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Jeffrey L. Bose *Editor*

The Genetic Manipulation of Staphylococci

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Preface

The ability to genetically manipulate pathogenic bacteria is essential to understanding their physiology, responses to environmental changes, and virulence. Genetic manipulation can come in many forms, including both directed and unbiased approaches. Historically, clinical isolates of *Staphylococcus* have posed a challenge for researchers to study due to their inability to acquire and maintain plasmids generated in other bacteria such as *E. coli*. This barrier involves bacteria-specific DNA recognition systems designed to prevent the inclusion of foreign DNA, a topic directly addressed in the Restriction Modifications Systems chapter and highlighted in many other chapters within this book. While these systems are potent barriers to genetic manipulation of the staphylococci, our increased understanding of these systems has greatly enhanced our ability to move plasmids into these bacteria. The second hurdle to overcome involves ways in which DNA is transferred into *Staphylococcus* cells. These bacteria are not naturally competent, nor can competence be induced. In addition, while possible, conjugation is rarely used for small plasmids. Furthermore, unlike *E. coli*, staphylococci cannot be transformed using a simple heat-shock method. To get plasmids into staphylococci, electrocompetent cells must be made. Often this is accomplished using the *S. aureus* laboratory isolate RN4220, originally created by Dr. Richard Novick's research group, a mutated strain that readily accepts DNA from *E. coli*. Once in RN4220, plasmids are often shuttled into strains of interest using bacteriophage, which can effectively be used to transfer both plasmids and chromosomal fragments between strains. This book contains review chapters and methods with vital information to accomplish all of these tasks.

Generating mutants of *Staphylococcus* can be accomplished in many ways, depending on the intended changes. To aid researchers, this book contains chapters using unbiased approaches such as chemical and transposon mutagenesis, as well as a protocol for allelic exchange to make targeted mutants. Using these methods, both our lab and others have been successful at making mutants that span individual single nucleotide changes in the chromosome to whole genome mutant libraries. The latter strategy provides a useful tool for high-throughput screening, while single nucleotide changes are an elegant way of teasing apart the importance of single nucleotides in gene expression or creating specific amino acid substitutions to examine protein function.

To complement the chapters directed toward actual genetic manipulation, this book includes additional chapters for nucleic acid analysis. qRT-PCR is a sensitive tool that can be used to examine specific gene expression changes, while newly developed RNAseq techniques provide a powerful means to examine the entire transcriptome. Both of these tools are essential to understanding gene expression and can serve as a beginning to direct mutant making or as a means to test the consequences of the generated mutant. Furthermore, the included chapters on RACE and EMSAs provide useful methods for mapping RNA and detecting interactions between DNA and proteins, respectively.

Genetic manipulation is often seen as a daunting task to those unfamiliar with the topic. Indeed, not too long ago our current capability to manipulate and study *Staphylococcus* was merely science fiction. We are indebted to those individuals and research groups who pioneered the methods/tools covered in this book. The goal for this book is to serve as a resource and guide to scientists in the staphylococcal community as they pursue their studies on these bacteria. While most protocols within this book are written with a particular species in mind, these methods should be adaptable to other species.

Kansas City, KS, USA ***Jeffrey L. Bose***

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Understanding Staphylococcal Nomenclature

Christina N. Krute and Jeffrey L. Bose

Abstract

Bacteria are often grouped by a variety of properties, including biochemical activity, appearance, and more recently, nucleic acid sequence differences. In the case of human pathogens, significant work goes into "typing" strains to understand relatedness. This is especially true when trying to understand the epidemiology of these organisms. In attempts to group Staphylococci, a variety of methods and nomenclatures have been employed, which can often serve as a point of confusion to those entering the field. Therefore, the intent of this chapter is to give a brief overview of some common methods and associated nomenclature used to type Staphylococci, with *S. aureus* as an example.

Keywords: Spa typing, MLST, PFGE, SCC*mec*, MLVA, *Staphylococcus* typing

1 Introduction

The genus *Staphylococcus* was originally grouped with *Micrococcus*, and was first named in 1883 by Alexander Ogston during a microscopic examination of pus associated with micrococcus poisoning. In his examination, Ogston described the existence of two distinct cell types, chained cocci and those that grouped similar to a bunch of grapes, which he termed *Staphylococcus* (Greek σταφυλή (staphulé), a bunch of grapes) [1]. The following year, Anton J. Rosenbach isolated and named two *Staphylococcus* species, *S. aureus* (gold) and *S. albus* (white), now called *S. epidermidis*, based on their pigmentation [2].

The genus *Staphylococcus* acquired its name due to its appearance as grape-like clusters, resulting from cell division occurring along two axes. As with most bacteria, the Staphylococci were originally (and still are) grouped biochemically. Generally speaking, but with exception, the genus is metabolically diverse and are catalase-positive, oxidase-negative facultative anaerobes. *Staphylococcus* cells are relatively small (0.5–1.0 μm in diameter) gram-positive cocci with low-GC-content (~30–40 % DNA) genomes ranging from 2.3 to 3.2 Mb. This wide range in possible genome size is due to complex diversity in mobile genetic elements, including transposons, bacteriophages, and antibiotic resistance cassettes, among differing Staphylococci strains [3].

There are currently greater than 40 recognized species of *Staphylococcus* and several subspecies that generally reside as normal flora of mammals and birds (reviewed in [4]). Despite this ubiquity, the most well-known species are *S. epidermidis* and *S. aureus* due to their importance in human disease. Because of their significance in human health, much work has gone into classifying or typing strains. Two common, yet not completely accurate, ways of discerning between these two species are the production of coagulase, a secreted protein which binds to host prothrombin [5], or staphyloxanthin, a membrane-associated carotenoid [6]. It is generally regarded that *S. aureus* is coagulase-positive, while non-*aureus* Staphylococci lack coagulase and are therefore categorically called coagulase-negative staphylococci or CoNS. While this is usually true, some *S. aureus* isolates do not produce coagulase and some other species have been shown to make this enzyme. Similarly, *S. aureus* is often singled out by its golden pigment, the result of staphyloxanthin production. However *S. aureus* isolates make greatly varying amounts of pigment, and often pigmentation is only obvious after prolonged growth.

In addition to the above analyses, there is a dizzying array of methods to type *Staphylococcus* isolates. The following sections are intended to serve as a brief introduction to orientate readers to some of the common nomenclature used, with *S. aureus* (the most complex) serving as an example. *S. aureus* isolates have come to be grouped by a variety of designations with no single typing method universally used. For example, the important clinical isolate FPR3757 is a CA-MRSA USA300 ST8 belonging to clonal complex CC8 with spa type t008 and SCCmec IV.

2 Antibiotic Resistance Status

As is the case with many human pathogens, the emergence or identification of antibiotic-resistant *S. aureus* generally appears in a short period of time following initial clinical or agricultural use of a particular antibiotic. This is not surprising since bacteria are efficient at horizontal gene transfer that allows for the acquisition of antibiotic resistance genes. In addition, while antibiotics are new to humans, bacteria have been engaging in this form of chemical warfare for millennia. The first major delineation of *S. aureus* strain designation based on an antibiotic resistance phenotype was in response to the antibiotic methicillin. Today, if no other characteristic is reported on an *S. aureus* isolate it will certainly be determined to be methicillin-resistant *S. aureus* (MRSA) or methicillin-susceptible *S. aureus* (MSSA). Of note, MRSA is often mistakenly called "multiple resistant." The antibiotic methicillin was first introduced in 1959 and just 2 years later, in 1961, the first clinical MRSA isolate was identified from a hospital [7]. It was

later found that methicillin resistance is due to the acquisition of the *mecA* gene, encoding the alternative penicillin-binding protein PBP2a. In addition, PBP2a confers resistance to other β-lactam antibiotics. Since its first emergence, MRSA has become a significant global health problem.

