HHS Public Access

Author manuscript

ACS Infect Dis. Author manuscript; available in PMC 2019 June 08.

Published in final edited form as:

ACS Infect Dis. 2018 June 08; 4(6): 1007–1018. doi:10.1021/acsinfecdis.8b00017.

Antibiotic combinations that enable one-step, targeted mutagenesis of chromosomal genes

Wonsik Lee[1], **Truc Do**[1], **Ge Zhang**[1,2], **Daniel Kahne**[2], **Timothy C. Meredith**[1,3], and **Suzanne Walker**[1,*]

[1]Department of Microbiology and Immunobiology, Harvard Medical School, 4 Blackfan Circle, Boston, Massachusetts, 02115

[2]Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, Massachusetts, 02138

[3]Department of Biochemistry and Molecular Biology, Pennsylvania State University, 206 South Frear Laboratory, University Park, Pennsylvania 16802

Abstract

Targeted modification of bacterial chromosomes is necessary to understand new drug targets, investigate virulence factors, elucidate cell physiology, and validate results of –omics-based approaches. For some bacteria, reverse genetics remains a major bottleneck to progress in research. Here we describe a compound-centric strategy that combines new negative selection markers with known positive selection markers to achieve simple, efficient one-step genome engineering of bacterial chromosomes. The method was inspired by the observation that certain non-essential metabolic pathways contain essential late steps, suggesting that antibiotics targeting a late step can be used to select for the absence of genes that control flux into the pathway. Guided by this hypothesis, we have identified antibiotic/counterselectable markers to accelerate reverse engineering of two increasingly antibiotic-resistant pathogens, *Staphylococcus aureus* and *Acinetobacter baumannii*. For *S. aureus*, we used wall teichoic acid biosynthesis inhibitors to select for the absence of *tarO* and for *A. baumannii*, we used colistin to select for the absence of *lpxC*. We have obtained desired gene deletions, gene fusions, and promoter swaps in a single plating step with perfect efficiency. Our method can also be adapted to generate markerless deletions of genes using FLP recombinase. The tools described here will accelerate research on two important pathogens, and the concept we outline can be readily adapted to any organism for which a suitable target pathway can be identified.

Graphical Abstract

*To whom correspondence should be addressed: suzanne_walker@hms.harvard.edu.

Author Contributions

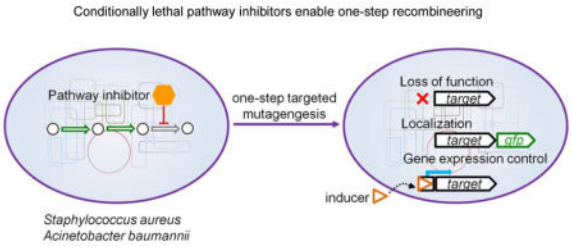
W. L. and S.W. conceived and designed the research. W.L. performed all experiments for marked deletion and chromosomal modification in *S. aureus* and *A. baumannii*. T.D. performed spa markerless deletion. G. Z., D.K., and T.C. M. made *Δlpx* strains. W.L., T.D., and S.W. wrote the manuscript with editorial input from all authors.

Notes

We have no competing financial interests to declare.

Supporting Information

Supplementary Figures S1–S10 and Tables S1–S3



Keywords

Homologous recombination; negative selection; bacterial genetics; pathway inhibitors; chemical genetics

The ready availability of bacterial genomic information has accelerated identification of new genes and enabled pathway-directed discovery of novel antibiotics. However, understanding gene function and validating possible new targets remains a major bottleneck due to a lack of suitable genome engineering tools for many bacterial organisms. For some organisms, such as *Escherichia coli*, tools that allow rapid and precise manipulation of chromosomal DNA are well established,[1–3] but for others, genome engineering remains a major challenge. *Staphylococcus aureus*, an important human pathogen responsible for a substantial proportion of fatal bloodstream infections, is notoriously difficult to genetically manipulate and it can take several weeks or longer to knock out a gene if the mutant is substantially less fit than the wildtype strain.[4] Understanding how different genes contribute to *S. aureus* physiology and pathogenesis is a high priority, and the lack of efficient methods to reengineer the chromosome greatly hampers progress.

Small molecules, including tool compounds identified in phenotypic or target-based screens, are invaluable tools in all areas of biology. Indeed, small molecules are widely used to discover or validate new drug targets, to probe biological pathways, to control gene expression and, notably, to facilitate rapid genome reengineering. A common feature of all reverse genetic tools is the use of genetic markers that, in combination with appropriate small molecules, enable selection for a desired mutational change in the chromosome. In bacterial genetics, antibiotic resistance genes are typically used to select for cells that have incorporated a vector, either as a stable plasmid or as a chromosomal integrant, and many different resistance gene-antibiotic combinations are available. For example, a common method to delete chromosomal genes in *S. aureus* makes use of the pKFC vector,[5] which carries an antibiotic resistance marker and also has a temperature-sensitive origin of replication. At elevated temperatures, the vector cannot independently replicate and so the marker can only confer resistance to antibiotic selection if the vector integrates into the chromosome (Figure 1). Integration at the desired chromosomal locus is directed by homology arms that flank the antibiotic resistance marker and enable homologous recombination. Because transformation efficiencies are low in *S. aureus*, the temperature-sensitive origin of replication is included as a means to enrich for this rare recombination event by permitting first the uptake of plasmid and amplification of cells containing vector prior to a temperature upshift.[4, 5] An undesired consequence of the need for clonal expansion of vector-containing cells is that single-step recombination resulting in the direct

allelic exchange of the target chromosomal locus for the antibiotic resistance marker is not possible.

The vast majority of cells in the clonal expansion undergo a single crossover upon temperature upshift, resulting in incorporation of the entire plasmid at the desired locus, and at this stage in the process both the mutant and the wildtype alleles are present (Figure 1). A second crossover in which the plasmid is excised from the chromosome is required to remove the wildtype allele, but deletion is disfavored if the mutant has a large fitness defect. Often, one must screen a great many individual colonies to identify those having the desired genotype, and in some cases colony screening can take weeks to months. The inclusion of a counterselectable marker in the plasmid that encodes a conditionally lethal phenotype would allow selection against cells that retain the integrated plasmid, enabling rapid identification of colonies that have undergone this rare second crossover and successful allelic exchange. Unfortunately, counterselectable marker-small molecule pairs are not as common as selectable marker-compound pairs.

In *S. aureus*, three counterselectable systems that also rely on vectors containing temperature-sensitive origins of replication have been reported. The pKOR1 method involves inducible expression of an antisense RNA that blocks expression of an essential *S. aureus* gene for counterselection,[6] while two others adapt counterselection methods commonly used in other organisms. The first reported of these exploits the *ura3*/5-fluoroorotic acid (5-FOA) marker-compound pair used for counterselection in yeast; the non-toxic compound 5-FOA is converted to a toxic metabolite by the *ura3* gene encoded uniquely on the plasmid.[7] The second of these uses an *rpsL*-streptomycin counterselection pair that exploits the fact that drug-sensitivity is dominant when both wildtype and mutant alleles of *rpsL* are present.[8] The pKOR1 method has been used extensively to make unmarked deletions and a major advantage is that it can be utilized in any *S. aureus* background.[6] A disadvantage is that extensive screening is required to identify the desired mutant when there is a substantial fitness difference between mutant and wildtype. The other counterselection methods are not widely used, although the *rpsL* method was only reported recently. A possible limitation of using *rpsL* for counterselection is that mutations in genes that compromise the membrane potential required for uptake of streptomycin would contribute to false positive colonies.[9, 10] More recently, Chen *et al.* reported a new strategy for constructing chromosomal mutations in *S. aureus* that does not make use of a counterselectable marker.[11] Instead, the method adapts CRISPR/Cas9 technology to introduce mutations at targeted sites of double-strand DNA break. This system will likely be advantageous for making single base substitutions. At present, however, application of CRISPR/Cas9 technology to *S. aureus* is limited by the low efficiency of homology-directed repair (HDR).[12] Although the more efficient strategy of non-homologous end joining (NHEJ) is commonly used to repair DNA breaks in eukaryotes, established bacterial CRISPR/Cas9 systems predominately use HDR because NHEJ is not well described. While HDR is sufficient to make markerless mutations, it can be difficult to insert large segments of exogenous DNA (*e.g.,* antibiotic resistance cassettes) to enable rapid transfer of modified loci to multiple strain backgrounds.[12, 13]

We sought a reliable one-step method that would permit the rapid construction of mutants containing gene deletions, fusions, or conditional promoters, and where the limitations of the method are predictable. Because many null mutations are temperature-sensitive and passaging at elevated temperatures may lead to acquisition of secondary mutations, it was important that the method not include a temperature upshift.[14, 15] Here, we report a strategy to make marked *S. aureus* mutants in a single step. The strategy addresses major bottlenecks in *S. aureus* reverse genetics because efficiencies of transformation and homologous recombination are greatly improved, and direct plating on small molecules to achieve simultaneous selection and counterselection is enabled. We also demonstrate that the counterselection approach we used for *S. aureus*, which exploits a pathway that is non-essential but contains an essential late step for which lethal inhibitors are available, can be adapted to *Acinetobacter baumannii,* which possesses a known pathway having similar properties. In principle, our negative selection approach is compatible with any organism possessing a metabolic pathway having non-essential early steps and essential late steps.

RESULTS AND DISCUSSION

Design of a counterselection strategy using pathway inhibitors

Our goal was to identify a reliable counterselection approach that would allow us to select against integrated plasmid and retention of the wildtype allele. In the course of screening for antibiotics that kill *S. aureus*, we identified two structurally unrelated compounds, targocil and targocil-II, which target a late step in wall teichoic acid (WTA) biosynthesis (Figures 2a and 2b).[16, 17] Identification of these compounds was accomplished by comparing the differential growth of a wildtype strain and a mutant strain lacking WTAs (Figure 2c). Wall teichoic acids are highly abundant peptidoglycan-linked glycopolymers that are synthesized inside the cell and exported to the outside via the ABC transporter TarGH.[18, 19] Targocil and targocil-II inhibit TarGH, preventing export (Figure 2a).[17] The lethality of a block in the WTA pathway is due to depletion of peptidoglycan precursors, which occurs because cellular pools of undecaprenyl phosphate carrier lipid are sequestered in wall teichoic acid intermediates. [20, 21] However, because the wall teichoic acid pathway is non-essential in vitro, targocil/targocil-II lethality can be suppressed by preventing expression of the gene encoding the first step in the pathway, *tarO*, and thus blocking flux into the pathway.[22, 23] Conversely, compound lethality is fully restored by complementation with an exogenous copy of *tarO* present on a plasmid or elsewhere on the chromosome (Figure 2c and Figure S1).

