Published in final edited form as:

Curr Protoc Microbiol. ; 32: Unit-9C.3.. doi:10.1002/9780471729259.mc09c03s32.

Genetic manipulation of *Staphylococcus aureus*

Olaf Schneewind and Dominique Missiakas

Department of Microbiology, University of Chicago, Chicago, Illinois 60637, USA

Abstract

Staphylococcus aureus is a facultative anaerobic Gram-positive coccus and a member of the normal skin flora as well as the nasal passages of humans. However, *S. aureus* can also gain entry into the host and cause life-threatening infections or persist as disease foci that develop into suppurative abscesses. While genetically tractable, the manipulation of *S. aureus* remains challenging. This unit describes methods developed in our laboratory for gene disruption by allelic replacement and transposition. We also provide a protocol for bacteriophage-mediated transduction of mutants marked with selectable alleles and describe plasmid utilization for complementation studies.

INTRODUCTION

Several challenges must be overcome for the genetic manipulation of *S. aureus*. First, the organism is endowed with a very thick peptidoglycan envelope (Giesbrecht et al., 1998) and is not naturally competent, although it carries a complete set of competence genes (Morikawa et al., 2003). The lack of competence activity can be reversed by gene duplication rearrangement to activate the cryptic *sigH* gene (Morikawa et al., 2012). This process occurs spontaneously at low frequency and may account for the acquisition of antibiotic resistance genes (Morikawa et al., 2012) but it has not yet been exploited toward genetic manipulations in the laboratory. Current laboratory methods for the transfer of DNA into *S. aureus* include electroporation or bacteriophage transduction. Second, many strains are resistant to multiple antibiotics, a phenotype that makes the use of selectable markers challenging (Missiakas and Schneewind, 2013). Third, due to host DNA restriction-modification systems, plasmid DNA prepared in *Escherichia coli* cannot be directly introduced into clinical *S. aureus* isolates but must first be passaged in *S. aureus* hosts lacking such restriction barriers while retaining the attribute of DNA modification. Fourth, molecular cloning in *S. aureus* requires shuttle vectors that can replicate and be selected for in both the Gram-negative bacterium *E. coli* and in Gram-positive hosts such as *S. aureus*. For more information on genetically tractable and relevant strains of *S. aureus* as well as methods for growing and storing such strains, the reader should consult Unit 9C.1 (Missiakas and Schneewind, 2013). In this Unit, we describe methods for *bursa aurealis* transposon mutagenesis, allelic replacement using plasmid pKOR1 and phage transduction. Protocols for the delivery of plasmid DNA by electroporation in *S. aureus*, preparation of

competent cells as well as mapping of transposon insertions on the chromosome are also provided.

CAUTION: *S. aureus* is a highly virulent and adaptable pathogen with the ability to colonize, infect, invade, persist, and replicate in all human tissue including skin, bone, visceral organs, or vasculature (Lowy, 1998). The organism has been categorized as a Risk Group Level 2 pathogen. All manipulations with *S. aureus* strains must be performed following biosafety level 2 (BSL2) practice. Guidelines for BSL2 practice can be obtained from the latest edition of *Biosafety in Microbiological and Biomedical Laboratories* (BMBL, 5th Edition) via the following CDC web link: www.cdc.gov/biosafety/publications/bmbl5/.

BASIC PROTOCOL 1: PREPARATION OF COMPETENT CELLS OF *S. aureus*

This protocol describes a method to render cells of *S. aureus* competent for the electroporation of DNA into the cytoplasm.

Materials

Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB)

Petri dishes, 15-mL culture tube, 1-L flask to grow culture

Sterile loop

37°C incubator

Spectrophotometer and plastic cuvette to measure cell density at 600 nm (A_{600})

Sterile 50-mL sterile centrifuge tubes (that can be centrifuged at up to 5,000 $\times g$)

Ice

Ice-cold sterile 0.5 M sucrose in deionized water

Sterile microcentrifuge tubes

Dry ice-ethanol bath

−80°C freezer

1. Streak *S. aureus* strain from a frozen stock on a TSA plate; incubate overnight at 37°C
2. Pick an isolated colony with a sterile loop; inoculate 1 mL TSB in a 15-mL culture tube.
3. Incubate overnight at 37°C with shaking.
4. Transfer culture to a 1-L sterile flask containing 100 mL TSB.
5. Grow cells with vigorous shaking for approximately 2.5 to 3 h until culture reaches $A_{600} \sim 0.5$.
6. Transfer culture into two 50-mL sterile centrifuge tubes and centrifuge at 5,000 $\times g$ for 15 min.

7. Discard supernatant and suspend cell pellet in a total volume of 20 mL ice-cold sterile 0.5 M sucrose prepared in de-ionized water.
8. Transfer cell suspension to a pre-chilled 50-mL sterile centrifuge tube (keep tubes containing cells on ice from there on).
9. Centrifuge tube at $8,000 \times g$ for 10 min at 4°C.
10. Discard supernatant and suspend cell pellet in 10 mL of the ice-cold 0.5 M sucrose solution as above.
11. Collect cells by centrifugation at $8,000 \times g$ for 10min at 4°C.
12. Repeat steps 10 and 11 once more.
13. Suspend cells in 1 mL ice-cold 0.5 M sucrose solution.
14. Aliquot cells by transferring volumes of 100- μ L to microcentrifuge tubes kept on ice.
15. Freeze tubes by plunging in a dry ice-ethanol bath and store at -80°C until needed (this protocol can be adapted to prepare larger volumes of competent cells).

BASIC PROTOCOL 2: ELECTROPORATION OF PLASMID DNA AND PLATING FOR SELECTION OF TRANSFORMANTS

This protocol describes a method to introduce DNA into the cytoplasm of *S. aureus* competent cells prepared as described in BASIC PROTOCOL 1.

Materials

Plasmid DNA (see BASIC PROTOCOL 6 and troubleshooting section)

Frozen competent cells

Ice

TSB

TSA plates containing either chloramphenicol at 5 or 10 $\mu\text{g/mL}$ or tetracycline at 2.5 $\mu\text{g/mL}$ (alone or in combination)

Sterile tips and pipetman

Electroporation cuvette

Electroporator

Sterile Eppendorf tube

Sterile spreader

30°C or 37°C incubator

1. Remove a tube of competent cells from freezer (BASIC PROTOCOL 1 *step 15*); place tube on ice and let cells thaw

2. Add 0.1 to 1 μg of plasmid DNA (preferentially in water to minimize electrical arcing).

Note that for the introduction of two plasmids as required for transposon mutagenesis, should be performed sequentially; only one plasmid should be electroporated at a time and new competent cells prepared for the electroporation of the second plasmid (see BASIC PROTOCOL 3).
3. Transfer the content of the tube into a 0.1-cm electroporation cuvette kept on ice (such cuvettes can be obtained from Bio-Rad, Hercules, CA).
4. Set the electroporator as follows: voltage = 2.5 kV, resistance = 100 Ω , capacity = 25 μF
5. Apply electrical pulse and immediately add 1 mL TSB kept at room temperature; then transfer the content from the electroporation cuvette to a sterile Eppendorf tube.
6. Close tube and incubate for an hour at 30°C (no shaking required).
7. Centrifuge tubes (8000 $\times$ g, 3 min, RT) and decant or remove supernatant with a sterile tip using a pipetman.
8. Suspend cell pellet in 50 to 100 μL of fresh TSB.
9. Spread cells on TSA plates using a sterile spreader as follows:
 - a. For selection of pBursa use TSA plates containing 5 $\mu\text{g}/\text{mL}$ chloramphenicol (TSA_{Chl5})
 - b. For selection of pKOR1 and pEC194-based plasmids use TSA plates containing 10 $\mu\text{g}/\text{mL}$ chloramphenicol (TSA_{Chl10})
 - c. For selection of pFA545 use a TSA plate containing 2.5 $\mu\text{g}/\text{mL}$ tetracycline (TSA_{Tet2.5})
10. Incubate plates at 30°C for experiments involving plasmids pBursa, pFA545 and pKOR1 and at 37°C for experiments involving pC194-based plasmids.