In response to the emergence and spread of MRSA, vancomycin was fast-tracked through the Food and Drug Administration in 1958, and became the drug of choice for serious and confirmed MRSA infections. In 1997, the second major antibiotic resistance designation for *S. aureus* emerged with the report of vancomycin-intermediate resistant *S. aureus* (VISA) strain Mu50 in Japan [8]. VISA strains are moderately resistant to vancomycin, which is due not to the acquisition of new genes, but instead correlates with mutations in existing genes that are often involved in affecting cell wall metabolism [9–11]. These mutants typically have a thicker cell wall and more D-Ala-D-Ala vancomycin targets, potentially diluting out the vancomycin to sub-inhibitory concentrations. It was not until 2002 that the first vancomycin-resistant *S. aureus* (VRSA) strain was isolated by the Michigan State Department of Health [12]. This strain had an MIC = 1024 μg/ml resulting from the acquisition of the *vanA* gene found on Tn*1546* from a vancomycin-resistant Enterococci during a coinfection. In 2006, the Clinical and Laboratory Standards Institute updated the vancomycin susceptibility breakpoint as ≤2 μg/ml for vancomycin-susceptible *S. aureus* (VSSA), 4–8 μg/ml for VISA, and ≥16 μg/ml for VRSA [13].

3 Acquisition of Infection

In addition to antibiotic resistance status, pathogenic Staphylococci are often designated based on the source of the infection. Historically, Staphylococci species, including *S. aureus*, were considered to be opportunistic pathogens, primarily associated with infections of immunocompromised individuals or those who undergo surgical procedures in hospitals. These isolates are often called hospital acquired or healthcare associated (HA). In contrast, in the early 1990s, strains emerged that cause outbreaks in the community, independent of hospital association. These community-associated (CA) *S. aureus* strains are easily transmissible, more readily lead to dangerous invasive diseases, and can infect otherwise healthy individuals. These infections are especially prevalent in military personnel, athletes, students, children, and prison inmates as they are in close contact with one another [14–17]. This increasing trend of CA-MRSA infections in otherwise healthy individuals leads to questioning of the use of the term "opportunist pathogen" for *S. aureus*. In addition, some epidemic CA-MRSA strains have spread quickly and account for many hospital-acquired infections, and as such are beginning to displace HA-MRSA in clinical settings [18].

In addition to HA-MRSA and CA-MRSA designations, it is not uncommon to find the LA-MRSA abbreviation in the literature, wherein LA refers to livestock-associated strains. This has been heightened by reports of human infections by LA-MRSA, which in some communities can account for 15 % of all deep skin and soft tissue infections [19]. LA-MRSA infections typically correlate with direct contact with animals; however, recent reports suggest that humans residing close to farms, but without direct contact, can become colonized as well. In addition, LA-MRSA strains have been found in raw meat [20] and milk [21], which could be contributing factors to the spread of these strains to humans.

4 PFGE

Pulsed-field gel electrophoresis (PFGE) is a highly discriminatory way to type *Staphylococcus* strains. This technique (see chapter of this book) relies on digesting genomic DNA with the infrequently cutting endonuclease SmaI and subsequent separation by agarose electrophoresis. Unlike conventional electrophoresis, the alternating voltage of PFGE allows for separation and resolution of large DNA fragments. Separation of fragments results in a unique banding pattern that can be used to identify *Staphylococcus* lineages when compared to known strains. To be consistent, a strict protocol is necessary in order to compare between laboratories. This technique has been well adopted in the USA at the national level and results in the reporting of a USA type, such as USA300.

5 Spa Locus

While PFGE has been used extensively for typing *S. aureus* strains, particularly for epidemiological studies of outbreaks, it does have limitations. Comparing DNA patterns by gel electrophoresis is complicated if one does not have the appropriate reference strains available. To counter this, single-locus sequencing of the *spa* gene was proposed in 1996 [22]. Spa typing involves sequencing the 3′ X-region of the *spa* gene, which contains a variable number of tandem repeat sequences. Upon sequencing, repeats are assigned a numerical code based on the order of the repeats. Spa typing is curated by SeqNet.org [23] via the *spa* server (www.spaserver.ridom.de), which harbors a large database (as of writing, 14,883 Spa types from thousands of strains and 57 countries). Sequencing of this single locus provides good discriminatory power at an affordable cost and reasonable workload when typing multiple strains.

6 MLST

Multilocus sequence typing (MLST) was first suggested for *S. aureus* as a means to more discriminately differentiate between epidemic methicillin-resistant *Staphylococcus aureus* (EMRSA) and virulent MSSA strains [24]. The technique involves sequencing 402–516 base pairs of seven housekeeping genes, *arc* (carbamate kinase), *aroE* (shikimate dehydrogenase), *glp* (glycerol kinase), *gmk* (guanylate kinase), *pta* (phosphate acetyltransferase), *tpi* (triose-phosphate isomerase), and *yqiL* (acetyl coenzyme A acetyltransferase). The resulting sequences are then deposited into the Multi Locus Sequence Typing home page (www.mlst.net). By comparing the sequences, isolates are assigned a sequence type (ST). Furthermore, STs that share 6/7 alleles can be assigned to clonal complexes (CC) using eBURST (eburst.mlst.net) [25]. For consistency, the MLST home page provides details on isolating DNA, primer sequences, and PCR conditions.

Much like *S. aureus*, *S. epidermidis* is most often typed by either PFGE or MLST. In this case, MLST sequencing is performed for the following genes: *arcC* (carbamate kinase), *aroE* (shikimate dehydrogenase), *gtr* (an ABC transporter), *mutS* (DNA repair protein), *pyrR* (regulator of pyrimidine operon), *tpiA* (triosephosphate isomerase), and *yqiL* (acetyl coenzyme A acetyltransferase) [26]. The resulting data is imported into the MLST home page (sepidermidis.mlst.net) and results in the assignment of an ST type.

7 SCC*mec*

The *mecA* gene is found on the mobile genetic island staphylococcal cassette chromosome *mec*, SCC*mec*. While the origins of SCC*mec* are unclear, it is believed to have originated in a non-*aureus Staphylococcus* species and then was acquired by *S. aureus* via horizontal gene transfer. Sequencing of the SCC*mec* led to the observation that this genetic island is highly variable between strains, and thus could serve as means of typing SCC*mec*-positive strains. To date, there are 11 recognized SCC*mec* types containing multiple subtypes based on several criteria. Due to the overall complex nature of SCC*mec* typing, it will not be covered in detail here since the International Working Group on the Staphylococcal Cassette Chromosome elements website (www.sccmec.org) provides a written and visual overview of SCC*mec* typing with strain examples.