We realized that *tarO*-targocil (or *tarO*-targocil II) might make an ideal counterselectable marker-compound pair because lethality due to a WTA pathway block can be prevented by turning off a single, non-essential gene that controls flux into this non-essential pathway (Figure 2). Having a single gene that induces lethality and is also dispensable is central for stringent counterselection. We therefore proceeded to explore whether we could select for double-crossover mutants in a single step by transforming cells with a non-replicating plasmid containing both a selectable antibiotic resistance marker and a counterselectable *tarO* marker and directly plating on the appropriate antibiotic and the WTA pathway

inhibitor targocil. Initial validation was performed with targocil and subsequent studies confirmed that targocil-II can also be used.

Increased transformation efficiency of *tarO* null strains is crucial for one-step gene modification

To achieve double-crossover mutants in a single step without plasmid replication using *tarO*-targocil, we first needed to assess transformation efficiency because double-crossover events are rare and DNA uptake must be sufficient for recombination using a non-replicating plasmid vector. Since our approach relies on *tarO* as a marker for counterselection, we initially performed these experiments in a Δ*tarO* strain. We compared the transformation efficiency of wildtype *S. aureus* RN4220 and an isogenic Δ*tarO* strain using as proxy an empty pKFC plasmid.[5] We chose to work in the restriction-defective RN4220 background due to its inherently higher transformation efficiency and because its genome is fully sequenced.[24] Using 100 ng of DNA for transformation, we obtained ~200 colonies for the wildtype strain and 15,000 colonies for the Δ*tarO* strain, showing that removing negatively charged wall teichoic acids, which account for as much as 50% of the cell wall by mass, results in an almost two log improvement in transformation efficiency, which is consistent with a previous observation.[25] Although the transformation efficiency of the *S. aureus* Δ*tarO* strain is still relatively low, we thought it might be sufficient to enable use of a non-replicating plasmid that recombines directly into the chromosome.

We next attempted to implement our strategy by separately deleting two genes, *rodA*[15] and *ezrA*,[26, 27] which we had previously found very difficult to remove using the pKFC method.[5] Both these genes contribute substantially to *S. aureus* fitness, and the fitness defects are more severe at the elevated temperatures needed for pKFC plasmid integration.[15, 27] We prepared a non-replicating plasmid vector, pTarKO, having the kanamycin resistance cassette for positive selection flanked by approximately 1-kb homology arms to direct recombination to the desired region of the chromosome, and we included a constitutively expressed copy of *tarO* in the vector to enable negative selection on targocil after transformation into the Δ*tarO* strain (Figure S2). No colonies were obtained after plating the transformation reactions directly on plates containing both kanamycin and targocil. In retrospect, this was not surprising given that chromosomal integration via single crossover using replicating plasmid vectors is typically performed only after expansion to ~10^8 vector-containing cells. As we obtained only ~10^4 plasmid-containing cells from direct transformation into the Δ*tarO* strain (see above), and two crossover events were required in order to accomplish a one-step selection, we clearly needed to increase the efficiency of recombination for a double-crossover approach to succeed.

tarO counterselection enables one-step chromosomal gene deletions

One way to increase recombination efficiency is to damage plasmid DNA using, *e.g.,* UV irradiation, because nicks or other damage stimulate recombinases and other DNA repair machinery.[28] In mycobacterial genetics, plasmids are routinely irradiated before transformation in order to stimulate recombination,[29] but to our knowledge irradiation has not been applied to other bacteria. We briefly irradiated the *rodA* deletion vector prior to transformation into the Δ*tarO* strain and plating on kanamycin and targocil (Figure 3a and

Figure S3). Under these conditions, we obtained 49 colonies from a single transformation of ~1 μg of DNA by the next day. All colonies contained the desired genotype resulting from a double crossover, with *rodA* replaced by the *kan*^{R} cassette (Figure 3b and Figure S9a). Irradiating the *ezrA* deletion vector resulted in a similar number of colonies, and all had the desired genotype, although two days were required for the growth-defective *ezrA* null colonies[27] to grow to a reasonable size. Importantly, our counterselection approach, which does not require a temperature upshift, enables deletion of genes that are essential for growth at elevated temperatures. These temperature-sensitive mutations include deletions of *rodA* and *ezrA* (Figure S4).[15] We applied the same strategy to make several other knockouts, and in each case, the desired mutants were obtained in a day (Figure 3b and Figure S9a). No false positive mutants due to spontaneous suppressor mutations that bypass the stringency of targocil counterselection were identified, and sequencing confirmed that UV irradiation did not introduce additional mutations to the regions of homology flanking the insertion. We repeated these experiments with the same success using a *S. aureus* strain containing a chromosomal copy of *tarO* under the control of an anhydrotetracycline-inducible promoter,[30] with the inducer withheld during transformation and selection to produce essentially a *tarO* null phenotype (*i.e., tarO*$_{off}$) (Figure S3). We also found that targocil-II was similarly effective as a small molecule for counterselection. We conclude that *tarO*-targocil and *tarO*-targocil II are useful counterselectable marker-compound pairs for accomplishing single-step inactivation of chromosomal genes in *S. aureus*. Notably, targocil-II and close analogs that inhibit the same target are commercially available.[17]

tarO counterselection can be used to modify genes that are synthetically sick with WTA inhibition

All counterselectable marker strategies have limitations, and in the case of *tarO*-targocil, a predictable limitation is that one cannot delete genes that are dispensable in a wildtype background but essential in a strain lacking wall teichoic acids.[31] From a genome-wide synthetic lethal transposon screen and other sources, we have identified 14 genes that become important or essential for survival in the absence of wall teichoic acids (Table S3).[30] These include genes in the lipoteichoic acid (LTA) biosynthetic pathway, which makes an extracellular, membrane-anchored, polyanionic polymer similar to wall teichoic acid (Figures 3c and 3d).[32, 33] We have found that blocking wall teichoic acid expression is completely lethal when *ugtP* (*SAOUHSC_00953*), involved in lipoteichoic acid membrane anchor biosynthesis, is deleted, and consistent with this finding, we could not knock out *ugtP* using our counterselection method (Figure S5). We were, however, able to delete *ltaA* (*SAOUHSC_00952*), which exports the lipoteichoic acid membrane anchor to the cell surface (Figure 3c). Mutants lacking *ltaA* are very sick when wall teichoic acid expression is blocked, but we found that by using a two-step selection procedure, we could delete this gene (Figures S6 and S9a). In this two-step process, we first selected colonies containing the pTarKO plasmid stably integrated into the chromosome on kanamycin and then grew them to high density before plating on targocil to select for a second crossover event leading to plasmid loss (Figure S6). Because only a small number of otherwise nonessential genes are strictly required for viability when the wall teichoic acid pathway is off, our method has broad scope for making gene deletions in *S. aureus*, particularly as the background due to spontaneous suppressors is negligible. In these few cases where *tarO*-targocil would fail due

to synthetic lethality between a desired gene deletion and the absence of WTAs in the *tarO*$_{off}$ background, other allelic replacement methods can be used.

Gene fusions and promoter swaps can also be obtained easily using *tarO* counterselection

Genetic changes other than gene deletions are also useful for probing biological function of genes and proteins in cells. For example, the ability to install fluorescent and affinity tags in a protein allows studies of protein localization, protein quantitation, and identification of protein-protein interaction partners. Similarly, the ability to swap native promoters for inducible promoters allows conditional gene expression. Although we were unable to delete *ugtP* using our counterselection method, we easily made a C-terminal GFP fusion protein by fusing *gfp* to the 3′ end of the gene (Figure 3e and Figure S9b). Viability of the mutant in the *tarO*$_{off}$ background showed that the GFP-tagged UgtP protein was functional, and after the fusion construct was transduced to a clean wildtype background to assess protein localization, we observed strong fluorescence. The ability to quickly generate fusions to genes at their native loci circumvents problems with gene dosage. When fusion proteins are instead expressed from replicative plasmids or plasmids integrated at ectopic chromosomal loci, their levels may be significantly different from native levels, and this difference can lead to misinterpretation of protein localization and function. We also swapped the native promoter of a gene of unknown function that is important for cell envelope integrity, *SAOUHSC_01050*, [34] for an anhydrotetracycline (aTc)-inducible promoter, and then transduced this modified locus into a wildtype RN4220 strain (Figure 3f and Figure S9b). We had previously found that *SAOUHSC_01050* becomes essential in the presence of a small molecule, amsacrine (AMSA), that inhibits *S. aureus* DltB, a polytopic membrane protein required for introducing positively charged D-alanyl esters into lipo- and wall teichoic acids.[30] Consistent with this finding, cells containing the conditional *SAOUHSC_01050* allele were unable to grow on amsacrine unless the inducer aTc was also present (Figure 3f). The ability to construct conditional alleles rapidly will make it possible to speedily validate functionally important genetic interactions, accelerating global analysis of gene networks.

