NTML with a DNA fragment with enhanced functionality.

The first component of this system is a markerless exchange plasmid, pTnT, which gives users of the NTML the ability to inactivate the *gfp* and *ermB* genes found within *bursa aurealis*. This allows for the construction of mutant strains that lack a selectable marker, easing the introduction of secondary mutations (either marked or unmarked) in other sites on the chromosome. A demonstration of the utility of this plasmid is shown in Fig. 2, which illustrates the replacement of the *bursa aurealis*-associated erythromycin resistance gene with an ~1-kb "scar" that lacks any resistance determinants but retains the alpha-toxin-defective phenotype. The incorporation of the unique NheI site in pTnT allows for easy cloning of other reporters or markers that a user may place on the chromosome. Importantly, this system provides nearly limitless flexibility when constructing strains containing multiple mutations. It is imperative that proper PCR screening be performed on resulting strains since each round of transfer has a possibility of leading to recombination between *bursa aurealis* fragments.

Second, we constructed a series of plasmids that enables the replacement of the erythromycin resistance gene in *bursa aurealis* with other selectable markers, which allows for up to four marked mutations to be introduced into a single strain. The exchange of the *hla*-associated transposon with other selectable markers, such as those encoding spectinomycin, kanamycin, and tetracycline resistance, was shown to confer resistance to these antibiotics despite the fact that these genes reside in single copy (Fig. 3). Thus, this system should be useful for the selection of combinations of insertion mutations within the chromosome.

Finally, we built a collection of plasmids that are useful for the generation of single-copy promoter fusion constructs using five different fluorescent proteins. The fluorescent proteins used here were chosen because they encompass a range of excitation wavelengths compatible with many epifluorescence and confocal microscopes; additionally, flow cytometry systems can detect most of these fluorophores. Furthermore, the near-infrared fluorescent protein eqFP650 has been demonstrated to work well with IVIS imaging systems (17), making this protein an extremely useful tool for researchers interested in performing live-animal studies to monitor infections. Moreover, the versatility of these fluorescent proteins will allow researchers to monitor expression of multiple promoters within a single population of cells and permit sorting of those populations using fluorescence-activated cell sorting. The utility of these reporters, when expressed from the chromosome, is aided by the presence of the TIR-optimized ribosome binding site (RBS), as well as the generation of staphylococcal codon-optimized genes encoding these proteins. Due to high sequence homology between *ecfp*, *eyfp*, and the native *gfp* in *bursa aurealis*, we were concerned about possible undesired recombination events. However, we were unable to detect any occurrences of recombination events during plasmid replication in *E. coli* or *S. aureus*, as identified by PCR and restriction endonuclease digestion. In addition, we never identified any undesired recombination in our