Note: plasmids pBursa, pFA545 and pKOR1 carry a thermosensitive replicon and are unstable at temperatures above 30°C.

BASIC PROTOCOL 3: TRANSPOSON MUTAGENESIS USING BURSA AUREALIS

Transposon mutagenesis has been very useful in isolating large collections of mutants. Most transposons are delivered on plasmids carrying a temperature replicon. The transposon and transposase genes may be carried on one or two plasmids. For example, the transposable element Tn917 from *Enterococcus faecalis* (Shaw and Clewell, 1985; Tomich et al., 1980) is delivered via a single plasmid that has been optimized by several groups and has been used for the mutagenesis of several Gram-positive bacteria (Bae et al., 2004; Camilli et al., 1990; Hartley and Paddon, 1986; Youngman et al., 1983). In collaboration with Fredrik Åslund,

our laboratory developed *bursa aurealis*, a mariner-based transposon that inserts into target DNA to generate TA duplications and does not exhibit sequence preference in the genome (Bae et al., 2004; Tam et al., 2006). *Bursa aurealis* has been used to generate several libraries of mutants in *S. aureus* including the Phoenix Library (Missiakas collection) and the Nebraska Library (made publicly available through NARSA: www.narsa.net). A target strain is first transformed with the plasmid-bearing transposon pBursa and transposase encoding plasmid pFA545 (Fig. 1A) at the permissive temperature (30°C) as described in BASIC PROTOCOLS 1–3. Next, bacterial cultures are grown at non-permissive temperature (39–43°C) and erythromycin resistant clones are selected (Bae et al., 2004).

Here, we describe a protocol for the isolation of transposon mutants on solid medium.

Materials

A target *S. aureus* strain carrying plasmids pBursa and pFA545, prepared as described in BASIC PROTOCOLS 1–2

Sterile water, TSB and TSA

Chloramphenicol, erythromycin, and tetracycline antibiotics used at the following final concentrations 5, 10, and 2.5 µg/mL, respectively

Incubators for plates and liquid cultures (30°C, 43°C)

Centrifuge and microcentrifuge

Sterile freezing solution for long-term storage of bacterial strains at 80°C: 5% monosodium glutamate, 5% bovine serum albumin

Cryotube (2-ml)

Sterile tips and pipetman

Sterile glass beads or spreader

Day 1:

1. The target strain carrying both plasmids, pBursa and pFA545, is obtained following electroporation of pBursa first. Transformants isolated on TSACHl5 at 30°C should be grown in TSBChl5 at 30°C to generate electrocompetent cells for subsequent electroporation with plasmid pFA545. Candidate transformants should be plated on TSACHl5Tet2.5 at 30°C as described in BASIC PROTOCOLS 1–2.

Note: the strain carrying both plasmids can be stored at –80°C; when starting from the frozen stock, an aliquot of frozen cells should be patched on TSA_{Tet2.5} Chl5 and incubated overnight for formation of isolated colonies at 30°C).

Day 2:

2. Keep a sterile flask containing 50 ml sterile water at 43°C.
3. Fill 10 sterile microcentrifuge tubes with 100 µL sterile water each and place in the 43°C incubator for 1 hour.

4. Use 10 TSA_{Erm10} plates and place in the 43°C incubator for 1 h.
5. With a sterile loop, pick a colony from the TSA_{Tet2.5 Chl5} plate (*step 1*) and inoculate into one of the microcentrifuge tubes. Vortex and repeat this procedure with the remaining tubes picking a new colony each time; keep tubes at 43°C.
6. Transfer 2 µL of the cell suspension (*step 5*) and 100 µL of prewarmed water (*step 2*) onto a prewarmed TSA_{Erm10} plate (*step 4*).
7. Add 7 to 15 sterile glass beads on the plate, shake to spread cells evenly, remove and recycle glass beads.
8. Place plates immediately at 43°C and incubate until colonies appear (up to 2 days).

Day 3 or 4:

9. Inoculate colonies single or in pools (*step 8*), in 5 ml TSB_{erm10} and incubate at 43°C overnight with shaking.

Day 5 or 6:

10. Freeze 1 ml of cultures at –80°C (*step 9*) in 50% sterile freezing solution (see BASIC PROTOCOL 3 Unit 9C.1). The remaining culture can be used to map transposon insertion site(s) (see BASIC PROTOCOL 4).

BASIC PROTOCOL 4: MAPPING BURSA AUREALIS INSERTIONS BY INVERSE PCR

This protocol is useful to sample a number of isolated mutant strains for quality control. Sampling is described below for the analysis of 96 strains but can be adapted to a smaller sample size. Other methods can also be used to map transposon insertions. Genomic DNA extracted from transposon mutant candidates (generated in Basic Protocol 6) is first digested with *Aci*I, and self-ligated. These products are then used as DNA templates for “inverse PCR” amplification using the primer set, Martn-F and Martn-erm-R (Fig. 1B).

Materials

Oligonucleotide primers Martn-F (5′-TTT ATG GTA CCA TT CAT TTT CCT GCT TTT TC) and Martn-ermR (5′-AAA CTG ATT TTT AGT AAA CAG TTG ACG ATA TTC).

Restriction enzyme *Aci*I, T4 DNA ligase, polymerase for PCR amplification (New England Biolabs or preferred commercial source)

37°C incubator

Eppendorf and PCR tubes, tips and pipetman

Qiagen MinElute 96 UF PCR purification Kit (or preferred kit for purification of DNA fragments)