Modified loci can be transduced into other *S. aureus* strains, including MRSA strains

Because *S. aureus* is a pathogen and strains can show phenotypic differences upon gene deletion, it is common to assess gene function in more than one strain background. This is most easily achieved by transducing marked genetic changes from one strain to another using staphylococcal phages. Transduction requires wall teichoic acids because these serve as phage receptors.[35] The genetic modifications we have described so far were made in RN4220 strains that lack wall teichoic acids (either a *tarO*$_{off}$ or Δ*tarO* background) as a prerequisite for targocil/targocil-II counterselection. In the conditional *tarO*$_{off}$ strain, the chromosomal copy of *tarO* is tightly controlled, permitting efficient counterselection, but it can be expressed by adding the inducer aTc. By inducing wall teichoic acid expression with aTc, we found that we could transduce every modified chromosomal locus constructed in the conditional *tarO*$_{off}$ strain into a *tarO* wildtype background, including RN4220, HG003, Newman, and the methicillin-resistant strains MW2 and USA300 (Figure S7 and Table S1). To exclude potential co-tranduction of known background mutations from the RN4220 donor strain, targeted sequencing of nearby loci can be performed. If desired, positive

selection markers can be removed after transduction using FLP recombinase-mediated excision of the markers, provided they are flanked by FRT (FLP recognition target) sites,[15] as we have demonstrated for the *spa* gene deletion; this will enable construction of multiple deletions in a single strain (Figure S8).

lpxC/colistin enables counterselection in *Acinetobacter baumannii*

Having shown that several types of genetic modifications can be rapidly constructed in *S. aureus* using an appropriate counterselectable marker-compound pair, we wondered whether the strategy could be extended to other bacteria. We have posited that the ideal gene-compound pair for counterselection exploits an unusual feature of certain pathways: namely, that a late block in the pathway prevents growth even though the pathway itself is non-essential in vitro. Therefore, we sought other examples in which the bioactivity of a lethal compound known to act at a late step in a biosynthetic pathway is suppressed by deleting a non-essential early gene in the same pathway. We found such an example for the Gram-negative organism *A. baumannii*, an important human pathogen that is challenging to reverse engineer.[36] Like other Gram-negative organisms, *A. baumannii* has an outer membrane that contains lipopolysaccharide (LPS), a glycan polymer of variable length that is anchored to the membrane through the highly conserved Lipid A component.[37] Lipid A also serves as an initial binding site for the structurally related antibiotics colistin and polymyxin to gain entry to the periplasm.[38] In *A. baumannii*, LPS is not essential in vitro,[39] and so the bioactivity of these antibiotics can be completely suppressed by preventing the expression of LPS; this can be accomplished by deleting *e.g., lpxC*, [40–42] which acts at an early step of biosynthesis (Figures 4a and 4b). Therefore, *lpxC*-colistin seemed like an appropriate gene marker-compound pair for counterselection in *A. baumannii.* Because deletion of *lpxC* causes a growth defect on solid agar medium,[43] we have isolated a Δ*lpxC* mutant that shows growth comparable to that of wildtype. Genome-sequencing identified only three background mutations (Table S1). As proof of concept, we chose to replace the genes encoding an efflux pump, *adeFGH* (5.8 kb), with an antibiotic marker in this Δ*lpxC* strain. As with pTarKO, we designed and constructed a non-replicating plasmid containing a constitutively expressed copy of *lpxC* for negative selection (pColKO) (Figure 4c), with an apramycin resistance gene flanked by homology arms to direct recombination at the *adeFGH* locus and positively select for recombinants. Here, we also took advantage of a high-efficiency electroporation method to enable direct transformation of pColKO into *A. baumannii*, thereby circumventing plasmid delivery by conjugation through *Escherichia coli*.[44] We UV-irradiated the pColKO plasmid (~1 μg), electroporated it into the *A. baumannii* Δ*lpxC* strain, and selected on a colistin-apramycin plate. We obtained ~21 colonies by the next day and all contained the desired replacement of *adeFGH* with the apramycin marker (Figure 4c and Figure S10).

Modified loci in the *A. baumannii* recombineering strain can be horizontally transferred to wildtype *A. baumannii*

In order to make the counterselection method for *A. baumannii* practically useful, we needed a good strategy to transfer modified loci from the Δ*lpxC* construction strain into a wildtype *lpxC* background. Unlike with *S. aureus*, the use of phage transduction to move marked loci from one *A. baumannii* strain to another has not been described. We thought it might be

possible, however, to use a mating-out approach in which genes are horizontally transferred between two strains (Figure 4d).[45] The Δ*lpxC* strain is 1200-fold more sensitive to the antibiotic rifampicin than the wildtype strain (minimal inhibitory concentration of rifampicin: Δ*lpxC* donor, 0.00025 μg/mL; wildtype recipient, 0.3 μg/mL), and this window can be exploited to select against survival of the Δ*lpxC* strain after mating.[46] We incubated wildtype *A. baumannii* with the Δ*lpxC* mutant strain containing the marked deletion (Δ*adeFGH*::apramycin^R) for several hours and selected colonies on apramycin and rifampicin. To verify that the marked *adeFGH* deletion was transferred to wildtype from the Δ*lpxC* strain, we performed whole genome sequencing of the donor, recipient, and the resultant mating-out strains. Based on single-nucleotide polymorphism analysis, we concluded that the Δ*adeFGH*::apramycin^R marker had moved into the wildtype strain, confirming unidirectional transfer. Coupled with this simple mating-out approach to move marked loci out of the Δ*lpxC* strain, the colistin-*lpxC* counterselection method may serve as a general tool to genetically manipulate *A. baumannii*. As with *tarO*-targocil counterselection in *S. aureus*, the use of *lpxC* here precludes deletion of genes that are synthetically lethal with lack of LPS, but these synthetic lethal interactions are not presently known. Moreover, it is possible that certain gene deletions will result in rifampicin hypersensitivity, which could affect mating-out. Nevertheless, the method worked as expected for our test case, providing proof-of-concept for the generality of a pathway-specific counterselection approach, and we are currently assessing practical limitations for its application to *A. baumannii*.

CONCLUSIONS

In this work, we have demonstrated the utility of a new one-step selection-counterselection approach for rapid reverse engineering of bacterial genomes. We focused on *S. aureus* due to the still extant need for methods to construct mutants rapidly in this organism, but we have shown that the approach can be extended to *A. baumannii*, and we expect it is general. In these organisms, arrayed transposon libraries are available for quick validation of mutant phenotypes, and the antibiotic resistance marker allows phage transduction of the insertion into other *S. aureus* strains. However, not all non-essential genes are represented in these libraries. For example, the *S. aureus* library lacks transposon insertions in ~500 non-essential genes. Furthermore, some transposon insertions do not result in full loss of protein function because one or more domains of a protein may still be preserved. Rapid methods to obtain clean marked deletions of genes or groups of genes arranged in an operon therefore remain useful. For *S. aureus*, we fully vetted the counterselection approach by showing that we can delete genes even for mutants that are sick or have a temperature-sensitive growth defect (Figure S4).[15] We also demonstrated rapid genome engineering to make fluorescent fusions and swap promoters for conditional gene expression. In most cases, we obtained the desired mutants in a single day after simultaneous selection-counterselection following transformation. Doing so required increasing recombination efficiency, which was accomplished by UV irradiation of the vector DNA prior to transformation. We suggest that UV irradiation of vectors will improve the efficiency of all bacterial genome-engineering methods that require chromosomal integration, although sequencing of flanking homology arms should be performed after phage transduction into a different background. The

RN4220 *tarO*$_{off}$ strain can be easily transduced to other strain backgrounds after induction of *tarO*. While this requires additional time (a few days), multiple strains can be transduced in parallel. Targeted sequencing of nearby genes can be performed following phage transduction to confirm that known mutations found in the RN4220 donor strain are not also transferred to the recipients; this is important when using mutant strains in virulence studies. Although markerless deletions cannot be generated in a single step with our system, the ability to identify rare double crossover mutants containing the desired marked deletion rapidly means that the method remains advantageous when there is a fitness cost associated with the mutation. For instance, with current recombineering strategies that rely on plasmid integration into the chromosome, the integrant is likely to resolve in favor of the wildtype allele, thereby requiring extensive screening for the unmarked mutant allele. Here, we used FLP recombinase to remove the positive selection marker, but other recombination systems such as the Cre-*lox* system could also be utilized.[47] Moreover, we demonstrated that we could readily construct deletions of two genes, *ezrA* and *rodA*, that are temperature-sensitive because our method does not require a temperature upshift.

Central to our one-step approach was the identification of a suitable counterselection strategy. We achieved simple and efficient counterselection by exploiting a metabolic pathway that contains non-essential early steps and essential late steps. Using a small molecule that inhibits a late step of the pathway in combination with a marker encoding an early gene in the same pathway enabled selection against integrated plasmid and retention of the wildtype allele. Notably, a pathway with these characteristics also lends itself readily to screens to discover bioactive compounds that block a late step in the pathway, as one can simply compare the differential growth of wildtype and a mutant lacking an early gene to identify hits (Figure 5).[17, 22, 48] Applying targocil and targocil-II, the compounds we previously discovered as inhibitors of TarG, the transmembrane component of the ABC transporter that exports wall teichoic acids to the cell surface of *S. aureus*,[17] we demonstrated stringent counterselection and direct allelic exchange. We have also shown that the counterselection concept we exploited for *S. aureus* can be readily extended to the Gram-negative pathogen *A. baumannii* despite the need for a different counterselectable marker-small molecule pair. In this organism, the lethality of the pathway-specific antibiotic colistin can be suppressed by deleting an early gene in the non-essential lipopolysaccharide biosynthesis pathway,[42] allowing the use of *lpxC*-colistin as a counterselectable marker-compound pair. Although we tested *lpxC*-colistin counterselection with only a single set of genes, this second example of the approach should instill confidence that the approach will work in a diverse set of bacterial organisms, justifying screening campaigns to find the necessary small molecule inhibitors if they do not already exist. We note in closing that there are pathways in other organisms for which late genes are essential and early genes are not, and it should be possible to identify small molecules that inhibit such pathways with the same screening approach used to discover targocil and targocil-II. For example, the pathways for O-antigen biosynthesis in *Pseudomonas aeruginosa* and *Salmonella enterica,* and for capsular polysaccharide biosynthesis in *Streptococcus pneumoniae* contain non-essential early genes and essential late genes and may provide suitable screening targets.[22, 49–51] Hence, this counterselection approach can be adapted for other organisms that are challenging to genetically manipulate.

MATERIALS AND METHODS

Reagents and General Methods

Antibiotics were obtained from Sigma. Targocil and targocil-II were synthesized as previously described[16, 17]. Targocil-II can also be purchased from Aurora Fine Chemicals LLC. Restriction enzymes were obtained from New England Biolabs. DNA concentrations were measured using a NanoDrop One Spectrophotometer (Thermo Scientific). Plasmid DNA was irradiated using a UV-Crosslinker (CX-2000, UVP). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, and PCR was performed with KOD Hotstart DNA polymerase (Novagen) on genomic DNA isolated from wildtype RN4220 *S. aureus*. PCR products and plasmids were purified using Qiagen kits. Primers were designed using DNASTAR-Lasergene, obtained from Integrated DNA Technologies (IDT), and are listed in Table S2. DNA sequencing was completed through Eton Bioscience and the Dana-Farber/Harvard Cancer Center DNA Sequencing Facility. For colony PCR, a colony was resuspended in 30 μL of buffer containing 40 mM NaOH and 0.2% SDS, and incubated at 98°C for 5 min followed by cooling on ice for 5 min. After dilution with 200 μL H_2O, 4 μL of this cell lysate was used for a 25 μL PCR reaction.