8 MLVA

Multilocus variable-number tandem repeat analysis seeks to provide good discriminatory power in a high-throughput and low-cost method. This typing method utilizes genomic regions containing natural variable repeats. The repeat regions are amplified by multiplex PCR using primers targeting *sdrCDE*, *clfA*, *clfB*, *sspA*, *spa*, *mecA*, and *fnBP*in *S. aureus* [27, 28]. For *S. epidermidis*, primers amplify regions of *sdrG*, *sdrH*, SERP0719, SERP2395, *aap*, and *mecA* [29, 30]. Following amplification, the products are separated by gel electrophoresis. Much like PFGE, the resulting banding pattern is indicative of strain relatedness. One advantage to this method is the ability for automation, making it attractive to clinical epidemiology.

9 LOOKING FORWARD

As is the case with most techniques, each typing method has advantages and disadvantages including time, cost, and feasibility. In addition, depending on ones' need, only a certain level of identity or phylogeny may be desired or needed. For a particular study or epidemiology analysis, it may not be necessary to have some or all of these typing details. Moving forward, those studies needing extremely detailed analysis will likely take advantage of the decreasing costs of whole-genome sequencing. Interestingly, a recent study of whole-genome sequences revealed that *S. aureus* contains 47 mutational hotspots consisting of ~2.5 % of the genome [31]. Sequencing of these hotspots showed good resolution of strains, but, not surprisingly, was not as strong as sequencing of the whole genome. Of note, while cost of whole-genome sequencing has rapidly decreased in recent years, perhaps the limiting factor of performing such detailed study is the bioinformatics time and computational power needed to handle a whole-genome sequencing project.

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Restriction–Modification Systems as a Barrier for Genetic Manipulation of *Staphylococcus aureus*

Marat R. Sadykov

Abstract

Genetic manipulation is a powerful approach to study fundamental aspects of bacterial physiology, metabolism, and pathogenesis. Most *Staphylococcus aureus* strains are remarkably difficult to genetically manipulate as they possess strong host defense mechanisms that protect bacteria from cellular invasion by foreign DNA. In *S. aureus* these bacterial "immunity" mechanisms against invading genomes are mainly associated with restriction–modification systems. To date, prokaryotic restriction–modification systems are classified into four different types (Type I–IV), all of which have been found in the sequenced *S. aureus* genomes. This chapter describes the roles, classification, mechanisms of action of different types of restriction–modification systems and the recent advances in the biology of restriction and modification in *S. aureus*.

Keywords: *Staphylococcus aureus*, RM systems, REase, MTase, REBASE, Genetic manipulation

1 Introduction

Staphylococcus aureus is a major human pathogen and a leading cause of both nosocomial and community-associated infections worldwide (1). Functional genomics and genetic manipulation of *S. aureus* are essential approaches for studying the fundamental principles of staphylococcal physiology, virulence, and pathogenesis, and for the discovery and development of new strategies to combat these multidrug-resistant bacteria. The majority of clinical isolates of *S. aureus* are notoriously difficult to manipulate genetically as they possess strong host defense mechanisms limiting horizontal gene transfer (HGT). In relationship to the genetic manipulation of staphylococci, this translates to a difficulty of transferring recombinant DNA from other bacteria like *Escherichia coli* or ligation products directly into the target cells. In *S. aureus* the defense mechanisms protecting bacteria from invader DNA are mainly associated with different types of restriction–modification (RM) systems. Understanding the biology of restriction and modification in various clinical isolates of *S. aureus* is critical for overcoming strain-specific RM barriers for DNA uptake and for developing better genetic engineering tools and techniques.

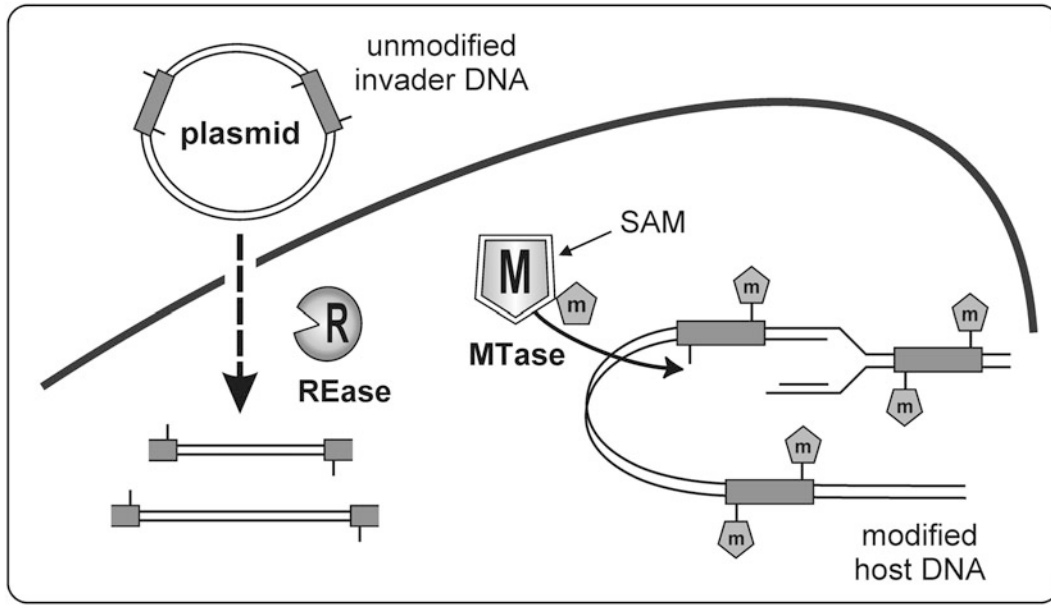

Fig. 1 The host defense function of restriction–modification systems. RM systems recognize the methylation status of DNA at their specific target sequence. Fully methylated DNA is recognized to be a part of the host genome and hemimethylated DNA is recognized as newly replicated host DNA. The methylation status of the genomic DNA is maintained by the methyltransferase (MTase). Incoming unmethylated DNA such as that of plasmid for example is recognized to be foreign and cleaved by the restriction endonuclease (REase)

Bacterial RM systems typically consist of enzymes responsible for two opposing activities and may function as primitive prokaryotic immune systems that attack foreign DNA entering the cell (2). Restriction activity is based on the action of a restriction endonuclease (REase), while modification activity is carried out by a site-specific DNA methyltransferase (MTase). Classical REses recognize foreign DNA such as that of bacteriophages, plasmids, and other mobile genetic elements by the absence of proper methylation within specific recognition sites and then inactivate invader genomes by endonucleolytic cleavage (Fig. 1) (2–4). Their cognate site-specific MTases protect DNA from cleavage by methylating cytosine or adenine bases within the same recognition sequence (Fig. 1) (2, 3). Generally, the hemimethylated state of DNA at the recognition sites confers protection to the cleavage, and host DNA is usually fully methylated by a resident MTase following replication while invading foreign DNA is not (Fig. 1). RM systems are widely spread among prokaryotic organisms, and different bacterial species and strains have their own combinations of restriction and modification enzymes. In addition, a single prokaryotic organism often retains multiple RM systems (http://rebase.neb.com/rebase/rebase.html).

The individual high specificity and the prevalence of RM enzymes in prokaryotic organisms as well as the often occurrence of tight linkage between cognate restriction and modification genes suggest their diverse biological functions. In addition to cellular defense and immigration control of DNA in bacteria (5), several important biological roles have been attributed to RM systems so far (see recent review (6)). Among others, these roles include the functioning of RM systems as selfish mobile genetic elements (7–9), stabilization of genomic islands and other mobile elements in the cell population (6, 10–13), involvement in recombination and genome rearrangements (14–19), and implications on instability and evolution of prokaryotic genomes (6, 20–23).