PCR machine

1. Use cultures isolated during *step 10* of BASIC PROTOCOL 3 and lyse cells to extract genomic DNA as described in BASIC PROTOCOL 6. If DNA was stored at sub-zero temperatures, bring to room temperature.
2. Reaction mix for digestion of DNA with *Aci*I in a 96-well plate assay:
 - 100 μ L 10 $\times$ buffer for *Aci*I
 - 75 μ L *Aci*I (10,000 units/ml)
 - 325 μ L waterTransfer 5 μ L of this mix to each well, add 5 μ L chromosomal DNA from 96 different isolates from Basic Protocol 3, step 10.
3. Incubate samples 1 h at 37°C (this incubation can be prolonged overnight).
4. Inactivate *Aci*I by incubating samples for 20 min at 65°C.
5. Ligation mix for the 96-well plate assay:
 - 7.9 mL water
 - 1.0 mL 10 $\times$ buffer for T4 DNA ligase
 - 0.1 mL T4 DNA ligase (New England Biolabs)Transfer 90 μ L of the ligation mix to each well.
6. Incubate ligation reactions for 3 h at room temperature (this incubation can be prolonged overnight).
7. Purify DNA with the Qiagen MinElute 96 UF PCR purification Kit (for 96-well sample) according to the manufacturer's protocol. Elute DNA in each well with 60 to 75 μ L of elution buffer or deionized water.
8. Transfer 5 μ L of ligated DNA for PCR reaction in a 25- μ L reaction volume (Taq enzyme and 10 $\times$ buffer from Promega are recommended; use primers at 1 μ M each).
9. For primers Martn-F and Martn-ermR, the following 40-cycle program is recommended:
 - 30 seconds at 94°C
 - 30 seconds at 63°C
 - 3 minutes at 72°C
10. Analyze products obtained in *step 9* as follows: (1) load 3 μ L of the PCR reaction on a 1% agarose gel electrophoresis and insure that the size distribution of the fragments varies greatly; (2) submit PCR products for DNA sequencing using Martn-F as a primer.

BASIC PROTOCOL 5: ALLELIC REPLACEMENT USING pKOR1

Allelic replacement has been used extensively to generate mutations in chromosomal genes. A mutated allele of a target gene is cloned into a plasmid that replicates conditionally, typically at permissive temperatures, owing to a temperature-sensitive replicon. Selection for the resistance trait encoded by the plasmid under non-permissive conditions, favors plasmid integration into the chromosome via homologous recombination (Foster, 1998). Plasmid resolution is achieved by growing cells under permissive condition (Bae et al., 2004). Without markers for counterselection, allelic replacement with plasmid loss can be a very rare event that involves extensive screening and often several weeks or months of work. In 2006, Taeok Bae in our laboratory developed plasmid pKOR1, a modified pTS1 plasmid with an inducible counter-selection system (Bae et al., 2004). Following pKOR1 integration into the bacterial chromosome at non-permissive temperature (43°C), anhydrotetracycline is added to the culture to induce pKOR1-encoded *secY* antisense transcripts via the *P_{xyl}/tetO* promoter. Expression of *secY* antisense transcript is not compatible with staphylococcal growth (Ji et al., 2001), and allows for the selection of clones in which the plasmid is lost following chromosomal excision.

This protocol describes how to introduce the target DNA (gene to be disrupted) into pKOR1 and how to perform allelic replacement of target DNA.

Materials

'Gateway manual' downloaded from www.invitrogen.com (consult as needed and see additional comments in the Commentary section below)

Cloning primers

[*Note:* Assuming that the target gene is to be deleted, primers should be designed for amplification of at least 1 kbp upstream and 1 kbp downstream of the region to be omitted. The left primer of the upstream sequence and right primer of the downstream sequence should be flanked with the following *attB1* and *attB2* sequences: GGGGACAAGTTTGTACAAAAAAGCAGGCT- (*attB1*) and GGGGACCACTTTGTACAAGAAAGCTGGGT- (*attB2*). The remaining two primers should be designed in such a way that the two fragments of DNA can be ligated with the possibility of introducing a resistance marker in between if necessary. See (Bae and Schneewind, 2006) for more details]

Polymerase for PCR amplification, T4 DNA ligase (restriction enzymes as needed for digestion of PCR products prior to ligation), BP Clonase enzyme mix (Invitrogen)

30°C, 37°C and 43°C incubators

Competent cells of *E. coli* DH5α and appropriate medium for growth

Eppendorf and PCR tubes, tips and pipetman

PCR machine

TSB and TSA to grow *S. aureus*

Ampicillin and chloramphenicol antibiotics used at the final concentrations of 100 and 10 µg/mL, for selection of *E. coli* and *S. aureus* carrying pKOR1, respectively.

Sterile spreader

Agarose and supply to pour and run agarose gel and visualize DNA in gel

Preferred kit for extraction of DNA from agarose gel

Day 1:

1. Amplify target DNA upstream and downstream sequences by PCR using template DNA extracted from *S. aureus* as described in BASIC PROTOCOL 6.
2. Run PCR products on an agarose gel, visualize and cut out band containing DNA of interest; extract DNA from gel using preferred commercial kit or in-house method.
3. Ligate the two DNA inserts to fuse upstream and downstream DNA fragments with the missing intervening target gene.

Day 2:

4. Perform recombination reaction between ligated PCR products (30–300 ng DNA) and pKOR1 (200 ng) using BP clonase, buffer, incubation time and temperature *as per* manufacturer's recommendations.

Day 3:

5. Transform the clonase products from *step 4* in *E. coli* DH5α (proceed as you would with any cloning and transformation of *E. coli*).
6. Select pKOR1 clones by plating cells on agar containing ampicillin (100 µg/ml)
Note: ampicillin is used for selection in E. coli and chloramphenicol for selection of pKOR1 transformants in S. aureus.

Day 4:

7. Analyze (ie. Restriction digest, PCR products, etc) candidate colonies for recombinant pKOR1 carrying the target DNA insert (extract plasmid DNA and perform restriction digest and/or DNA sequencing to ensure the identify and integrity of the inserted fragments).

Day 5:

8. Transfer DNA to *S. aureus* following BASIC PROTOCOLS 1–2; plasmid DNA should be passaged in strain RN4220 (or preferred strain lacking a restriction modification system, $r^- m^+$ genotype) and selected by plating transformants on TSB_{CH10} at 30°C. Extract plasmid DNA from RN4220 and electroporate target strain; select transformants by plating on TSB_{CH10} at 30°C.

Day 6:

9. To select for integration of plasmid pKOR1 into the genome of target strain, pick an isolated colony from transformant plates (*step 8*) and inoculate into a 15-ml culture tube containing 3 ml TSB_{Chl10} pre-warmed at 43°C.
10. Incubate tubes at 43°C overnight with vigorous shaking to favor plasmid integration on the chromosome (selection for this integration event is pursued in steps 11 through 13).

Day 7:

11. Plate culture on TSA_{Chl10} pre-warmed at 43°C and incubate overnight at 43°C.

Day 8:

12. Pick a large colony and inoculate into a 15-ml culture tube containing 3 ml TSB_{Chl10}; incubate at 30°C overnight or until growth is observed (but no more than 36 h).

Day 9:

13. Transfer 10 µl of culture into a fresh 15-ml culture tube containing 3 ml TSB_{Chl10}; incubate at 30°C overnight or until growth is observed.
14. Use 1-ml of the step 13 culture to extract genomic DNA and assess the integration of the plasmid on the chromosome (lyse cells to extract genomic DNA as described in BASIC PROTOCOL 6 and analyze by PCR with appropriate primers, *i.e.* primers flanking the target region cloned in pKOR1).
15. If the plasmid has not integrated on the chromosome, begin the experiment over starting at step 9. When reaching step 12 inoculate the large colony into a 15-ml culture tube containing 3 ml TSB_{Chl10}; incubate at 43°C overnight as described in step 10 and proceed with the remaining steps as described.