Bacterial Strains, Plasmids, and Growth Conditions

All bacterial strains and plasmids used in this study are listed in Table S1. *S. aureus* strains were maintained in trypticase soy broth (TSB) at 30°C, and *Escherichia coli* and *A. baumannii* strains were maintained in Luria-Bertani (LB) broth at 37°C. Antibiotics were added as indicated at the final concentrations: 50 μg/mL kanamycin, 50 μg/mL neomycin, 3 μg/mL tetracycline, 5 μg/mL chloramphenicol, and 10 μg/mL targocil (or targocil-II) for *S. aureus*; 100 μg/mL carbenicillin for *E. coli*; 100 μg/mL apramycin and 10 μg/mL colistin for *A. baumannii*.

Susceptibility tests of Δ*tarO* and Δ*lpxC* strains to targocil and colistin, respectively

To generate an inducible *tarO* strain, the *tarO* gene and its native ribosomal binding site (−10) were amplified by PCR using primers *tarO*-F/*tarO*-R and cloned into KpnI and EcoRl restriction sites of the staphylococcal integration vector pTP63, which contains an anhydrotetracycline (aTc) inducible tetR-promoter. pTP63-*tarO* was electroporated into RN4220 *ΔtarO*/pTP44,[30] resulting in an inducible *tarO* strain ($tarO_{on/off}$). The strains were tested for antibiotic susceptibility on TSB agar containing 5 μg/mL, 10 μg/mL, and 25 μg/mL of targocil, or 10 μg/mL targocil-II in the presence of 0.4 μM aTc. Overnight cultures of *S. aureus* strains were inoculated in TSB medium and incubated at 30°C until they reached mid-log phase. The cultures were normalized to OD_{600}=0.5, five ten-fold dilutions were prepared, and 5 μL of each dilution was spotted on TSB containing targocil and incubated at 30°C.

As the *ΔlpxC A. baumannii* ATCC 19606 strain has a growth defect on solid agar medium,[43] the strain was passaged multiple times to isolate less defective mutants. One of the *ΔlpxC* derivatives from 6 passages (pass-*ΔlpxC*) was found to have a growth rate comparable to wildtype and was used for these studies. Mutations present in the passaged strain are described in Table S1. To generate a complemented strain, the *lpxC* gene and its native

promoter were amplified using PCR and cloned into SalI and HindIII restriction sites of pWH1266, generating p*lpxC*. The p*lpxC* plasmid was transformed into the pass-*ΔlpxC* strain. To test susceptibility, the strains were assayed on a four-fold diluted LB medium (dLB) containing 1.5% agar and 10 μg/mL colistin. Overnight cultures of *A. baumannii* strains were inoculated in LB broth and incubated at 37°C until they reached mid-log phase. The cultures were normalized to OD_{600}=0.5, five ten-fold dilutions were prepared, and 5 μL of each dilution was spotted on dLB agar containing colistin and incubated at 37°C.

Construction of pTarKO and pColKO plasmids

To generate pTarKO and pColKO recombineering vectors, we removed the temperature-sensitive replication origin from the *S. aureus* pKFC vector by digestion with AatII.[5] A DNA fragment containing both *tarO* and its native promoter (or both *lpxC* and its native promoter) were amplified by PCR and cloned between the EcoRI and KasI restriction sites of the pKFC vector. The constructed plasmids, pTarKO (for *tarO* counterselection) and pColKO (for *lpxC* counterselection), were confirmed by sequencing.

Electroporation of DNA

To prepare electrocompetent *S. aureus* cells, an overnight culture of an RN4220 strain was inoculated into 100 mL of TSB and cultured at 30°C. When the cultures reached OD_{600}~0.8, the cells were harvested and washed with ice-cold dH_2O twice followed by washing with ice-cold 10% glycerol twice, and finally resuspended in 1 mL of 10% glycerol. Prior to electroporation, 0.5~1 μg of DNA was irradiated with UV-320 nm at 80,000 μJoul/cm^2 and mixed with 50 μL of the electrocompetent cells in a 2-mm electroporation cuvette on ice. The plasmid and cell mixture was pulsed at 29 kV/cm, 100 Ω, and 25 μF followed by immediate addition of 1 mL of TSB. The cells were recovered at 30°C with shaking at 100 rpm for 1.5 hours and plated on TSB agar containing 50 μg/mL kanamycin, 50 μg/mL neomycin and 10 μg/mL targocil. Plates were incubated at 30°C for 1–2 days. In the case of *mreDC* deletion, the cells were plated on TSB agar containing 3 μg/mL tetracycline and 10 μg/mL targocil.

As previously described,[44, 52] for *A. baumannii*, an overnight culture of the pass-*ΔlpxC* strain was inoculated into 100 mL LB media and incubated at 37°C until the culture reached OD_{600}~0.8. The cells were collected and washed with dH_2O once at room temperature followed by washing with 10% glycerol twice at room temperature. The cells were then resuspended with 1 mL of 10% glycerol. For electroporation, 50 μL of competent cells was mixed with 0.5–1 μg of UV-irradiated DNA in a 2-mm electroporation cuvette and pulsed at 25 kV/cm, 100 Ω, and 25 μF followed by the addition of 1 mL of LB media. The cells were recovered at 37°C with shaking at 100 rpm for 1.5 hours and plated on LB agar containing 100 μg/mL apramycin and 10 μg/mL colistin. Plates were incubated at 37°C for 1–2 days. Colonies that appeared following transformation were restreaked on plates containing the appropriate (i.e., kanamycin or tetracycline) antibiotic to confirm their antibiotic resistance phenotype, and the exchange of wildtype alleles for an antibiotic resistance cassette was verified by PCR. No mutations in both upper and lower homology arms were identified by sequencing.

Construction of targeting plasmids for making marked deletion mutants in *S. aureus*

rodA, ezrA, gdpS, mreDC, ltaA, and *SAOUHSC_01649*. To generate targeting plasmids, approximately 0.8–1 kb regions upstream and downstream of a target gene were amplified by PCR using wildtype *S. aureus* genomic DNA. A PCR-amplified antibiotic resistance cassette encoding resistance to either kanamycin (*aphA3*) or tetracycline (*tetM*) was incorporated in between these upstream and downstream homology arms by overlap PCR. The adjoined DNA fragment was cloned between the BamHI and SalI restriction sites of the pTarKO vector. Then, as described above, the targeting plasmid was irradiated with UV and electroporated into the *ΔtarO* RN4220 strain.

Unmarked deletion of *spa* in *S. aureus*

A markerless deletion of the *spa* gene encoding surface protein A was constructed by flipping out the marker after allelic exchange and phage transduction. The ~1000 bp region immediately upstream of the *spa* ORF (upper homology arm) was amplified from *S. aureus* RN4220 genomic DNA using primers spaAA and spaAB. The ~1000 bp region directly downstream of the *spa* ORF (lower homology arm) was amplified from *S. aureus* RN4220 genomic DNA using primers spaBA and spaBB. A kanamycin-resistance cassette (*aphA3)* flanked by FRT (FLP recognition target) sites was amplified using primers Kan-FRT-F and Kan-FRT-R from pTM204*attLint*.[15] The FRT-flanked *Kan*^{R} marker was ligated in between the upper and lower homology arms of *spa* to generate a ~3.5-kb sequence for cloning into pTarKO digested with BamHI and SalI restriction enzymes, resulting in pTarKO-*spa*. The pTarKO-*spa* targeting plasmid was electroporated into RN4220 *ΔtarO*$_{off}$ electrocompetent cells carrying an anhydrotetracycline-inducible copy of *tarO*, and transformants were selected on TSB agar plates supplemented with 50 μg/mL kanamycin, 50 μg/mL neomycin, and 10 μg/mL targocil. Colony PCR was performed to screen several colonies that appeared on the transformation plate after overnight incubation to confirm replacement of the wildtype *spa* allele with the *Δspa*::FRT-*Kan*^{R}-FRT marker, which was then transduced to a clean wildtype HG003 background using the generalized transducing phage phi85. HG003 cells containing the FRT-flanked, *Kan*^{R}-marked *spa* deletion was transduced with pFLP[15] constitutively expressing FLP recombinase to flip out the kanamycin-resistance cassette by recombination between the two FRT sites. After transformants were passaged on non-selective TSB agar plates for two rounds to provide time for FLP-mediated recombination, colony PCR was performed to confirm loss of the *Kan*^{R} marker and resultant markerless deletion of *spa*.

Construction of a promoter-swapped strain of *S. aureus: SAOUHSC_01050*

To exchange the native promoter of *SAOUHSC_01050* with an inducible promoter, an upper homology arm containing ~ 1 kb upstream of the start codon (ATG) and a lower homology arm containing ~ 1 kb downstream of ATG were PCR-amplified from genomic DNA. An aTc inducible promoter (815 bp) was amplified from pTP63[30] and linked to a kanamycin resistance cassette (*aphA3*) by PCR. The resultant PCR product was incorporated in between the upper and lower homology arms of *SAOUHSC_01050* by PCR, generating a DNA fragment having the aTc inducible promoter facing in the same direction as the *SAOUHSC_01050* ORF. The DNA fragment was digested with HindIII and SalI restriction

enzymes and ligated into pTarKO. As described above, the targeting plasmid was irradiated with UV and electroporated into the *ΔtarO* RN4220 strain. Allelic exchange of the native promoter for the inducible promoter in these mutants was confirmed by PCR.