2 Types of RM Systems

Restriction–modification systems are currently classified into four types based on their subunit composition, cofactor requirements, target recognition, and DNA cleavage mechanism (24).

2.1 Type I Systems

Type I RM systems are hetero-oligomeric complexes that are composed of three different types of subunits (S, M, and R), which are encoded by the *hsdS* (*hsd* for *h*ost *s*pecificity *d*eterminant), *hsdM*, and *hsdR* genes (Table 1). The specificity subunit (S) is required for the recognition of a specific DNA sequence, the MTase subunit (M) catalyzes DNA methylation and the REase subunit (R) is essential for DNA cleavage (25, 26). Type I RM systems exist as two functional complexes: a trimer of 1S and 2M subunits acts solely as a methyltransferase and a pentamer of 1S, 2M, and 2R subunits that exhibits both methyltransferase and endonuclease activities (27–29). DNA sequence specificity of Type I RM systems is determined by two target recognition domains (TRDs) in the S subunit. Each of the two TRDs recognizes one half of a bipartite target sequence separated by a gap. For example, the sequence recognized by the EcoKI is AAC(N6)GTGC where N = any nucleotide (25, 30). Depending upon the methylation status of DNA at the recognition sequences, Type I restriction–modification enzymes have two modes of action. If DNA is fully methylated, the enzyme complex does not bind to the recognition sequence and DNA is immune to restriction (31). When the target site is hemimethylated, the methyltransferase complex catalyzes modification of the other strand at the N6 position of adenine within the recognition sequence, using S-adenosyl methionine (SAM) as a methyl group donor (32). When DNA is unmethylated the restriction enzyme complex binds to the recognition sequence and translocates DNA bidirectionally through the bound complex in a reaction coupled to ATP hydrolysis (33–36). The restriction reaction requires ATP, SAM (with the exception for R.EcoR124I) and

Table 1
Characteristics and organization of the genes and subunits of the four types of RM systems

	Type I	Type II	Type III	Type IV
Features	Hetero-oligomeric REase and MTase complex	Separate REase and MTase or REase-MTase in one polypeptide (e.g., subtypes IIC, IIG)	Hetero-oligomeric REase and MTase complex	Methylation-dependent REase
Example	EcoKI	EcoRI	EcoP1I	EcoKMcrBC
Genes	*hsdM*, *hsdR*, *hsdS*	*ecoRIR*, *ecoRIM*	*mod*, *res*	*mcrBC*
Subunits	3 different subunits (R, M, and S)	2 different subunits (R and M)	2 different subunits (Mod and Res)	2 different subunits (McrB and McrC)
Enzyme activities	MTase: S + 2M (± 2R) REase: S + 2M + 2R ATPase (required for restriction)	Orthodox MTase: M Orthodox REase: 2R	MTase: 2M (± 2R) REase: 1 or 2R + 2M ATPase (required for restriction)	REase: varies GTPase or ATPase (required for restriction)
Cofactors	ATP, SAM, Mg^{2+}	SAM, Mg^{2+}	ATP, SAM, Mg^{2+}	GTP (ATP, dATP), Mg^{2+}
Recognition sequence	Asymmetric and bipartite; e.g., EcoKI, 5′AAC(N_6)GTGC[a]	Symmetric and asymmetric (methylated for subtype IIM); e.g., EcoRI, 5′GAATTC	Asymmetric; e.g., EcoP1I, 5′AGACC	Modified; Bipartite and methylated; e.g., EcoKMcrBC, 5′RmC($N_{30–3000}$)RmC[a]
Cleavage site	Variable locations, often far (1,000 bp) from recognition site	Fixed location within or close to recognition site	Fixed location 24–28 bp from recognition site	Variable locations from modified (e.g., m6A, m4C, m5C, hm5C, ghm5C) recognition site
DNA translocation	Yes	No	Yes	Yes
Example in *S. aureus*	Sau1 (79)	Sau3AI (68)	SauKLT6ORF138600P (REBASE) (82);	SauUSI (83, 77)
Occurrence in *S. aureus*[b]	72	42	2	112

[a]N = any nucleotide; R = A or G
[b]Current number of *S. aureus* strains containing particular type of RM system among 125 isolates where RM systems have been identified

Mg^{2+} ions, and occurs at a site that is distant from the recognition sequence (25, 30, 37–41). Cleavage is triggered by collision and stalling of two restriction enzyme complexes during translocation along a DNA chain or by stalling of a single enzyme on a single-site circular substrate following DNA translocation (24, 30, 34, 42). Based on genetic complementation and biochemical studies, Type I RM systems are currently divided into five different subclasses (IA–IE) (24, 43, 44). Type I RM systems are present in the vast majority of sequenced bacterial genomes and nearly 6,000 putative Type I RM systems have been identified so far (http://rebase.neb.com/rebase/statlist.html).

2.2 Type II Systems

Type II RM systems represent the most abundant group of characterized RM enzymes that are classified into 11 overlapping subtypes (http://rebase.neb.com/cgi-bin/sublist) (24). The majority, but not all, of Type II RM systems consist of separate REase and MTase proteins that recognize the same, often palindromic (4–8 base pairs), DNA sequence (Table 1) (24). The MTases share several conserved amino acid motifs and generally act as monomers to modify a specific base at their recognition sequences. Type II MTases catalyze modification of cytosine at either the N4 or the C5 position, and methylation of adenine at the N6 position using SAM as a methyl group donor (41). The REase proteins consist of a distinct and dissimilar amino acid sequences and usually act independently of their cognate MTases (4, 24). Type II REases typically require Mg^{2+} ions as a cofactor and can act as monomers, dimers, or even tetramers (4). Type II REases cleave both DNA strands at fixed positions either within or close (generally within 20 bp) to the recognition site generating 5′-phosphates and 3′-hydroxyls (4). Because of their ability to cleave DNA molecules at precise positions, Type II REases are widely used as tools in recombinant DNA technology. Type II REases encompass many well-known restriction endonucleases such as BamHI, EcoRI, and HindIII. Nearly 4,000 Type II restriction enzymes have been discovered so far, and many of them are available commercially (45) (http://rebase.neb.com/rebase/statlist.html).

2.3 Type III Systems

Type III RM systems are hetero-oligomeric complexes composed of Mod (for *mod*ification) and Res (for *res*triction) protein subunits encoded by *mod* and *res* genes, respectively (Table 1) (24, 26). The Mod subunit is required for substrate recognition and DNA methylation and the Res subunit is essential for DNA cleavage. The Mod subunit can methylate the recognition site independently (as a dimer Mod_2) or in complex with the restriction endonuclease subunit Res (as an oligomer Mod_2Res_1 or Mod_2Res_2) (46, 47). Type III MTases require SAM as a methyl group donor and catalyze methylation of only one strand of an unmodified 5–6 bp asymmetric recognition sequence at the N6 position of adenine to produce a

hemimethylated DNA molecule (24, 47). This methylation is sufficient for protection of host DNA since only completely unmethylated DNA can be cut by Type III REases. Restriction activity requires both the Mod and Res subunits (Mod_2Res_1 or Mod_2Res_2), Mg^{2+} ions as a cofactor and has an absolute requirement for ATP hydrolysis (24, 46, 47). In most cases DNA cleavage requires interaction of Type III RM enzymes with two separate inversely oriented asymmetric recognition sequences in the same DNA molecule and is preceded by ATP hydrolysis, which is required for the long-distance communication between the recognition sites (46–49). DNA cleavage is occurred at a defined location that is 24–28 bp downstream of one of the recognition sites (47). The exact cleavage mechanism is not yet clear, and different models (e.g., the collision cleavage model and the random walk sliding model) have been proposed to explain how the long-distance interaction between the two recognition sites takes place (26, 47, 49–54). The most studied examples of Type III enzymes are EcoP1I and EcoP15I (47). To date, over 2,000 putative Type III RM systems have been identified throughout sequenced bacterial genomes suggesting that these systems are widely spread in these organisms (47)(http://rebase.neb.com/rebase/statlist.html).