Day 10:

16. Diluted culture from *step 13* (assuming formation of co-integrants has been achieved) with sterile water (10^4 dilution), and plate 10 and 100 µl on two TSA plates containing anhydrotetracycline at 100 ng/ml; as a control, plate the same volume of the culture on TSA plates without anhydrotetracycline.

Note: steps 16–18 are performed for the selection of bacteria where the plasmid has excised.

17. Incubate plates at 30°C

Day 11:

18. Bacteria should grow in a lawn on TSA plates whereas only 100 to 200 colonies should be observed on plates containing anhydrotetracycline. If so, pick 10 large colonies and inoculate into individual 15-ml culture tubes containing 3 ml TSB; incubate at 37°C overnight.

Day 12:

19. Remove culture aliquots from each tube and extract genomic DNA to check for replacement of target gene with the new allele using PCR and flanking primers designed outside the region used for cloning into pKOR1 (use same primers as in *step 14*).

BASIC PROTOCOL 6: PREPARING *S. aureus* LYSATES FOR EXTRACTION OF DNA

The following method describes a protocol for preparation of protoplasts of *S. aureus* using lysostaphin, an endopeptidase specific for the pentaglycine cross-bridge that links peptidoglycan strands of staphylococci. Once converted to protoplasts, staphylococci are susceptible to lysis and DNA extraction can be performed by using commercially available kits. For example, the Wizard Genomic DNA Purification Kit from Promega and QIAGEN Plasmid Kits can be used to extract genomic DNA or plasmids, respectively. DNA should be kept in water or a low salt buffer (Tris-HCl 20–50 mM pH7–8) and stored at –20°C.

Materials

Culture tubes and TSB
Centrifuge
Tubes for centrifugation
Pipettes, tips and pipetman
Lysostaphin (commercially obtained)
TSM buffer

Day 1:

1. Grow target strain for DNA extraction overnight by inoculating an isolated colony from a plate or an aliquot of a frozen stock (see Unit 9C.1 BASIC PROTOCOL 1) into a 15-ml culture tube containing 1.5 ml TSB and antibiotics as needed.

Day 2:

2. Transfer culture to an eppendorf tube and sediment bacterial cells by centrifugation at 6,000 $\times g$ for 3 min.
3. Remove supernatant.
4. Suspend cells in 100 μ L of TSM containing 2.5% (v/v) lysostaphin working solution (2 mg/mL).
5. Incubate tubes at 37°C for 15 minutes.
6. Sediment protoplasts by centrifugation at 6,000 $\times g$ for 3 min.

7. Remove supernatant carefully; the protoplasts are fragile and a bit sticky and can be lysed readily with “lysis solutions” provided in commercial kits.

BASIC PROTOCOL 7: TRANSDUCTION

Some bacteriophages (phages) package DNA exclusively based on size and digest host DNA during the infectious cycle. As a result, fragments of the genomic DNA may become encapsulated in phage particles. The resulting virus is known as a transducing particle, an infective vehicle that carries DNA and can transfer it to a recipient cell, a process known as transduction and first described by Joshua Lederberg and Norton Zinder in 1951 (Lederberg et al., 1951). Phage transduction is a rapid and useful mode of genetic transfer between bacteria and can be used in the laboratory to transfer selectable alleles between *S. aureus* isolates. Here, we describe a protocol for transducing (crossing) *bursa aurealis* alleles back into the wild type background using $\Phi 11$ or $\Phi 85$ phages. This process ensures that any phenotype observed for a given allele is truly associated with the insertional mutation. Transduction is also a fast method to combine selectable alleles in a single strain background.

Materials

Phage lysate amplified on a sensitive strain with no resistance marker (phage titer should be $\sim 10^9$ - 10^{10} plaque forming units, pfu)

The donor strain (Donor) that carries a mutation in a target gene marked with a selectable marker (such as *bursa aurealis* insertion)

A recipient strain, preferentially the parent strain used for *bursa aurealis* mutagenesis (Recipient)

TSB, TSA

CaCl₂ 5 mM

Na-Citrate 40 mM kept on ice

Culture tubes, pipettes, tips, pipetman, tubes for centrifugation

30°C, 37°C incubators

0.2 mm filter sterilizing units

15-ml screw cap plastic tubes

Centrifuge

Petri dishes

Spreader

Generate a phage lysate on the Donor strain

Day 1:

1. Grow Donor strain in TSB in the presence of CaCl₂ 5 mM overnight (this will favor phage adsorption later).

Day 2:

2. Dilute culture (1:100) in 20 ml TSB CaCl₂ 5 mM and grow until A₆₀₀ 0.4 (~ 10⁸ cells/ml).
3. Add phage stock at a multiplicity of infection of 1:1. Incubate at 37°C until lysis is observed (up to 16 h).

Day 3:

4. Transfer culture from *step 3* to a centrifuge tube and spin at 3,000 $\times g$ for 10 min; carefully transfer supernatant to a fresh tube (leave 1–2 ml behind to avoid contaminating with bacteria).

Note: this lysate contains both infective and transducing particles and should have a very high titer (~10¹⁰ pfu/ml); it can be filter sterilized using a 0.2 μ m filter-syringe and stored at 4°C for several months.

Transduction into the Recipient strain**Day 1:**

5. Start an overnight culture of the recipient strain in 2 ml TSB CaCl₂ 5 mM.

Day 2:

6. Transfer 1 ml of culture from *step 5* to a sterile eppendorf tube and add phage lysate (from *step 4*) at a multiplicity of infection of 1 phage particle to 10 bacteria. Incubate for 30 min at 37°C without shaking.
7. Spin tube at 3,000 $\times g$ for 3 min, remove supernatant completely by pipetting.
8. Suspend cells in 1 ml of 40 mM Na Citrate (chelates Ca⁺, inhibiting further phage adsorption and cell lysis) and repeat *step 7*.
9. Repeat *step 8* two more times.
10. Following the last wash in citrate buffer, suspend cell pellet in 50–100 μ l Na Citrate 40 mM and plate on TSA_{Erm10} (if selecting for *bursa aurealis*) containing 40 mM Na Citrate .
11. Incubate plates at 30°C or 37°C for up to 48 h.

Day 3 or 4:

12. Isolated colonies on plates (*step 11*) should be streaked on a new citrate containing plate (TSA_{erm10}) to eliminate residual lytic phage (repeat this step once more).
13. Candidate transductants should be verified by PCR or inverse PCR (BASIC PROTOCOL 5) and stored frozen at –80°C (Unit 9C. 1 BASIC PROTOCOL 3).