Construction of a GFP-labeled strain of *S. aureus: ugtP* (*SAOUHSC_00953*)

To label UgtP with a green fluorescence protein (GFP) at its C-terminus, an upper homology arm containing ~ 1 kb upstream of the *ugtP* stop codon and a lower homology arm containing ~ 1 kb downstream of the stop codon were PCR-amplified from genomic DNA. A green fluorescence protein sequence for mNeonGreen[53] was codon optimized for *S. aureus* and synthesized (711 bp) by IDT (gBlock) (mNeonGFP sequence in Supporting Information). This DNA sequence, designated as mNeonGFP, was adjoined to a kanamycin-resistance cassette and incorporated in between the upper and lower homology arms of *ugtP* by PCR, thus generating a fragment containing *ugtP* linked to mNeonGFP. An 8 amino acid spacer (Gly-Gly-Gly-Ser-Ala-Gly-Gly-Ala) between UgtP and mNeonGFP was used to promote proper folding of these two proteins.[54] The DNA fragment was digested with HindIII and BamHI restriction sites and ligated into pTarKO. As described above, the targeting plasmid was irradiated with UV and electroporated into the *ΔtarO* RN4220 strain. Allelic exchange of wildtype *ugtP* for *ugtP* C-terminally fused to mNeonGFP was confirmed by PCR and sequencing.

Fluorescence microscopy of GFP-labeled UgtP in *S. aureus*

As previously described,[55] a 1-mL culture of the GFP-labeled UgtP strain was grown to an OD_{600} of 0.5 and pelleted by centrifugation at 3500 ×g for 2 min. The cells were resuspended in 30 μL of pH 7.4 phosphate-buffered saline (PBS). 5 μL of the cell suspension was immobilized on a pad of 2% agarose in PBS spotted on a microscope slide. Phase-contrast and fluorescence visualization of the live cells was performed using a Nikon TE2000 inverted microscope equipped with a Nikon Plan Apo ×100 oil objective lens (NA 1.4). For observing the GFP fluorophore, an Ex475/28 filter set was used. Image acquisition was carried out using a CoolSnapHQ2 CCD camera (Photometrics) attached to the microscope and the Nikon NIS-Elements Software. Image analyses were performed using ImageJ software.

Construction of Δ*rodA* RN4220 using pKFC

As previously described,[5] approximately 1-kb regions upstream and downstream of *rodA* were amplified by PCR using wildtype *S. aureus* RN4220 genomic DNA. A PCR-amplified kanamycin (*aphA3*) resistance cassette (kan^R) was incorporated in between these upstream and downstream homology arms by overlap PCR. The DNA fragment was then cloned into pTarKO between the BamHI and SalI sites, generating pKFC-*rodA*-kan^R. The pKFC-*rodA*-kan^R plasmid was electroporated into wildtype RN4220 strain as described above. After recovering for 1 hour at 30°C, the cells were plated on TSA agar containing 5 μg/mL chloramphenicol (Cm), and incubated at 30°C for 1–2 days. Eight colonies were retested on TSA agar containing 5 μg/mL Cm at 30°C, and a confirmed resistant colony was grown at 30°C in TSA containing 5 μg/mL Cm until OD_{600}=0.2. The culture was then shifted to a 43°C shaking incubator and further incubated at 43°C for 6 hours. 100 μL of different dilutions (10^2 or 10^3-fold) of the culture was plated on TSA agar containing 5 μg/mL Cm

and incubated at 43°C for overnight (12–16 hours). Approximately 8–16 colonies from the plate at 43°C were re-tested on TSA agar containing 5 μg/mL Cm and chromosomal integration of the pKFC-*rodA*-kan^R plasmid via a single crossover event was confirmed by PCR using these primers, *rodA*-CA/KFC-R and *rodA*-CB/KFC-F. To induce the second crossover, 4–8 confirmed colonies containing the plasmid integrant were passaged multiple times on TSA at 30°C, and 100–300 colonies from each passage were plated and screened for the *rodA* deletion by colony PCR using primers *rodA*-CA/*rodA*-CB.

Phage transduction in *S. aureus*

As previously described, to prepare *S. aureus* phage lysates, deletion mutants constructed in the *ΔtarO$_{off}$* strain background were grown in 2 mL of TSB containing 0.4 μM aTc until mid-log phase.[30] 500 μL of diluted cultures (OD_{600}=0.2) were mixed with 100 μL of staphylococcal phage ϕ11 FRT (10^6 PFU/mL) and 6 μL of 1 M $CaCl_2$. After 30 min of incubation at 30°C, 10 mL of pre-warmed (45°C) top agar containing 10 mM $CaCl_2$, 0.4 μM aTc, and 0.65% agar in TSB was added to each tube, and the mixture was poured on a TSB agar plate containing 0.4 μM aTc and 10 mM $CaCl_2$. The plates were incubated at 30°C overnight, and 10 mL of TSB was added to the plates the next day. After 4 hours of rocking at room temperature, the media containing the phage lysate was collected and filtered through a 0.4-μm syringe filter. To transduce into wildtype RN4220, a 1-mL overnight culture of wildtype was spun down and resuspended in 100 μL of TSB. 50 μL of the resuspension was mixed with 100 μL of LB media containing 20 mM $CaCl_2$ and 50 μL of the donor phage lysate. The mixture of cells and phage lysate was incubated at 30°C for 25 min followed by 15 min incubation at 30°C with shaking (100 rpm). The mixture was mixed with 100 μL of ice-cold 20 mM citrate and incubated on ice for 5 min followed by centrifugation at 9000 ×g for 10 min. The pellet was resuspended in 300 μL of 20 mM citrate and incubated on ice for 2 hours. The cells were plated on TSB agar containing 10% citrate and the appropriate antibiotic (*i.e.,* kanamycin), and plates were incubated at 30°C for 1–2 days.

Construction of complementation strains for Δ*ltaA* and Δ*ugtP* mutations

For p*ltaA*, the genomic locus encoding *ltaA* (*SAOUHSC_00952*) and its 300-bp upstream region was amplified by PCR using primers ltaA-F-SalI and ltaA-R-BamHI. The PCR product was cloned into pLOW [56] using SalI and BamHI sites. For p*ugtP*, the genomic locus containing *ltaA*-*ugtP* (*SAOUHSC_00952*, *SAOUSHC_00953*) and its 300-bp upstream region was amplified by PCR using primers ltaA-F-SalI and ugtP-R-BamHI, digested with SalI and BamHI, and cloned into pLOW. The plasmids were confirmed by sequencing. The p*ltaA* and *pugtP* plasmids were then electroporated into *ΔltaA* and *ΔugtP* RN4220, respectively. After recovering at 30°C for 1 hour, cells were plated on TSB agar containing 10 μg/mL erythromycin followed by incubation at 30°C for 1–2 days to obtain transformants.

Construction of an *apr*^{R}-marked Δ*adeFGH* deletion strain of *A. baumannii*

To target the *adeFGH* locus in *A. baumannii*, DNA fragments (~1000 bp) both upstream and downstream of the target locus were amplified and adjoined to an apramycin resistance gene (*apr*^{R}) by PCR. The resultant PCR product was digested with HindIII and SalI and cloned

into pColKO. The pColKO-*ΔadeFGH-apr*^{R} targeting plasmid was UV-irradiated as described above for *S. aureus* and electroporated into the pass-*ΔlpxC* strain. Obtained colonies were confirmed by testing their resistance phenotype on selective agar plates containing 100 μg/mL apramycin, and the deletion of the *adeFGH* gene locus was verified by PCR. No mutations in both upper and lower homology arms were identified by sequencing.

Mating-out assay for *A. baumannii* strains

To transfer the *ΔadeFGH*::apr^R marked deletion into a wildtype *A. baumannii* background, a mating-out assay was performed as previously described[45] using *ΔadeFGH*, pass-*ΔlpxC A. baumannii* as the donor strain and wildtype *A. baumannii* 19606 as the recipient strain. After overnight growth in TSB medium, both cultures were diluted 10-fold in TSB medium. The two cultures were then incubated for 1 hour at 37°C followed by mixing at a ratio of 1 to 4 (donor:recipient) in a total volume of 2 mL. Subsequently, this mixed culture was grown for 3 hours at 37°C, and 200 μL of the culture was plated on Mueller-Hinton 2 agar containing both 100 μg/mL apramycin and 0.04 μg/mL rifampicin. Obtained colonies were confirmed by testing their resistance phenotype on selective agar plates containing apramycin, and the deletion of the *adeFGH* gene locus in the recipient strain was verified by PCR.

Determination of MICs of rifampicin for pass-Δ*lpxC and wildtype A. baumannii*

Overnight cultures grown at 37°C in LB media to stationary phase were normalized to OD_{600}=1.0, and then diluted further to OD_{600}=0.0001. 100 μL of these diluted cultures were dispensed using a multi-channel pipette into 96-well plates. Rifampicin was dispensed using a D300e digital dispenser (Tecan) over a concentration range. Plates were incubated without shaking at 37°C for 24 hours. Following incubation, MICs were determined as the lowest concentrations at which the OD_{600} remained at baseline.

Whole genome sequencing of *A. baumannii* strains

To confirm directionality and exclude the exchange of possible background mutations during the mating-out assay, we performed whole genome sequencing with genomic DNA from wildtype, *ΔlpxC*, pass-*ΔlpxC*, pass-*ΔlpxCΔadeFGH*, and *ΔadeFGH* strains, as previously described.[55] 1 μL of 0.5–1 ng of genomic DNA from each strain was used in a 2.5 μL Tagmentation reaction to generate adapter-labeled DNA fragments of the appropriate size range (Illumina). The 3.5 μL reaction was incubated at 55°C for 10 minutes followed by incubation at 4°C in a PCR machine. The tagmented DNA was amplified by PCR using 11.2 μL of KAPA Library Amplification Kit master mix (Kapa Biosystems) and 4.4 μL of each indexing primer (5 μM) with the following PCR conditions: 72°C for 3 min/98°C for 5 min/13 cycles (98°C for 10s/62°C for 30s/72°C for 30s)/72°C for 5 min/4°C. To clean up the library DNA and remove short library fragments, 15 μL of the PCR product was mixed with 12 μL of bead (Agencourt AMPure XP beads, Beckman Coulter) and 30 μL of buffer (10 mM pH 8.0 Tris-Cl, 1 mM EDTA, and 0.05% Tween-20). The DNA bound on the bead was washed with 200 μL of 80% ethanol and eluted with 40 μL of H_2O. The DNA concentration was measured by Qubit (Thermo Fisher Scientific Inc.) and equal amounts of DNA from each sample were pooled, analyzed on TapeStation (Biopolymers Facility Biopolymer, Harvard Medical School), and sequenced using the MiSeq platform (Illumina). Sequencing

reads were analyzed using Geneious version 11.03. Single nucleotide polymorphisms (SNP) and deletions were identified by comparing the sequence of the mutants to the parental strains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH Grants U19AI109764 (S.W. and D.K.) P01AI083214 (S.W.), and a fellowship NSF GRFP grant DGE1144152 (T.D.).