2.4 Type IV Systems

Type IV systems are modification-dependent REases that cleave DNA substrates only when their recognition sites at the N4 or the C5 positions of cytosine or at the N6 position of adenine have been modified, e.g., methylated (m), hydroxymethylated (hm), or glucosyl-hydroxymethylated (ghm) (Table 1) (24). Type IV REases recognize modified DNA with low sequence specificity, and their recognition sites have usually not been well defined (24, 55). Type IV enzymes currently comprise a highly diverse family, and only a few of them have been characterized in any detail (44). The best studied example within this group is EcoKMcrBC (55–58), which is composed of two subunits, McrB and McrC. The McrB subunit is responsible for specific DNA binding and GTP hydrolysis, whereas the McrC harbors the catalytic center for DNA cleavage (55, 58–60). EcoKMcrBC recognizes two copies of a dinucleotide sequence, consisting of a purine followed by a modified cytosine at either the N4 or the C5 position, which are separated by anywhere from 30 to 3,000 bases (2, 24, 55, 61). The recognition sites may be on either DNA strand and thus do not need to be in a particular orientation (57, 62). Like Type I RM systems, the EcoKMcrBC endonuclease translocates DNA and remains bound to its recognition sites (63). DNA translocation and cleavage by the EcoKMcrBC enzyme requires GTP hydrolysis in the presence of Mg^{2+} ions (57, 59, 63). DNA cleavage results in a double-strand break and preferentially takes place ~30–35 bp away from one of the modified RmC sites (R = A or G) (55, 62, 64). Cleavage requires cooperation of two sites and takes place when translocation

is blocked by collision of translocating complexes or by topological barriers such as nucleoprotein complexes for example (55, 61–63). Cleavage will also occur when recognition elements are located on opposite sides of a DNA replication fork (65). Type IV modification-dependent restriction endonucleases provide powerful tools to determine the methylation status of CG islands and to study epigenetic mechanisms in mammals and plants (55, 66, 67). To date, over 5,000 putative Type IV enzymes have been identified among sequenced prokaryotic genomes (http://rebase.neb.com/rebase/statlist.html).

3 RM Systems in *S. aureus*

RM systems are widely spread in *S. aureus*. To date, all four types of RM systems have been identified throughout 125 whole and partly sequenced *S. aureus* isolates (http://tools.neb.com/~vincze/genomes/enz_count.php). Some of these RM systems have been characterized in detail (22, 68–77). It is noteworthy that the majority of *S. aureus* isolates contain more than one RM system (http://tools.neb.com/~vincze/genomes/enz_count.php). Whole genome sequencing of 48 *S. aureus* strains revealed simultaneous presence of at least two RM systems of different types in their genomes with the most frequent co-occurrence of Type I and Type IV systems (http://tools.neb.com/~vincze/genomes/index.php?page=S).

Type I RM systems represent one of the most abundant groups of RM enzymes in *S. aureus*. Currently Type I RM systems have been found in all 48 completed *S. aureus* genomes (http://tools.neb.com/~vincze/genomes/index.php?page=S) and in the majority (72 out of 125) of the *S. aureus* isolates where RM enzymes have been identified (http://tools.neb.com/~vincze/genomes/enz_count.php) (Table 1). Many of the *S. aureus* strains, e.g., LGA251, M013, and MSSA476, contain more than one Type I RM system in their genomes (78) (http://tools.neb.com/~vincze/genomes/index.php?page=S). The most frequent Type I RM system found in *S. aureus* was given the generic name of Sau1 (22, 79). Among 48 *S. aureus* isolates with completely sequenced genomes 40 strains contain Sau1 system (http://tools.neb.com/~vincze/genomes/index.php?page=S). The Sau1 system is unusual as it consists of a single *hsdR* gene and two copies of both *hsdM* and *hsdS* genes (10, 22, 79). The two sets of *hsdMS* genes of the Sau1 RM system are lying within two different genomic islands and they are distantly located on the chromosome from each other and from the *hsdR* gene (10, 79). The R and M subunits of the Sau1 RM system exhibit homology to the R and M subunits of the EcoR124I system which belongs to the Type IC family (10, 22). However, the levels of identity between them were considered not

being sufficient enough for the members of the same Type IC group and it was suggested that Sau1 RM systems might form their own family (22). The Sau1 system is tightly associated with the lineages or clonal complexes (CC) into which *S. aureus* strains are divided (22, 79). The HsdR and HsdM proteins of the Sau1 system are nearly identical between different CC groups, while the HsdS proteins are highly conserved among strains of the same clonal complex and differ substantially between lineages (79, 80). Also, two copies of Sau1 *hsdS* genes within the same genome usually have different sequences that allow each lineage to recognize two different DNA target sites (22). The lineage specific sequence variation of the HsdS proteins is localized to the two TRD domains that flanked by highly conserved amino acid sequences (22). Recently the specific DNA target sites recognized by the Sau1 RM system have been identified for the dominant MRSA lineages (CC1, CC5, CC8, and ST239) (22). Interestingly, the *hsdS* genes of CC8 are identical to those of ST239 and these two lineages share one *hsdS* sequence with CC1 isolates, and the other with CC5 (22, 80).

Type II RM systems are less abundant among *S. aureus* isolates. To date, genes encoding Type II RM systems and/or Type II orphan DNA methyltransferases have been identified in 42 out of 125 *S. aureus* isolates with partly sequenced genomes and in 27 out of 48 completed *S. aureus* genomes (Table 1) (http://tools.neb.com/~vincze/genomes/enz_count.php; http://tools.neb.com/~vincze/genomes/index.php?page=S). Interestingly, several of those strains, e.g., NRS100, M1, and COL, carry inactive Type IIG RM enzymes which contain frameshift mutations within their genes. The most studied Type II RM systems in *S. aureus* are Sau3AI and Sau96I. Sau3AI REase belongs to the subtypes E, P (http://rebase.neb.com/cgi-bin/sublist) and cleaves both DNA strands of the unmodified palindromic GATC sequence just 5′ to the guanine nucleotides (68). Its cognate MTase, M.Sau3AI, protects DNA from cleavage by methylating the recognition site at the C5 position of cytosine (71). Sau96I REase belongs to the subtype P (http://rebase.neb.com/cgi-bin/sublist) and cleaves both DNA strands of the unmodified palindromic GGNCC sequences between two guanine nucleotides, while its cognate MTase, M.Sau96I, protects these sites by methylating the internal cytosine at the C5 position (69, 72).