Optional: How to titer a Phage Stock

14. Grow a culture of *S. aureus* as described in *step 2* of this protocol.

15. Prepare several TSA plates without antibiotic.
16. Mix one part of melted TSA with one part warm TSB (v/v) to decrease the amount of agar by half (alternatively prepare a soft agar medium at 0.7% agarose instead of 1.5%), keep the TSA/TSB mix melted but not hot.
17. Add 0.5 ml of the culture from *step 1* to 4.5 ml of TSA/TSB medium and immediately pour over a freshly prepared TSA plate (*step 2*); let the top agar solidify.
18. Make a serial dilution of your phage lysate (10-fold) and spot 5 μ l of each dilution on the plate. Let dry and incubate upright at 37°C overnight and next day enumerate visible plaques.
19. Calculate titers by taking into account the volume plated and dilution factor from the original stock.
20. Note: this “solid” method can be used to obtain and amplify phage stocks instead of the “liquid” method described above (*steps 1–3*). Bacteriophage plaques can be excised from agar with a sterile scalpel or loop and transferred to sterile culture tube containing TSB. The agar should be chopped to small pieces and the tubes vortexed vigorously. Solid materials should be removed by centrifugation and cleared supernatants containing phage stocks should be filtered and stored as described in *step 4*.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps.

Cryopreservation solution, 1x or 2x

To prepare the 1x solution, combine 5% (w/v) mono-sodium glutamate and 5% (w/v) bovine serum albumin with water. To generate a 2x solution, combine 10% (w/v) mono-sodium glutamate and 10% (w/v) bovine serum albumin with water.

Stir gently using a magnetic bar and magnetic stirrer (avoid generating foam).

Sterilize with a 0.22- μ m filter and store for up to 1 year at 4°C.

Plate preparation—All solid media can be re-dissolved in a microwave oven. Let the medium cool before adding any antibiotics. Pour into sterile petri plates near a sterilizing flame (standard Bunsen burner), and then pass flame over the surface of agar to remove any bubbles. Replace the lid. Allow the plates to dry overnight (agar-side down) at room temperature. Re-sleeve, invert, and store up to 1 month at 4°C.

Tryptic soy medium—For solid medium, dissolve 20 g Difco tryptic soy agar (soybean-casein digest agar) (Becton Dickinson, cat. no. 236920) with 500 ml H₂O in a 1000-ml Pyrex bottle.

For liquid medium, dissolve 30 g Bacto tryptic soy broth (soybean-casein digest medium) (Becton Dickinson, cat. no. 211822) with 1,000 ml H₂O in a 1000-ml Pyrex bottle. Autoclave all media at 121°C for 15 min. Store up to 6 months at room temperature.

Lysostaphin—Prepare a stock solution at 10 mg/ml in 20mM sodium acetate, pH 4.5, and keep frozen at –80°C or at 4°C for 4 weeks. The working solution is diluted in 200 mM Tris-HCl, pH 8.0, and contains lysostaphin at a final concentration of 2 mg/mL.

TSM Buffer and ice-cold sterile 0.5 M Sucrose—TSM buffer consists of 50 mM Tris-HCl pH 7.5, 0.5 M sucrose, 10 mM MgCl₂. For a 1 L solution, weight Tris Base powder (6 g), dissolve in 0.8 L water and adjust to pH 7.5. Add sucrose (171 g) and MgCl₂ · 6H₂O (2 g) powders and adjust to the final volume.

To prepare the 0.5 M sucrose solution, dissolve 171 g sucrose in water and adjust to 1-L final volume.

Filter-sterilize TSM buffer and the 0.5 M sucrose solution using a 0.45-μm filter and store at room temperature and at 4°C, respectively for up to 1 year.

Na Citrate—Dissolve 11.8 g Citric acid trisodium salt dihydrate (HOC(COONa)(CH₂COONa)₂ · 2H₂O) in 0.9 L H₂O. Adjust volume to 1 L, filter-sterilize using a 0.45-μm filter and store at room temperature for 1 year.

COMMENTARY

Background information

Hundreds of *S. aureus* isolates have been sequenced and the data have been deposited in the freely accessible GenBank. These genomic data reveal that *S. aureus* encodes roughly 2500–2900 genes with approximately 90–95% sequence conservation. Mutant alleles can thus be crossed between strains to interrogate the universal requirement of non-essential genes for specific phenotypes. This is particularly useful to assess genetic redundancy as is often observed for virulence factors of *S. aureus*. For example, replication in specific niches may favor the expression of fibrinogen-binding factor X over fibrinogen-binding factor Y in strain A and the converse may occur in strain B. It is important to remember that allelic exchange between strains, especially when performed by transduction, is accompanied by recombination of large DNA segments resulting in mosaic rearrangements. Thus, it is important to be aware of the genome sequence surrounding target genes in both donor and recipient strains.

To fully attribute the phenotypic defect of a mutant allele Z to the cognate gene Z it is critical to perform complementation studies. This Unit provides protocols for extraction and electroporation of plasmids in *S. aureus*. Plasmids used for complementation studies are often shuttle plasmids that result from ligation of two vectors, to provide replicons and selective markers that are compatible for both *E. coli* and *S. aureus*. Typically, *E. coli* replicons of shuttle vectors have a high copy number facilitating cloning and are small in size. In our laboratory, we use plasmid pOS1 (Schneewind et al., 1992), a hybrid of *E. coli*

pUC9 (Vieira and Messing, 1982) and *S. aureus* pC194 (Novick, 1991). pC194 is a small plasmid of 2.9 kbp that carries a chloramphenicol resistance marker and is maintained at 15 copies per cell (Novick, 1991). Wade W. Williams developed pWWW412, a derivative of pOS1 that provides the promoter and ribosome-binding site of the constitutively expressed *hprK* gene (Bubeck Wardenburg et al., 2006). We recommend the use of pC194-based plasmids for complementation studies in mice as we have used derivatives of both pWWW412 and pOS1 successfully by simply adding Chloramphenicol palmitate to the drinking water of animals (Bubeck Wardenburg et al., 2006; McAdow et al., 2011). Other commonly used staphylococcal plasmids for genetic manipulations and complementation studies include plasmids pT181, pSN2 and pE194 (Novick, 1991).

Plasmids with conditional replication phenotypes have been isolated by mutagenesis and screening for temperature sensitive replication and are used for allelic replacement (Greene et al., 1995; Novick et al., 1986). For example, the shuttle plasmid pTS1 (O'Connell et al., 1993) carries a thermosensitive pE194*ts* replicon (Youngman, 1987) and chloramphenicol resistance determinant for plasmid replication and selection in *S. aureus* and is fused to *E. coli* pUC18 (Yanisch-Perron et al., 1985). The pKOR1 plasmid used for allelic replacement in our laboratory is a modified version of pTS1 that encodes the antisense *secY* RNA for counter-selection during allelic exchange and the lambda recombination cassette (Gateway Technology, Invitrogen) that permits rapid cloning of mutant alleles without the use of restriction enzymes and ligases (Hartley et al., 2000). The recombination cassette encompasses two important features, the *attP* sites and *ccdB* gene. In the presence of bacteriophage lambda integrase and integration host factor (BP clonase enzyme mix, Invitrogen), *attP* sequences recombine with *attB* sequences of DNA inserts leading to the loss of *ccdD*. In non-recombinant pKOR1 plasmids, *ccdB* is not lost and the resulting product inhibits *E. coli* gyrase (Bernard and Couturier, 1992; Miki et al., 1992).