ABBREVIATIONS

WTA	wall teichoic acid
LTA	lipoteichoic acid
GlcNac	N-Acetylglucosamine
ManNac	N-Acetylmannosamine
Und-PP	undecaprenyl pyrophosphate
AMSA	amsacrine
aTc	anhydrotetracycline
tuni	tunicamycin
pass-Δ*lpxC*	one of the *ΔlpxC* derivatives from 6 passages
***kan*^{R}**	kanamycin resistance cassette
***apr*^{R}**	apramycin resistance gene
LPS	lipopolysaccharide
MIC	minimal inhibitory concentration
FRT	FLP recognition target
HDR	homology-directed repair
NHEJ	non-homologous end joining
SNP	Single nucleotide polymorphisms
Cm	chloramphenicol
TSB	trypticase soy broth
LB	luria-Bertani

dLB	a four-fold diluted LB medium
PBS	phosphate-buffered saline

References

1. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A. 2000; 97:6640–6645. DOI: 10.1073/pnas.120163297 [PubMed: 10829079]
2. Link AJ, Phillips D, Church GM. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. J Bacteriol. 1997; 179:6228–6237. [PubMed: 9335267]
3. Yu BJ, Kang KH, Lee JH, Sung BH, Kim MS, Kim SC. Rapid and efficient construction of markerless deletions in the *Escherichia coli* genome. Nucleic Acids Res. 2008; 36:e84.doi: 10.1093/nar/gkn359 [PubMed: 18567910]
4. Prax M, Lee CY, Bertram R. An update on the molecular genetics toolbox for staphylococci. Microbiology. 2013; 159:421–435. DOI: 10.1099/mic.0.061705-0 [PubMed: 23378573]
5. Kato F, Sugai M. A simple method of markerless gene deletion in Staphylococcus aureus. J Microbiol Methods. 2011; 87:76–81. DOI: 10.1016/j.mimet.2011.07.010 [PubMed: 21801759]
6. Bae T, Schneewind O. Allelic replacement in *Staphylococcus aureus* with inducible counter-selection. Plasmid. 2006; 55:58–63. DOI: 10.1016/j.plasmid.2005.05.005 [PubMed: 16051359]
7. Redder P, Linder P. New range of vectors with a stringent 5-fluoroorotic acid-based counterselection system for generating mutants by allelic replacement in *Staphylococcus aureus*. Appl Environ Microbiol. 2012; 78:3846–3854. DOI: 10.1128/AEM.00202-12 [PubMed: 22447609]
8. Chen J, Ram G, Yoong P, Penades JR, Shopsin B, Novick RP. An *rpsL*-based allelic exchange vector for *Staphylococcus aureus*. Plasmid. 2015; 79:8–14. DOI: 10.1016/j.plasmid.2015.02.002 [PubMed: 25659529]
9. Bryan LE, Kwan S. Roles of ribosomal binding, membrane potential, and electron transport in bacterial uptake of streptomycin and gentamicin. Antimicrob Agents Chemother. 1983; 23:835–845. [PubMed: 6351731]
10. Damper PD, Epstein W. Role of the membrane potential in bacterial resistance to aminoglycoside antibiotics. Antimicrob Agents Chemother. 1981; 20:803–808. [PubMed: 6173015]
11. Chen W, Zhang Y, Yeo WS, Bae T, Ji Q. Rapid and Efficient Genome Editing in *Staphylococcus aureus* by Using an Engineered CRISPR/Cas9 System. J Am Chem Soc. 2017; 139:3790–3795. DOI: 10.1021/jacs.6b13317 [PubMed: 28218837]
12. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell. 2014; 157:1262–1278. DOI: 10.1016/j.cell.2014.05.010 [PubMed: 24906146]
13. He X, Tan C, Wang F, Wang Y, Zhou R, Cui D, You W, Zhao H, Ren J, Feng B. Knock-in of large reporter genes in human cells via CRISPR/Cas9-induced homology-dependent and independent DNA repair. Nucleic Acids Res. 2016; 44:e85.doi: 10.1093/nar/gkw064 [PubMed: 26850641]
14. Sun F, Cho H, Jeong DW, Li C, He C, Bae T. Aureusimines in *Staphylococcus aureus* are not involved in virulence. PLoS One. 2010; 5:e15703.doi: 10.1371/journal.pone.0015703 [PubMed: 21209955]
15. Santiago M, Matano LM, Moussa SH, Gilmore MS, Walker S, Meredith TC. A new platform for ultra-high density Staphylococcus aureus transposon libraries. BMC Genomics. 2015; 16:252.doi: 10.1186/s12864-015-1361-3 [PubMed: 25888466]
16. Lee K, Campbell J, Swoboda JG, Cuny GD, Walker S. Development of improved inhibitors of wall teichoic acid biosynthesis with potent activity against *Staphylococcus aureus*. Bioorg Med Chem Lett. 2010; 20:1767–1770. DOI: 10.1016/j.bmcl.2010.01.036 [PubMed: 20138521]
17. Matano LM, Morris HG, Hesser AR, Martin SES, Lee W, Owens TW, Laney E, Nakaminami H, Hooper D, Meredith TC, Walker S. Antibiotic That Inhibits the ATPase Activity of an ATP-Binding Cassette Transporter by Binding to a Remote Extracellular Site. J Am Chem Soc. 2017; 139:10597–10600. DOI: 10.1021/jacs.7b04726 [PubMed: 28727445]

18. Brown S, Santa Maria JP Jr, Walker S. Wall teichoic acids of gram-positive bacteria. Annu Rev Microbiol. 2013; 67:313–336. DOI: 10.1146/annurev-micro-092412-155620 [PubMed: 24024634]
19. Sewell EW, Brown ED. Taking aim at wall teichoic acid synthesis: new biology and new leads for antibiotics. J Antibiot (Tokyo). 2014; 67:43–51. DOI: 10.1038/ja.2013.100 [PubMed: 24169797]
20. Lee W, Schaefer K, Qiao Y, Srisuknimit V, Steinmetz H, Muller R, Kahne D, Walker S. The Mechanism of Action of Lysobactin. J Am Chem Soc. 2016; 138:100–103. DOI: 10.1021/jacs.5b11807 [PubMed: 26683668]
21. D'Elia MA, Millar KE, Bhavsar AP, Tomljenovic AM, Hutter B, Schaab C, Moreno-Hagelsieb G, Brown ED. Probing teichoic acid genetics with bioactive molecules reveals new interactions among diverse processes in bacterial cell wall biogenesis. Chem Biol. 2009; 16:548–556. DOI: 10.1016/j.chembiol.2009.04.009 [PubMed: 19477419]
22. Swoboda JG, Meredith TC, Campbell J, Brown S, Suzuki T, Bollenbach T, Malhowski AJ, Kishony R, Gilmore MS, Walker S. Discovery of a small molecule that blocks wall teichoic acid biosynthesis in *Staphylococcus aureus*. ACS Chem Biol. 2009; 4:875–883. DOI: 10.1021/cb900151k [PubMed: 19689117]
23. D'Elia MA, Pereira MP, Chung YS, Zhao W, Chau A, Kenney TJ, Sulavik MC, Black TA, Brown ED. Lesions in teichoic acid biosynthesis in *Staphylococcus aureus* lead to a lethal gain of function in the otherwise dispensable pathway. J Bacteriol. 2006; 188:4183–4189. DOI: 10.1128/JB.00197-06 [PubMed: 16740924]
24. Nair D, Memmi G, Hernandez D, Bard J, Beaume M, Gill S, Francois P, Cheung AL. Whole-genome sequencing of *Staphylococcus aureus* strain RN4220, a key laboratory strain used in virulence research, identifies mutations that affect not only virulence factors but also the fitness of the strain. J Bacteriol. 2011; 193:2332–2335. DOI: 10.1128/JB.00027-11 [PubMed: 21378186]
25. Holland LM, Conlon B, O'Gara JP. Mutation of tagO reveals an essential role for wall teichoic acids in *Staphylococcus epidermidis* biofilm development. Microbiology. 2011; 157:408–418. DOI: 10.1099/mic.0.042234-0 [PubMed: 21051486]
26. Jorge AM, Hoiczyk E, Gomes JP, Pinho MG. EzrA contributes to the regulation of cell size in *Staphylococcus aureus*. PLoS One. 2011; 6:e27542.doi: 10.1371/journal.pone.0027542 [PubMed: 22110668]
27. Steele VR, Bottomley AL, Garcia-Lara J, Kasturiarachchi J, Foster SJ. Multiple essential roles for EzrA in cell division of *Staphylococcus aureus*. Mol Microbiol. 2011; 80:542–555. DOI: 10.1111/j.1365-2958.2011.07591.x [PubMed: 21401734]
28. Hinds J, Mahenthiralingam E, Kempsell KE, Duncan K, Stokes RW, Parish T, Stoker NG. Enhanced gene replacement in mycobacteria. Microbiology. 1999; 145(Pt 3):519–527. DOI: 10.1099/13500872-145-3-519 [PubMed: 10217485]
29. Lee W, VanderVen BC, Fahey RJ, Russell DG. Intracellular *Mycobacterium tuberculosis* exploits host-derived fatty acids to limit metabolic stress. J Biol Chem. 2013; 288:6788–6800. DOI: 10.1074/jbc.M112.445056 [PubMed: 23306194]
30. Pasquina L, Santa Maria JP Jr, McKay Wood B, Moussa SH, Matano LM, Santiago M, Martin SE, Lee W, Meredith TC, Walker S. A synthetic lethal approach for compound and target identification in *Staphylococcus aureus*. Nat Chem Biol. 2016; 12:40–45. DOI: 10.1038/nchembio.1967 [PubMed: 26619249]
31. Santa Maria JP Jr, Sadaka A, Moussa SH, Brown S, Zhang YJ, Rubin EJ, Gilmore MS, Walker S. Compound-gene interaction mapping reveals distinct roles for *Staphylococcus aureus* teichoic acids. Proc Natl Acad Sci U S A. 2014; 111:12510–12515. DOI: 10.1073/pnas.1404099111 [PubMed: 25104751]
32. Rajagopal M, Walker S. Envelope Structures of Gram-Positive Bacteria. Curr Top Microbiol Immunol. 2017; 404:1–44. DOI: 10.1007/82_2015_5021 [PubMed: 26919863]
33. Reichmann NT, Grundling A. Location, synthesis and function of glycolipids and polyglycerolphosphate lipoteichoic acid in Gram-positive bacteria of the phylum Firmicutes. FEMS Microbiol Lett. 2011; 319:97–105. DOI: 10.1111/j.1574-6968.2011.02260.x [PubMed: 21388439]