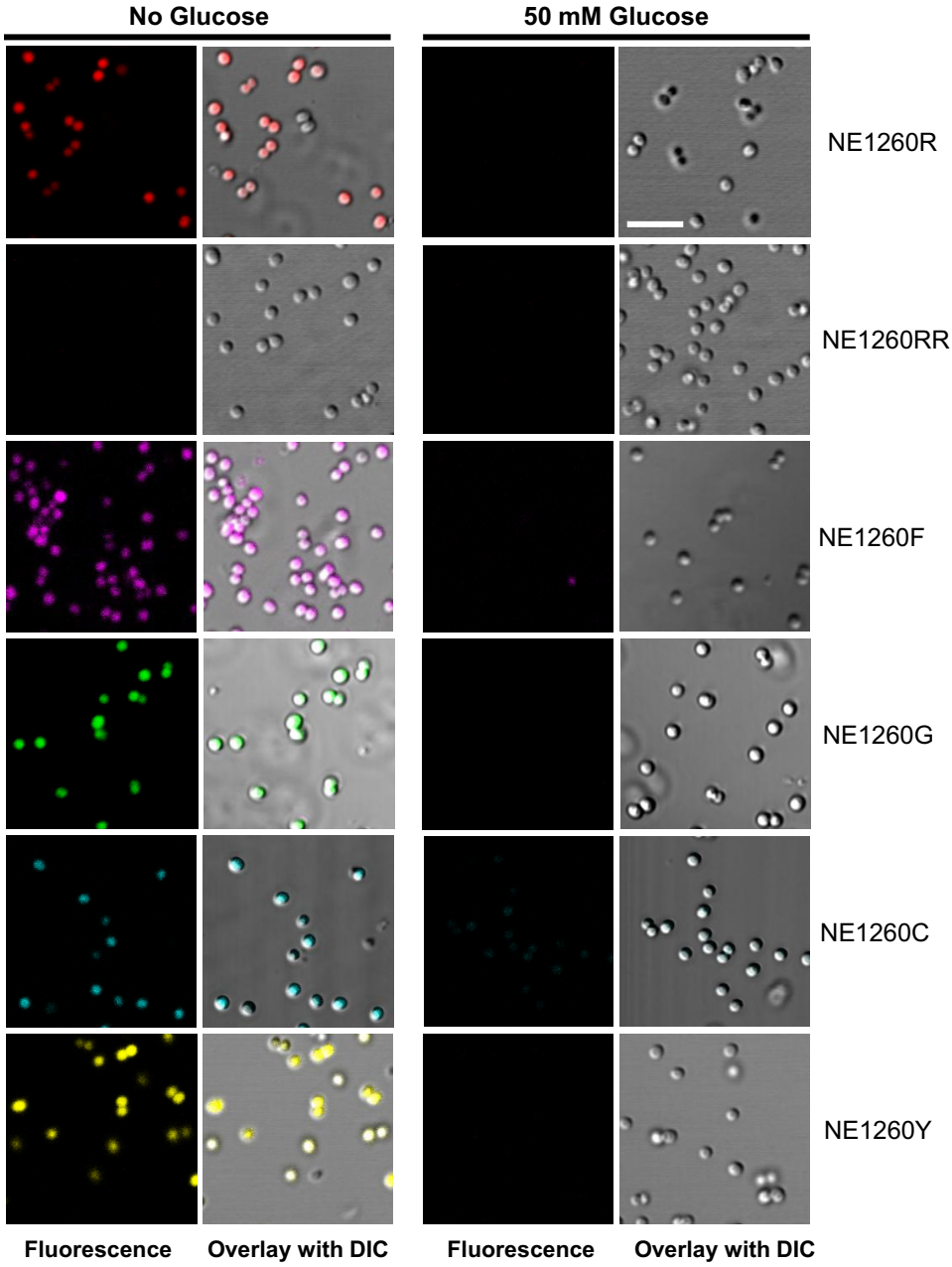

FIG 4 Expression of fluorescent reporters. Representative images of cells grown overnight on TSA with or without 50 mM glucose that were resuspended in PBS and visualized using a Zeiss LSM 710 microscope are shown. Scale bar (top right panel), 5 μm. DIC, differential interference contrast.

final mutants, as determined by multiple PCRs. Despite these findings, we would suggest that users of this system ensure proper allelic exchange by screening mutants via PCR. Furthermore, to ensure that any phenotypes associated with an allelic-exchange event are not a result of unanticipated secondary-site mutations, transfer of engineered mutations to a clean background and complementation studies (using plasmids such as pLI50 [20], pCN51 [21], and pRN8298 [21]) should be performed.

The goal of this project was to make a useful genetic "toolbox" that enhances the capabilities of *bursa aurealis* libraries. In addition to the NTML, this system is compatible with a previously generated library in *B. anthracis* (11) and the Phoenix library in *S. aureus* Newman (10) and can be used with future *bursa aurealis* libraries generated in other bacteria. Overall, combining the power of these libraries with this diverse set of tools should greatly enhance the ability to study gene function and regulation in *S. aureus* and other important Gram-positive pathogens.

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