The *bursa aurealis* transposable element developed in our laboratory was also cloned in plasmid pTS2 (Fitzgerald and Foster, 2000) that carries the temperature-sensitive replicon (pE194*ts*) and chloramphenicol resistance gene (Iordanescu, 1975; Villafane et al., 1987). *Bursa aurealis* itself is derived from the Himar 1 (mariner) transposon and encompasses short inverted repeats of the horn fly transposon (Robertson and Lampe, 1995), the *ermC* resistance marker (Trieu-Cuot et al., 1990) and the R6K replication origin (*oriV*) (the promoterless *Aequorea victoria* green fluorescent protein gene is also included in the cassette but unfortunately does not get expressed). The R6K replication origin allows rescue of transposon inserts along with the adjacent DNA fragments via cloning in *E. coli* λ pir Tn10; it is also the only *E. coli* strain that can support replication of R6K-based plasmids (Metcalf et al., 1994). Plasmid pFA545 is a derivative of vector pSPT181 (Janzon and Arvidson, 1990) and encodes the Himar 1 transposase (Lampe et al., 1996). While the original intention was to induce expression of the transposase gene, we found that the procedure worked best when the inducer was omitted. The parent vector pSPT181 is a shuttle vector with a ColE1-based replicon and ampicillin-resistance marker for replication in *E. coli* (Melton et al., 1984), and pRN8103 (Novick et al., 1986), a temperature-sensitive derivative of pT181 (Iordanescu et al., 1978) that replicates in Gram-positive bacteria and carries the tetracycline resistance marker. The complete sequences and maps of plasmids

pBursa and pFA545 can be retrieved from the databank using accession numbers AY672109.3 and AY672108, respectively.

Critical Parameters and Troubleshooting

Preparation of competent cells and electroporation: It is important that cells are still growing in the exponential phase at the time of harvest (*step 5* BASIC PROTOCOL 1) and kept on ice (*steps 7–15* BASIC PROTOCOL 1 and *steps 1–3* BASIC PROTOCOL 2). The sucrose solution used in BASIC PROTOCOL 1 should also be kept ice cold. Between 0.1 to 1 µg of plasmid DNA should be used for electroporation in a volume that does not exceed 10% of the volume of the competent cells. DNA should be concentrated if needed. Arcing (loud popping sound) during electroporation indicates the presence of too much salt in the DNA preparation or cells. Shuttle plasmids extracted from *E. coli* should first be transformed in the NCTC8325 derivative RN4220 developed for the genetic manipulation of plasmid DNA (Kreiwirth et al., 1983). Unlike clinical isolates, RN4220 can accept *E. coli* propagated plasmid DNA due to nitrosoguanidine-induced mutation(s) in its restriction-modification system (Novick, 1990), specifically the *sauI* *hsdR* gene (Waldron and Lindsay, 2006). Alternatively, plasmid DNA can be transferred directly from the *E. coli* *dcm* strain (DC10B) that has no restriction barrier (Monk et al., 2012).

Transposon mutagenesis and mapping: One of the main problems in generating a transposon-based library is a potential disproportionate amplification of cells carrying the same transposon insertion or so-called siblings. This unwanted process can be minimized by isolating mutants on solid medium as described in BASIC PROTOCOL 4. Nevertheless, the use of liquid culture remains an acceptable and a more rapid alternative.

A successful mutagenesis following *bursa aurealis* transposition should yield approximately 50 colonies per plate (transposition occurs at a frequency of $\sim 10^{-6}$). The colonies should be large with perhaps smaller, slow-growing colonies that should not be picked. When more colonies (>200) appear on a plate, the investigator should ensure that this is not due to amplification of siblings, or plasmid integration, or incomplete loss of plasmid by sampling a dozen of candidates by inverse PCR. Amplification of siblings may occur when a transposition event was selected immediately after transformation of pBursa and pFA545 at 30°C. Plasmid integration may occur by homologous recombination in some strains that share common DNA sequences with either one of the plasmids. In both cases, the investigator should repeat transformation with pBursa and pFA545. Incomplete loss of plasmids should be resolved by carefully assessing the optimal temperature for plasmid loss and strain viability. Repeated and prolonged incubations at elevated temperature are required for complete loss of both plasmids but lead to the unwanted acquisition of extragenic mutations to cope with temperature stress. This problem can be resolved by crossing *bursa aurealis* back to the wild type background by transduction.

For inverse PCR, two critical factors should be considered: (1) the choice of restriction site and enzyme used to digest genomic DNA and (2) the design of primers for amplification. We use the four-nucleotide restriction site (CCGC) recognized by the *Aci* I enzyme because the *S. aureus* genome displays a rather low GC content (32%). The closest *Aci* I site within

Bursa aurealis is positioned 256 bp away from the insertion site of the transposon, thus the expected size of digestion products will in all cases to 256 bp.

Allelic replacement using pKOR1: Plasmid pKOR1 should be propagated in the *E. coli* strain DB3.1 carrying the *gyrA462* that enables *ccdB* containing plasmid propagation (Bernard and Couturier, 1992) (DB3.1 can be obtained from Invitrogen). Recombination leading to excision of *ccdB* occurs in other backgrounds (wild type *gyrA*) preventing the use of *attB* sites for cloning. As with transposon mutagenesis, switch to high temperatures may result in acquisition of additional extragenic suppressors and target strains should be tested for viability at higher temperature. A temperature range between 37°–43°C is acceptable for loss of pKOR1 replication. Unless, the target gene is essential, the use of pKOR1 for allelic replacement should yield the expected mutant.

Transduction: Φ11, Φ80α, Φ85 are prototypical group B transducing phages of *S. aureus* with bursting size of approximately 250 plaque-forming units and low lysogenization frequency (Novick, 1967; Novick, 1991; Xia and Wolz, 2013). Most strains are lysogenic, *i.e.* they carry prophages in their genome as documented by whole genome sequence analysis. Prophages can be amplified following UV-induced excision of lysogenic strains (Miller, 1992). Φ11 for example can be amplified from strain NCTC8325 as described by Novick (Novick, 1991).

For successful transduction experiments, it is important to work with phage stocks with high titers and strictly use the multiplicity of infection (ratio of phage to bacteria) indicated in the protocol. The failure to observe transduction is primarily caused by low phage titers, intrinsic resistance of the recipient cell to a given bacteriophage or inappropriate quenching of lytic activity by the phage. Titers can be improved by generating new stock lysates or concentrating them using for example polyethyleneglycol (PEG) in a high salt solution (Miller, 1992). If the recipient cell is already a lysogene for the phage employed in the transduction experiment, it will be resistant to lysis and thus transduction. A different phage should be used. Finally, chelation with Na Citrate is critical for disruption of the lytic cycle of infecting bacteriophage. Wash steps with Na Citrate should be performed rapidly and extensively. If lysis persists (no transductants are observed), the incubation time between recipient cell and phage should be reduced.

Lysis of *S. aureus* for DNA extraction: *S. aureus* is exquisitely sensitive to lysostaphin. Any failure to observe lysis is most certainly due to an inactive stock of enzyme or overgrowth of a contaminant in the bacterial culture.