Type III RM systems appear to be extremely rare in *S. aureus*. To date, genes encoding putative Res and Mod subunits of Type III RM systems have only been identified in two *S. aureus* isolates, i.e., KLT6 and 118 (Table 1) (81, 82) (http://tools.neb.com/~vincze/genomes/enz_count.php). None of the *S. aureus* Type III RM systems have been characterized yet.

Type IV modification-dependent REases represent another most abundant group of RM enzymes in *S. aureus*. Currently Type IV

systems have been found in 44 out of 48 *S. aureus* isolates with fully sequenced genomes and in 112 out of 125 strains where RM enzymes have been identified (http://tools.neb.com/~vincze/genomes/enz_count.php; http://tools.neb.com/~vincze/genomes/index.php?page=S) (Table 1). Approximately one third of those strains carry more than one Type IV REse in their genomes. The only well-characterized Type IV system in *S. aureus* is SauUSI (77, 83). SauUSI REase is a multi-domain protein that contains a PLD-family endonuclease catalytic site at the N-terminus, an ATPase and DNA helicase motifs in the middle part of the protein, and a presumed m5C/hm5C TRD domain located at the C-terminus (77). SauUSI REase predominantly cleaves modified DNA containing methylated and/or hydroxymethylated cytosines at the C5 position (77). The preferred recognition sites for SauUSI are Sm5CNGS (where S = C or G) (77). Similar to McrBC, efficient cleavage by SauUSI requires interaction of the enzyme with more than one recognition site (77). DNA cleavage usually takes place ~2–18 bp away from one of the modified recognition sequences (77). The endonuclease activity requires ATP or dATP hydrolysis in the presence of divalent cations such as Mg^{2+}, Mn^{2+}, Ca^{2+}, or Co^{2+} (77). SauUSI REase is highly conserved and widely spread among *S. aureus* isolates, BLASTp analysis of SauUSI to *S. aureus* proteins revealed presence of the SauUSI homologs with at least 97 % of amino acids identity in 40 different *S. aureus* isolates. Interestingly, some of the *S. aureus* isolates, e.g., N315, Mu3, and Mu50, contain a nonsense mutation in the middle of the *sauUSI* gene (83).

4 Overcoming Restriction Barriers in *S. aureus*

The big question, as it pertains to this book, is what does all of these mean in the ability to genetically manipulate *Staphylococcus* and how does one circumvent such a barrier? As previously stated, RM enzymes are abundant in *S. aureus* and most of the *S. aureus* isolates contain multiple RM systems of different types within their genomes. Consequently, the number and diversity of the RM systems within particular *S. aureus* strains will have a large impact on the choice of DNA transfer approach and the transformation success rate. The common strategies to overcome restriction barriers in bacteria include deletion of the restriction sites from recombinant DNA, the usage of helper plasmids that carry specific or multiple MTases, in vitro site-specific methylation by cell-free extracts or by specific MTases, propagation of plasmids in methylation deficient *E. coli* strains and construction of mutant strains lacking REases (83–93). The last approach was successfully applied for genetic manipulation of staphylococci over 30 years ago by generation (via UV and nitrosoguanidine mutagenesis of 8325-4 strain) of the restriction-deficient but modification-proficient strain

designated as RN4220 (94). Since its construction, this strain has been one of the most extensively used in staphylococcal studies because of its ability to accept and modify DNA from *E. coli* cells that allows its application as an intermediate cloning strain (85). In spite of that, the use of RN4220 as an intermediate also has its limitations as it provides an efficient DNA transfer only into a limited number of closely related strains (CC8 lineage), whereas transfer of DNA into many clinical isolates of *S. aureus*, such as UAMS-1 (CC30) for example, still remains quite a difficult task (80, 95). Whole-genome sequencing of RN4220 revealed a multitude of single nucleotide polymorphisms (SNPs) by which it differs from the parental 8325 strain, and a nonsense mutation within the *hsdR* gene of lineage-specific Sau1 Type I RM system was one of them (96, 97). In support of the importance of the Sau1 system as one of the major restriction barriers limiting HGT in *S. aureus*, complementation of RN4220 strain with full-length *sau1hsdR* gene prevented transformation of plasmids from *E. coli*, inhibited transduction with phages isolated from distant lineages, and reduced conjugative transfer from enterococci (79). Later experiments, however, showed that inactivation of the *sau1hsdR* gene in three different *S. aureus* strains (8325-4, SH100, and COL) was not sufficient to make them readily transformable, suggesting the presence of an additional restriction barrier in *S. aureus* (98). This second major barrier preventing transformation of plasmids from *E. coli*, as it has been recently demonstrated, is based on the presence of a Type IV modification-dependent REase, SauUSI (77, 83). Importantly, in addition to the mutation within the *sau1hsdR* gene, RN4220 strain also contains premature stop codon in the *sauUSI* gene which explains its efficient transformation by plasmids propagated in *E. coli* background (96). Moreover, restoration of the wild type allele of the *sauUSI* gene in RN4220 significantly reduced transformation efficiency of this strain (99). As it was noted above, SauUSI REase recognizes and cleaves modified DNA containing methylated cytosines at the C5 position (77). Thus, propagation of the plasmids in bacteria lacking cytosine methylation will facilitate DNA transfer into *S. aureus* isolates containing SauUSI REase. Correspondingly, for overcoming Type IV barrier in *S. aureus* isolates, an improved transformation protocol that includes propagation of plasmids in a DNA cytosine methyltransferase mutant (*dcm*$^-$) of the high-efficiency *E. coli* cloning strain DH10B designated as DC10B has been recently developed (99).

As it is clear now, the major restriction barriers limiting DNA transfer in *S. aureus* isolates are based on the presence of two different types of RM systems, i.e., a lineage-specific Type I RM system Sau1 and Type IV modification-dependent REase SauUSI. A lineage-specific Sau1 RM system restricts HGT between different clonal complexes of *S. aureus* and limits transfer of unmodified recombinant DNA, while SauUSI REase confines transfer of modified recombinant DNA from *E. coli* and other bacteria and might

limit HGT from certain *S. aureus* isolates, containing Sau96I Type II RM system for example. The Sau1 and SauUSI RM systems are the most abundant in *S. aureus* and often simultaneously occur within the same isolates. The ability of these RM systems to coexist within the same cell is based on the differences in the recognition of the site specific methylation patterns, i.e., Sau1 systems methylate its recognition sites at the N6 position of adenine while SauUSI cleaves DNA with methylated cytosines at the C5 position. Consequently, inactivation of the *sau1hsdR* gene and propagation of the recombinant DNA in the m5C modification deficient background will be often sufficient for the successful DNA transfer into *S. aureus* isolates containing these two RM systems.

5 Outlook and Concluding Remarks

RM systems are a very diverse group of enzymes that are widespread among *S. aureus* isolates. The presence of various and often multiple restriction–modification systems in most of the *S. aureus* isolates creates strong strain-specific barriers that limit functional genomic analyses of this bacterium. For a long time a confined HGT between different clonal complexes of *S. aureus* and a difficulty of transferring recombinant DNA from other bacteria into most of the *S. aureus* isolates had restricted genetic manipulation of this bacterium to a limited number of strains. Major advances in understanding the biology of staphylococcal restriction and modification have occurred in post-genomic era with characterization of a linage-specific Type I RM system (Sau1) and a type IV modification-dependent REase (SauUSI), which were shown to be dominant barriers limiting foreign DNA uptake by *S. aureus*. Construction of the SauI restriction deficient mutants in different *S. aureus* lineages and usage of *dcm* negative *E. coli* strains for plasmids propagation will facilitate HGT between different clonal complexes as well as DNA transfer into many previously untransformable strains. This in turn will allow rapid and more sophisticated genetic manipulation of clinically relevant *S. aureus* isolates, which will significantly improve our knowledge of this medically important pathogen.