34. Rajagopal M, Martin MJ, Santiago M, Lee W, Kos VN, Meredith T, Gilmore MS, Walker S. Multidrug Intrinsic Resistance Factors in *Staphylococcus aureus* Identified by Profiling Fitness within High-Diversity Transposon Libraries. MBio. 2016; 7doi: 10.1128/mBio.00950-16
35. Xia G, Corrigan RM, Winstel V, Goerke C, Grundling A, Peschel A. Wall teichoic Acid-dependent adsorption of staphylococcal siphovirus and myovirus. J Bacteriol. 2011; 193:4006–4009. DOI: 10.1128/JB.01412-10 [PubMed: 21642458]
36. Biswas I. Genetic tools for manipulating *Acinetobacter baumannii* genome: an overview. J Med Microbiol. 2015; 64:657–669. DOI: 10.1099/jmm.0.000081 [PubMed: 25948809]
37. Zhang G, Meredith TC, Kahne D. On the essentiality of lipopolysaccharide to Gram-negative bacteria. Curr Opin Microbiol. 2013; 16:779–785. DOI: 10.1016/j.mib.2013.09.007 [PubMed: 24148302]
38. Olaitan AO, Morand S, Rolain JM. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. Front Microbiol. 2014; 5:643.doi: 10.3389/fmicb.2014.00643 [PubMed: 25505462]
39. Powers MJ, Trent MS. Expanding the Paradigm for the Outer Membrane: *Acinetobacter baumannii* in the Absence of Endotoxin. Mol Microbiol. 2017; doi: 10.1111/mmi.13872
40. Lopez-Rojas R, McConnell MJ, Jimenez-Mejias ME, Dominguez-Herrera J, Fernandez-Cuenca F, Pachon J. Colistin resistance in a clinical *Acinetobacter baumannii* strain appearing after colistin treatment: effect on virulence and bacterial fitness. Antimicrob Agents Chemother. 2013; 57:4587–4589. DOI: 10.1128/AAC.00543-13 [PubMed: 23836165]
41. Beceiro A, Moreno A, Fernandez N, Vallejo JA, Aranda J, Adler B, Harper M, Boyce JD, Bou G. Biological cost of different mechanisms of colistin resistance and their impact on virulence in *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2014; 58:518–526. DOI: 10.1128/AAC.01597-13 [PubMed: 24189257]
42. Moffatt JH, Harper M, Harrison P, Hale JD, Vinogradov E, Seemann T, Henry R, Crane B, St Michael F, Cox AD, Adler B, Nation RL, Li J, Boyce JD. Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. Antimicrob Agents Chemother. 2010; 54:4971–4977. DOI: 10.1128/AAC.00834-10 [PubMed: 20855724]
43. Moison E, Xie R, Zhang G, Lebar MD, Meredith TC, Kahne D. A Fluorescent Probe Distinguishes between Inhibition of Early and Late Steps of Lipopolysaccharide Biogenesis in Whole Cells. ACS Chem Biol. 2017; 12:928–932. DOI: 10.1021/acschembio.7b00159 [PubMed: 28248483]
44. Yildirim S, Thompson MG, Jacobs AC, Zurawski DV, Kirkup BC. Evaluation of Parameters for High Efficiency Transformation of *Acinetobacter baumannii*. Sci Rep. 2016; 6:22110.doi: 10.1038/srep22110 [PubMed: 26911658]
45. Krahn T, Wibberg D, Maus I, Winkler A, Bontron S, Sczyrba A, Nordmann P, Puhler A, Poirel L, Schluter A. Intraspecies Transfer of the Chromosomal *Acinetobacter baumannii blaNDM-1* Carbapenemase Gene. Antimicrob Agents Chemother. 2016; 60:3032–3040. DOI: 10.1128/AAC.00124-16 [PubMed: 26953198]
46. Garcia-Quintanilla M, Caro-Vega JM, Pulido MR, Moreno-Martinez P, Pachon J, McConnell MJ. Inhibition of LpxC Increases Antibiotic Susceptibility in *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2016; 60:5076–5079. DOI: 10.1128/AAC.00407-16 [PubMed: 27270288]
47. Leibig M, Krismer B, Kolb M, Friede A, Gotz F, Bertram R. Marker removal in staphylococci via Cre recombinase and different lox sites. Appl Environ Microbiol. 2008; 74:1316–1323. DOI: 10.1128/AEM.02424-07 [PubMed: 18165371]
48. Matano LM, Morris HG, Wood BM, Meredith TC, Walker S. Accelerating the discovery of antibacterial compounds using pathway-directed whole cell screening. Bioorg Med Chem. 2016; 24:6307–6314. DOI: 10.1016/j.bmc.2016.08.003 [PubMed: 27594549]
49. Xayarath B, Yother J. Mutations blocking side chain assembly, polymerization, or transport of a Wzy-dependent *Streptococcus pneumoniae* capsule are lethal in the absence of suppressor mutations and can affect polymer transfer to the cell wall. J Bacteriol. 2007; 189:3369–3381. DOI: 10.1128/JB.01938-06 [PubMed: 17322316]
50. Burrows LL, Lam JS. Effect of *wzx* (*rfbX*) mutations on A-band and B-band lipopolysaccharide biosynthesis in *Pseudomonas aeruginosa* O5. J Bacteriol. 1999; 181:973–980. [PubMed: 9922263]

51. Makela, P., Stocker, B. Genetics of Lipopolysaccharide. In: Rietschel, E., editor. Handbook of Endotoxin. Elsevier; Amsterdam: 1984. p. 59-137.

52. Amin IM, Richmond GE, Sen P, Koh TH, Piddock LJ, Chua KL. A method for generating marker-less gene deletions in multidrug-resistant *Acinetobacter baumannii*. BMC Microbiol. 2013; 13:158.doi: 10.1186/1471-2180-13-158 [PubMed: 23848834]

53. Cho H, Wivagg CN, Kapoor M, Barry Z, Rohs PD, Suh H, Marto JA, Garner EC, Bernhardt TG. Bacterial cell wall biogenesis is mediated by SEDS and PBP polymerase families functioning semi-autonomously. Nat Microbiol. 2016; :16172.doi: 10.1038/nmicrobiol.2016.172 [PubMed: 27643381]

54. Chen X, Zaro JL, Shen WC. Fusion protein linkers: property, design and functionality. Adv Drug Deliv Rev. 2013; 65:1357–1369. DOI: 10.1016/j.addr.2012.09.039 [PubMed: 23026637]

55. Pang T, Wang X, Lim HC, Bernhardt TG, Rudner DZ. The nucleoid occlusion factor Noc controls DNA replication initiation in *Staphylococcus aureus*. PLoS Genet. 2017; 13:e1006908.doi: 10.1371/journal.pgen.1006908 [PubMed: 28723932]

56. Liew AT, Theis T, Jensen SO, Garcia-Lara J, Foster SJ, Firth N, Lewis PJ, Harry EJ. A simple plasmid-based system that allows rapid generation of tightly controlled gene expression in *Staphylococcus aureus*. Microbiology. 2011; 157:666–676. DOI: 10.1099/mic.0.045146-0 [PubMed: 21109562]

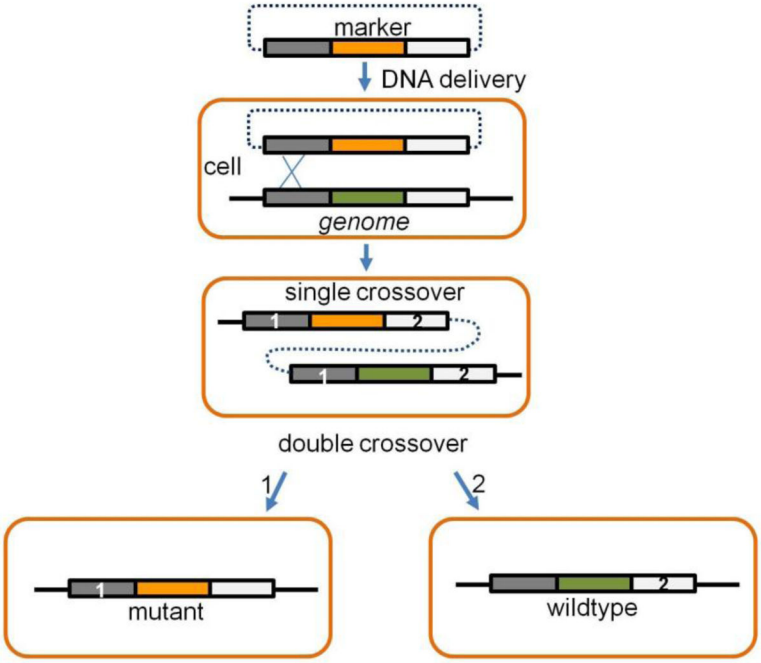


Figure 1. Recombineering strategy for allelic exchange using positive selection

After transformation of a vector containing flanking homology arms to direct recombination to a particular chromosomal locus, a first crossover event leads to integration of the vector into the chromosome. The vector recombines out through a second crossover event, producing a mutant allele or regenerating the wildtype allele depending on whether the upstream or downstream homology region is used for recombination. pKFC-mediated deletion of chromosomal genes utilizes this recombineering strategy.