Anticipated Results and Time Considerations

Preparation of competent cells and electroporation: A successful electroporation into RN4220 using plasmid DNA extracted from *E. coli* should yield >100 colonies per plate following overnight incubation. For thermosensitive plasmids and selection at 30°C, allow longer incubation times (up to 48 h). For transformation of clinical isolates using plasmid DNA extracted from RN4220, expect 10–100 colonies. Gram-positive replicons are maintained at a significantly lower copy number per cells as compared to Gram-negative

replicons and thus, the yield of plasmid DNA extracted from *S. aureus* is always lower (10–50 times less per volume of culture). For example, a 1–3 ml *E. coli* culture carrying pOS1 yields approximately the same amount of DNA as a 20–50 ml culture of RN4220 carrying the same plasmid.

Transposon mutagenesis and mapping: As mentioned above, successful mutagenesis with *bursa aurealis* should yield approximately 50 colonies per plate following 8–12 h incubation at the non-permissive temperatures. Times for each step have been provided in the protocol. When using Martn-R for DNA sequencing of transposon candidates following inverse PCR, the investigator should expect to read a matching DNA sequence of approximately 160 bp toward the end of the transposon and terminating with the sequence CCTGTTA at the TA junction. All subsequent sequences are in the target DNA and matches can be found by searching the databank.

Allelic replacement: Mutagenesis using plasmid pKOR1 is a guaranteed success unless the target gene is essential. In this case, the limiting step would be to generate a merodiploid strain (for example with an additional copy of the wild type gene on a plasmid) prior to allele replacement. pKOR1 can also be used to replace a target promoter with an inducible promoter.

Transduction: Successful transduction of marked alleles should yield approximately 10–100 colonies per plate following 16–48 h incubation at permissive temperature.

Lysis of *S. aureus* for DNA extraction: Lysis of staphylococci using lysostaphin is achieved rapidly. Note that lysostaphin incubation in TSM buffer yields intact cells with digested murein sacculi. Replacement of the high-density sucrose-containing buffer (TSM) with a low osmolarity solution is accompanied by cell lysis. Protoplasts prepared in TSM are also useful to study biological processes such as protein secretion and trafficking (Burts et al., 2005; Schneewind et al., 1992).

ACKNOWLEDGEMENTS

The authors thank members of their laboratory for critical discussion. This work was supported by grants from the U.S. National Institute of Allergy and Infectious Diseases (NIAID), Infectious Diseases Branch (AI052474 and AI038897 to O.S. and AI075258 to D.M.). D.M. and O.S. acknowledge membership within and support from the Region V Great Lakes Regional Center of Excellence in Biodefense and Emerging Infectious Diseases Consortium (National Institutes of Health award 1-U54-AI-057153).

LITERATURE CITED

- Bae T, Banger AK, Wallace A, Glass EM, Aslund F, Schneewind O, and Missiakas DM 2004 *Staphylococcus aureus* virulence genes identified by bursa aurealis mutagenesis and nematode killing. Proc. Natl. Acad. Sci. USA 101:12312–12317. [PubMed: 15304642]
- Bae T, and Schneewind O 2006 Allelic replacement in *Staphylococcus aureus* with inducible counter-selection. Plasmid 55:58–63. [PubMed: 16051359]
- Bernard P, and Couturier M 1992 Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes. J Mol Biol 226:735–745. [PubMed: 1324324]

- Bubeck Wardenburg J, Williams WA, and Missiakas D 2006 Host defenses against *Staphylococcus aureus* infection require recognition of bacterial lipoproteins. *Proc Natl Acad Sci U S A* 103:13831–13836. [PubMed: 16954184]
- Burts ML, Williams WA, DeBord K, and Missiakas DM 2005 EsxA and EsxB are secreted by an ESAT-6-like system that is required for the pathogenesis of *Staphylococcus aureus* infections. *Proc Natl Acad Sci U S A* 102:1169–1174. [PubMed: 15657139]
- Camilli A, Portnoy A, and Youngman P 1990 Insertional mutagenesis of *Listeria monocytogenes* with a novel Tn917 derivative that allows direct cloning of DNA flanking transposon insertions. *J Bacteriol* 172:3738–3744. [PubMed: 2163385]
- Fitzgerald SN, and Foster TJ 2000 Molecular analysis of the tagF gene, encoding CDP-Glycerol:Poly(glycerophosphate) glycerophosphotransferase of *Staphylococcus epidermidis* ATCC 14990. *J Bacteriol* 182:1046–1052. [PubMed: 10648531]
- Foster TJ 1998 Molecular genetic analysis of staphylococcal virulence. *Methods Microbiol* 27:432–454.
- Giesbrecht P, Kersten T, Maidhof H, and Wecke J 1998 Staphylococcal cell wall: morphogenesis and fatal variations in the presence of penicillin. *Microbiol Mol Biol Rev* 62:1371–1414. [PubMed: 9841676]
- Greene C, McDevitt D, Francois P, Vaudaux PE, Lew DP, and Foster TJ 1995 Adhesion properties of mutants of *Staphylococcus aureus* defective in fibronectin-binding proteins and studies on the expression of fnb genes. *Mol Microbiol* 17:1143–1152. [PubMed: 8594333]
- Hartley JL, Temple GF, and Brasch MA 2000 DNA cloning using in vitro site-specific recombination. *Genome Res* 10:1788–1795. [PubMed: 11076863]
- Hartley RW, and Paddon CJ 1986 Use of plasmid pTV1 in transposon mutagenesis and gene cloning in *Bacillus amyloliquefaciens*. *Plasmid* 16:45–51. [PubMed: 3016781]
- Iordanescu S 1975 Recombinant plasmid obtained from two different, compatible staphylococcal plasmids. *J Bacteriol* 124:597–601. [PubMed: 1184573]
- Iordanescu S, Surdeanu M, Della Latta P, and Novick R 1978 Incompatibility and molecular relationships between small *Staphylococcal* plasmids carrying the same resistance marker. *Plasmid* 1:468–479. [PubMed: 748954]
- Janzon L, and Arvidson S 1990 The role of the delta-lysin gene (hld) in the regulation of virulence genes by the accessory gene regulator (agr) in *Staphylococcus aureus*. *EMBO J* 9:1391–1399. [PubMed: 2328718]
- Ji Y, Zhang B, Van SF, Horn, Warren P, Woodnutt G, Burnham MK, and Rosenberg M 2001 Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA. *Science* 293:2266–2269. [PubMed: 11567142]
- Kreiswirth BN, Lofdahl S, Betley MJ, O'Reilly M, Schlievert PM, Bergdoll MS, and Novick RP 1983 The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature* 305:709–712. [PubMed: 6226876]
- Lampe DJ, Churchill ME, and Robertson HM 1996 A purified mariner transposase is sufficient to mediate transposition in vitro. *EMBO J* 15:5470–5479. [PubMed: 8895590]
- Lederberg J, Lederberg EM, Zinder ND, and Lively ER 1951 Recombination analysis of bacterial heredity. *Cold Spring Harb Symp Quant Biol* 16:413–443. [PubMed: 14942753]
- Lowy FD 1998 *Staphylococcus aureus* infections. *New Engl. J. Med* 339:520–532. [PubMed: 9709046]
- McAdow M, Kim HK, Dedent AC, Hendrickx AP, Schneewind O, and Missiakas DM 2011 Preventing *Staphylococcus aureus* sepsis through the inhibition of its agglutination in blood. *PLoS Pathog* 7:e1002307. [PubMed: 22028651]
- Melton DA, Krieg PA, Rebagliati MR, Maniatis T, Zinn K, and Green MR 1984 Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res* 12:7035–7056. [PubMed: 6091052]
- Metcalfe WW, Jiang W, and Wanner BL 1994 Use of the rep technique for allele replacement to construct new *Escherichia coli* hosts for maintenance of R6K gamma origin plasmids at different copy numbers. *Gene* 138:1–7. [PubMed: 8125283]