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Complementation Plasmids, Inducible Gene-Expression Systems, and Reporters for Staphylococci

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Abstract

A cornucopia of methods and molecular tools is available for genetic modification of staphylococci, as shown for at least ten different species to date (Prax et al. Microbiology 159:421–435, 2013). This chapter reviews a number of frequently used vectors for complementation purposes that usually replicate in *E. coli* and staphylococci and differ in parameters including copy number, mode of replication, and sequence length. Systems for the artificial control of gene expression are described that are modulated by low-molecular-weight effectors such as metal cations, carbohydrates, and antibiotics. Finally, the usefulness of reporter proteins that exhibit enzymatic or autofluorescent characteristics in staphylococci is highlighted.

Keywords: *Staphylococcus aureus*, Staphylococci, Reporter genes, Plasmids, Complementation, Inducible gene regulation

1 A Brief Overview of Frequently Used Complementation Plasmids

Most plasmids exploited for *Staphylococcus* genetics are derived from the naturally occurring vectors pC194, pE194, pT181, and pUB110 that are replicated by the rolling circle mechanism, or pI258 and pSK1, copied by the theta-mode. Key plasmids applicable in both *Staphylococcus* and *Escherichia coli* are described in the following, overviews covering also other well-established *Staphylococcus* vectors have been provided before (1–3). Early on, the ori (origin of replication) of small rolling circle plasmids, such as pUB110, was employed for hybrid plasmid vectors (4, 5). An enhanced segregational and structural stability has been attributed to derivatives of plasmid pSK1 (6, 7). A popular series of -shuttle-vectors is represented by the pCN series, which is designed in a modular fashion (8). This allows for choosing between a number of sequences for plasmid replication (based upon wild-type or modified pT181-ori or pI258-ori), antibiotic selection and maintenance in *E. coli*. These vectors vary in copy numbers, temperature sensitivity, replication, and ability to integrate into defined chromosomal loci.

2 Systems for Artificial Gene Regulation

A number of systems for artificial transcriptional induction have been adapted for use in staphylococci (Table 1). More than 2 decades ago, promoters from plasmid pI258 have been used to induce gene expression in *S. aureus* (8–10). These are inducible by β-lactam compounds, arsenite or Cd^{2+}, the latter of which serves as an inducer in some of the aforementioned pCN vectors. Carbohydrate responsive systems are continuously being applied in staphylococci. A xylose inducible promoter of *S. xylosus* is found on a set of vectors termed pCX, pKX, and pTX, which mostly differ in resistance markers, plasmid backbone, copy number, and regulation capacities (11–14). Notably, induction by xylose (e.g., 0.5 % w/v) can be counteracted by repression by glucose (e.g., 0.5 % w/v) due to carbon catabolite repression (15, 16). Inducible gene expression in the staphylococcal vector pPSHG3 is based upon the transcriptional repressor GalR of *S. carnosus*. An *S. carnosus* Δ*galRKET* strain, incapable of metabolizing galactose, aids in high-level production of heterologous cytoplasmic or secreted proteins (17). The P_{spac} hybrid promoter that is repressed by the *lac* operon regulator LacI and induced by IPTG is also functional in staphylococci (18). In terms of high-level induction, P_{spac} is outperformed by another artificial hybrid promoter known as $P_{xyl/tet}$ (19, 20). The original $P_{xyl/tet}$ represents the *B. subtilis* P_{xylA} promoter containing a *tet* operator (*tetO*) sequence between the −35 and the −10 elements. *tetO* is the cognate DNA binding site of the dimeric tetracycline (Tc) repressor TetR that is applied in dozens of bacterial species to control inducible gene expression (21). Anhydro-Tc (ATc), a potent effector of TetR, is commonly supplemented at a final concentration of 0.4 μM (approximately 200 ng/ml) to ensure complete induction, whereas lower amounts of ATc (e.g., 100 ng/ml) may be sufficient, particularly, when growth inhibition should be avoided. Notably, the $P_{xyl/tet}$ system can be regulated under in vivo condition, as reflected by experiments with *S. aureus*-infected mice that can be fed with Tc-supplemented drinking water (22). The *tet*-regulatory system has also been used in single copy level integrated into the chromosome or to express antisense RNA fragments (22–24). Popular plasmids exploiting *tet*-dependent target gene expression are pALC2073 (25), pRMC2 (26), and pRAB11 (27), which differ in the number and position of *tetO* sequences within the *tet*-control region. $P_{xyl/tet}$ variants harboring one to four mutations are generally less active in both the induced and the repressed state (27, 28). Also the reverse TetR (revTetR) system was applied in *S. aureus* (29). There, two amino acid mutations within each TetR monomer change ATc into a corepressor (30, 31). This reversed allostery can be exploited to achieve a rapid shutdown of the promoter upon interaction of ATc with revTetR.

Table 1
Inducible gene expression systems

Target promoter	Regulator	Effector(s)	Origins of genetic components	Remarks	Reference[a]
P-*blaZ*	BlaI	Carboxyphenylbenzoyl-aminopenicillanic acid	*Staphylococcus* plasmids pI524 and pI258	–	(10)
P_{cad}	CadC	Cd^{2+}	*Staphylococcus* plasmid pI258	–	(9)
P_{ars}	ArsR	Arsenite	*Staphylococcus* plasmid pI258	–	(9)
P_{xylA}	XylR	Xylose, glucose	*S. xylosus*	Induced by xylose, repressed by glucose	(52)
P_{galKET}	GalR	Galactose	*S. carnosus*	Improved efficacy in *S. carnosus* Δ*galRKET*	(17)
P_{spac}	LacI	IPTG	*E. coli*, hybrid promoter	–	(53)
$P_{xyl/tet}$	TetR, revTetR	Anhydrotetracycline (ATc), TetR-inducing-peptide (Tip)	Gram-negative Tc resistance determinants, hybrid or synthetic (rev)TetR variants, hybrid promoters	TetR: induction with ATc or Tip ("Tet-ON"), revTetR: corepression with ATc ("Tet-OFF")	(22, 54)
Pro3	C1 repressor	Thermal shift, 31–42 °C	Bacteriophage P1, synthetic promoters	Repressed at 31 °C, induced at 42 °C	(32)
P_{T7}	T7 RNAP	–[b]	Bacteriophage T7	–	(55)

[a]Reference in regard to first published application in *Staphylococcus*
[b]Transcriptional induction of the T7 RNA polymerase (RNAP) encoding gene by IPTG

In contrast to systems requiring chemical induction, triggering the bacteriophage P1 temperature-sensitive C1 repressor based induction system is achieved by a thermal shift. A promoter equipped with two C1 binding sites can be repressed at 31 °C and induced at 42 °C (32). Also the popular T7 induction system which uses a bacteriophage's RNA polymerase has been established in *S. aureus* (33). The polymerase-encoding gene was integrated into the chromosome together with a P*spac* promoter and a constitutively expressed *lacI* gene which encodes the Lac repressor to control P*spac*.