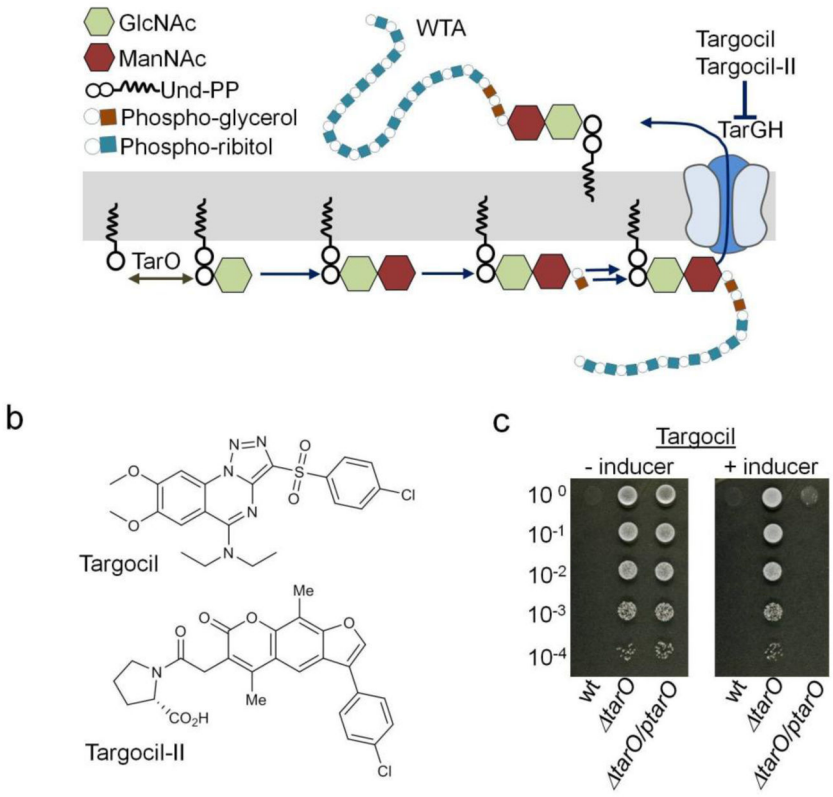
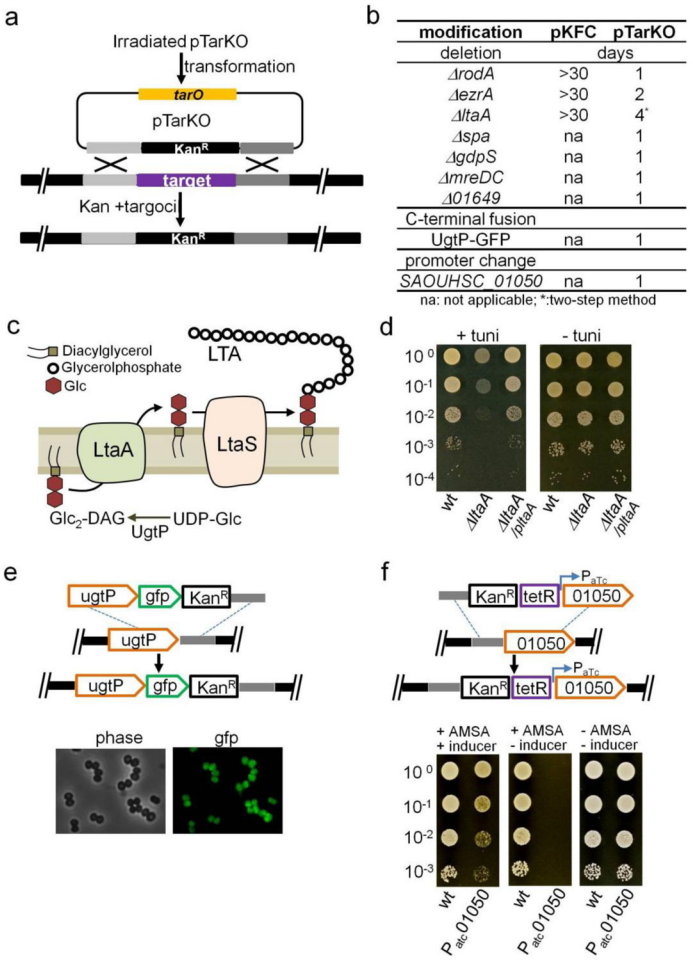


Figure 2. Modulation of WTA pathway with Δ*tarO* and targocil/targocil-II provides selection pressure in *S. aureus*

(a) Schematic of the pathway for WTA biosynthesis and target of targocil/targocil-II in the WTA pathway. **(b)** Structures of two WTA inhibitors that target TarGH. **(C)** A *ΔtarO* strain is resistant to 10 μg/mL targocil, a dose that is lethal to wildtype. Complementation with p*tarO* (pTP63-P_{aTc}-*tarO*) restored the targocil-susceptible phenotype in the presence of the inducer, 0.4 μM aTc.



modification	pKFC	pTarKO
deletion		days
ΔrodA	>30	1
ΔezrA	>30	2
ΔltaA	>30	4*
Δspa	na	1
ΔgdpS	na	1
ΔmreDC	na	1
Δ01649	na	1
C-terminal fusion		
UgtP-GFP	na	1
promoter change		
SAOUHSC_01050	na	1

na: not applicable; *:two-step method

Figure 3. The pTarKO system enables various chromosomal modifications for reverse genetic analysis in *S. aureus*

(a) Schematic of the method and targeting plasmid used for allelic exchange of a target locus in *S. aureus*. **(b)** Table summarizing chromosomal modification of various targets in *S. aureus.* Days required to obtain some mutants using the pKFC vector is shown for comparison. **(c)** Schematic of the pathway for biosynthesis of lipoteichoic acid. **(d)** Genes involved in the lipoteichoic acid pathway are synthetically sick or lethal with the WTA pathway. A *ΔltaA* strain is partially viable in the presence of 1 μg/mL tunicamycin (tuni), which inhibits TarO in the WTA pathway. **(e)** The C-terminus of UgtP was fused to GFP protein in the chromosome. **(f)** The native promoter of *SAOUHSC_01050* was swapped with the inducible tetR promoter that is controlled with anhydrotetracycline. In the presence of 1 μg/mL amsacrine (AMSA), which inhibits D-alanylation, the mutant is only viable if gene expression is induced.

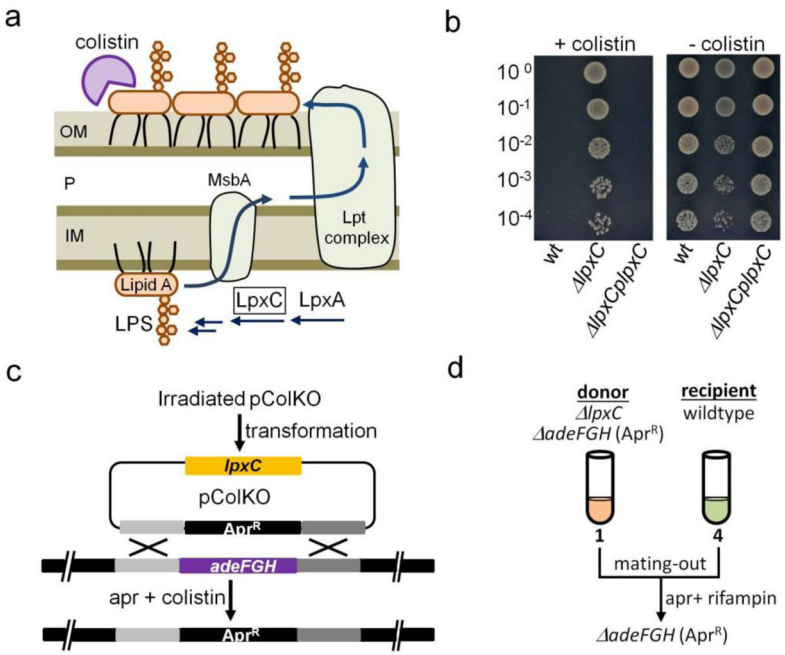


Figure 4. Counterselection with a pathway inhibitor is also applicable to *A. baumannii*

(a) Schematic of the pathway for LPS biogenesis and the target of colistin in the pathway. **(b)** A *ΔlpxC* strain is resistant to 10 μg/mL colistin, which is lethal to wildtype. Complementation with p*lpxC* (pWH1266-P_{native}-*lpxC*) restored the colistin-susceptible phenotype. **(c)** Schematic of the method and targeting plasmid used for allelic exchange of the efflux pump *adeFGH* locus in *A. baumannii.* **(d)** Schematic of the method used for mating-out for *A. baumannii* strains. The donor and recipient cultures were harvested at early-log phase (OD_{600}=0.2) and mixed at a volumetric ratio of 1:4.

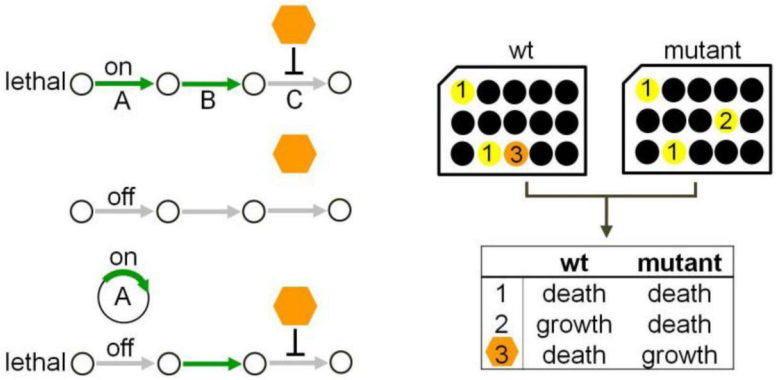


Figure 5. A counterselection strategy utilizing mutations of early genes in a non-essential pathway combined with late-stage inhibitors of the same pathway is applicable as a tool for bacterial genetics

Pathway inhibitors useful for counterselection can be identified in a high-throughput screen by comparing differential growth of wildtype and pathway mutant strains. In the first scenario, compounds that inhibit essential cellular processes will lead to death of both the wildtype and mutant. In the second scenario, compounds that are synthetically lethal with the mutant will only inhibit growth of the mutant. A pathway inhibitor will be lethal to wildtype, but growth inhibition is alleviated when the pathway is turned off via deletion of a non-essential, early gene and restored via ectopic expression of that gene. Differential bacterial growth is colored as black (full growth) and yellow (no growth); identification of a pathway inhibitor is denoted in orange.