- Miki T, Park JA, Nagao K, Murayama N, and Horiuchi T 1992 Control of segregation of chromosomal DNA by sex factor F in *Escherichia coli*. Mutants of DNA gyrase subunit A suppress *letD* (*ccdB*) product growth inhibition. *J Mol Biol* 225:39–52. [PubMed: 1316444]
- Miller JH 1992 A short course in bacterial genetics (New York, Cold Spring Harbor Laboratory Press).
- Missiakas DM, and Schneewind O 2013 Growth and laboratory maintenance of *Staphylococcus aureus*. *Curr Protoc Microbiol* Chapter 9:Unit 9C 1.
- Monk IR, Shah IM, Xu M, Tan MW, and Foster TJ 2012 Transforming the untransformable: application of direct transformation to manipulate genetically *Staphylococcus aureus* and *Staphylococcus epidermidis*. *MBio* 3.
- Morikawa K, Inose Y, Okamura H, Maruyama A, Hayashi H, Takeyasu K, and Ohta T 2003 A new staphylococcal sigma factor in the conserved gene cassette: functional significance and implication for the evolutionary processes. *Genes Cells* 8:699–712. [PubMed: 12875655]
- Morikawa K, Takemura AJ, Inose Y, Tsai M, Nguyen Thi le T, Ohta T, and Msadek T 2012 Expression of a cryptic secondary sigma factor gene unveils natural competence for DNA transformation in *Staphylococcus aureus*. *PLoS Pathog* 8:e1003003. [PubMed: 23133387]
- Novick R 1967 Properties of a cryptic high-frequency transducing phage in *Staphylococcus aureus*. *Virology* 33:155–166. [PubMed: 4227577]
- Novick RP (1990). The *Staphylococcus* as a molecular genetic system. In *Molecular Biology of the Staphylococci*, Novick RP, ed. (New York, VCH Publishers), pp. 1–40.
- Novick RP 1991 Genetic systems in staphylococci. *Methods Enzymol* 204:587–636. [PubMed: 1658572]
- Novick RP, Edelman I, and Lofdahl S 1986 Small *Staphylococcus aureus* plasmids are transduced as linear multimers that are formed and resolved by replicative processes. *J Mol Biol* 192:209–220. [PubMed: 2951524]
- O’Connell D, Pattee PA, and Foster TJ 1993 Sequence and mapping of the *aroA* gene of *Staphylococcus aureus* 8325–4. *J. Gen. Microbiol* 139:1449–1460. [PubMed: 8371108]
- Robertson HM, and Lampe DJ 1995 Recent horizontal transfer of a mariner transposable element among and between Diptera and Neuroptera. *Mol Biol Evol* 12:850–862. [PubMed: 7476131]
- Schneewind O, Model P, and Fischetti VA 1992 Sorting of protein A to the staphylococcal cell wall. *Cell* 70:267–281. [PubMed: 1638631]
- Shaw JH, and Clewell DB 1985 Complete nucleotide sequence of macrolide-lincosamide-streptogramin B-resistance transposon Tn917 in *Streptococcus faecalis*. *J Bacteriol* 164:782–796. [PubMed: 2997130]
- Tam C, Glass EM, Anderson DM, and Missiakas D 2006 Transposon mutagenesis of *Bacillus anthracis* strain Sterne using *Bursa aurealis*. Plasmid [Epub ahead of print].
- Tomich PK, An FY, and Clewell DB 1980 Properties of erythromycin-inducible transposon Tn917 in *Streptococcus faecalis*. *J Bacteriol* 141:1366–1374. [PubMed: 6245068]
- Trieu-Cuot P, Poyart-Salmeron C, Carlier C, and Courvalin P 1990 Nucleotide sequence of the erythromycin resistance gene of the conjugative transposon Tn1545. *Nucleic Acids Res* 18:3660. [PubMed: 2163525]
- Vieira J, and Messing J 1982 The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* 19:259–268. [PubMed: 6295879]
- Villafane R, Bechhofer DH, Narayanan CS, and Dubnau D 1987 Replication control genes of plasmid pE194. *J Bacteriol* 169:4822–4829. [PubMed: 2443486]
- Waldron DE, and Lindsay JA 2006 SauI: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into *Staphylococcus aureus* and between *S. aureus* isolates of different lineages. *J Bacteriol* 188:5578–5585. [PubMed: 16855248]
- Xia G, and Wolz C 2013 Phages of *Staphylococcus aureus* and their impact on host evolution. *Infect Genet Evol*.
- Yanisch-Perron C, Vieira J, and Messing J 1985 Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33:103–119. [PubMed: 2985470]

- Youngman P (1987). Plasmid vectors for recovering and exploiting Tn917 transpositions in *Bacillus* and other Gram-positive bacteria. In *Plasmids: a Practical Approach*, Hardy KG, ed. (Oxford, IRL Press), pp. 79–103.
- Youngman PJ, Perkins JB, and Losick R 1983 Genetic transposition and insertional mutagenesis in *Bacillus subtilis* with *Streptococcus faecalis* transposon Tn917. *Proc Natl Acad Sci U S A* 80:2305–2309. [PubMed: 6300908]

Figure 1. Map of transposon *bursa aurealis* and plasmids used for delivery along with summary of steps involved in the mapping of insertion sites by inverse PCR.

(A) *Bursa aurealis* (3.2 kbp), a mini-mariner transposable element, was cloned into pTS2, with a temperature-sensitive plasmid replicon (*repts*) and chloramphenicol resistance gene *cat* to generate pBursa (7,383 bp). *Bursa aurealis* encompasses mariner terminal inverted repeats (TIR), R6K replication origin (*oriV*) for replication in *E. coli*, and erythromycin-resistance determinant *ermC*, an rRNA methylase that allows selection in both *E. coli* and *S. aureus*. The position of the most terminal site for the restriction enzyme *Acil* is indicated as well as the site of hybridization and nucleotide sequence of primer Martn-F (F). Plasmid pFA545 (10,079 bp) encodes the mariner transposase *tnp* and is a derivative of pSPT181, a shuttle vector consisting of pSP64 with ampicillin resistance (*bla*) for replication and selection in *E. coli*, and pRN8103, a temperature-sensitive derivative of pT181 (*repts*) and tetracycline-resistance marker (*tetB tetD*). The presence of *repts* and *tetBD* allows for replication of pFA545 in *S. aureus* and other Gram-positive bacteria.

(B) Mapping insertion sites by inverse PCR. Genome DNA from a candidate mutant strain is isolated and digested with *Acil*. Next, fragment self-ligation and inverse PCR are performed using DNA ligase and primers Martn-F (F) and Martn-ermR (R). PCR products are subjected to DNA sequence analysis using primer Martn-F (F).