3 Reporter Systems

Reporters are useful to track and/or quantify the activity of genes or proteins of interest via transcriptional or translational fusions. Reporter genes used since the dawn of *Staphylococcus* genetics include *xylE* of *Pseudomonas putida*, *lip* of *S. hyicus*, *cat* of plasmid pC194, *blaZ* of pI258, and *lacZ* of *E. coli* (Table 2). Due to their ease of monitoring, proteins or enzymes with fluorescent or luminescent properties are frequently applied nowadays. Whereas the *Photinus pyralis* firefly luciferase (*luc*) and the bacterial *luxAB*-encoded luciferase require *n*-decyl aldehydes, the inclusion of *luxCDE* genes eliminates the need for exogenous substrate for the LuxAB luciferase reporter in *Staphylococcus* (9, 34–38). The *lux* system is suitable to trace an *S. aureus* infection in mice due to the infective strain's bioluminescence (39). Exploiting cofactor- and substrate-independent fluorescent proteins drastically improves the spatial resolution capacity of a reporter system. The green-fluorescent protein (GFP) is a well-approved reporter in bacteria to study promoter activity or protein localization (40) and in staphylococci mutant versions such as GFP_{UV}, GFP3, or Gfpmut3.1 have been employed (41–44). Fluorescence microscopy facilitates locating single GFP-tagged cytoplasmic proteins (45) and during infection, fluorescent protein tagged bacteria can be traced subcutaneously in mice (46). A number of new fluorescent reporters with different emission wavelengths (mCherry, YFP, GFP, CFP (40, 45)) and excitation maxima (GFP_{UVR} (47)) found their way into staphylococci. In some cases, these genes or proteins are optimized in codon usage, are activated by light of a certain wavelength (48, 49) or exhibit different folding characteristics (50, 51). In order to facilitate time-resolved expression studies, GFPmut3 derivatives with short peptide tags prone to proteolytic degradation decrease protein stability reflected by weaker fluorescence (43).

Table 2
Reporters

Gene(s)	Function	Substrate	Origin	Remarks[a]	Reference[b]
xylE	Catechol 2,3-dioxygenase	Catechol	*P. putida*	Generally requires cell disruption	(56)
lip	Lipase	Tributyrin or tween	*S. hyicus*	–	(14)
blaZ	β-Lactamase	Nitrocefin	*Staphylococcus* plasmid pI258	–	(57)
cat	Chloramphenicol acetyl transferase	Chloramphenicol	*Staphylococcus* plasmid pUB112	Requires cell disruption	(58)
lacZ	β-Galactosidase	X-Gal	*E. coli*	–	(59)
bgaB	β-Galactosidase	X-Gal	*B. stearothermophilus*	Used for qualitative assays only	(60)
luc	Luciferase	Luciferin	*Photinus pyralis*	–	(36)
luxAB/luxABCDE	Luciferase	*n*-Decyl aldehydes/–	*Vibrio harveyi* or *Photorhabdus luminescens*	No exogenous substrate required, when *luxABCDE* genes used	(9, 38)
gfp (variants)	Auto-fluorescent protein	–	*Aequorea victoria* and synthetic variants	Numerous derivatives with altered properties, substrate independent	(41)

[a]All listed reporters allow for a direct or coupled photometric readout in a mostly quantitative fashion (exceptions marked)
[b]Reference in regard to first published application in *Staphylococcus*

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De Novo Assembly of Plasmids Using Yeast Recombinational Cloning

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Abstract

Molecular cloning is a cornerstone of modern biology laboratories. However, traditional cloning can be time-consuming and problematic. We outline herein a method that utilizes the endogenous gap repair system of yeast cells to clone and assemble DNA constructs. This system is simple, cheap, and requires minimal reagents. It can be used for the assembly of both simple (single DNA fragments) and complex (multiple DNA fragments) constructs into plasmids.

Keywords: Yeast, Molecular cloning, *Staphylococcus*, Homologous recombination, Genetics, Molecular biology

1 Introduction

Molecular cloning serves as a crucial fulcrum in multiple scientific pursuits. However, cloning, and especially restriction enzyme-dependent cloning, can at times be problematic and time-consuming. Not surprisingly, multiple techniques have focused on improving cloning processes to increase efficiency. Gap repair cloning using *Saccharomyces cerevisiae* is among the more widely used techniques (also referred to as yeast recombinational cloning; YRC) [1–5]. This method utilizes the ability of yeast cells to take up and recombine DNA fragments, thereby bypassing the need for vector ligation [1, 4–7]. In recent years the introduction of YRC has made a significant impact in the fields of bacterial genetics and molecular biology, including its use in the assembly of the first synthetic genome.

YRC technology is based upon mechanistic studies showing that when linear DNA fragments containing homologous ends are transformed into yeast cells the DNA fragments are united by homologous recombination in vivo [5]. Thus, de novo assembly of plasmids containing DNA inserts of choice is readily achieved using YRC. Traditionally, a linearized vector containing both a selectable marker and an origin of replication for *S. cerevisiae* is cotransformed into yeast with a DNA sequence(s) of interest [1, 3]. Homology to the

linearized vector is incorporated into the DNA sequence to be cloned using a PCR-mediated reaction. Traditionally, ~50 base pairs of homology were required to promote efficient YRC; however, studies found that 20–30 base pairs of homology are sufficient to facilitate YRC [1].

Alongside collaborators, we recently designed and created a "yeast cloning cassette (YCC)" [6]. The YCC contains the *ura3* gene and 2 μm origin of replication facilitating plasmid selection and maintenance, respectively, in *S. cerevisiae*. The utility of our approach is highlighted in the fact that it allows the cloning of DNA fragments into any plasmid of choice (with the sole exception of plasmids native to *S. cerevisiae*) [6]. In our study, we demonstrated that recombinational cloning, using YCC, allows one to clone DNA fragments into any vector that can replicate in *Escherichia coli* (AGAP cloning). Therefore, the YCC can be incorporated into any *Staphylococcus aureus/E. coli* shuttle vector. The YCC can be cloned in tandem with the DNA fragments of interest or at a site elsewhere in the vector. For the purposes of this protocol we outline a procedure for cloning a gene of interest in the commonly used *S. aureus* single-integration vector pLL39.

2 Materials

1. Reagents that we commonly use for YRC include: Yeast extract; Peptone; Dextrose; Agar; Yeast Nitrogen Base; Lithium acetate; Polyethylene glycol (molecular weight ~3350); Salmon sperm DNA; Tryptic soy broth; Oligonucleotides; dNTPs; Restriction enzymes; DNA polymerase; *Escherichia coli* cloning strain; *S. cerevisiae* strain FY2 (*ura3* minus strain); Tris-buffered phenol/chlorophorm; DMSO; Glass beads (212–300 μm); Sodium acetate; Ethanol and Agarose.

   Unless otherwise noted, all buffers are prepared with deionized/distilled water and all media is prepared with distilled water and sterilized by autoclaving. Unless otherwise mentioned all buffers and media are routinely stored at room temperature and stabilities at this temperature are mentioned below.
2. DNA analysis software design software: We use DNAstar Lasergene 11, but theoretically any word processing software will suffice.
3. YPD media: 1 % yeast extract, 2 % peptone, 2 % dextrose. Take 500 mL of distilled water in a beaker and add 5 g yeast extract, 10 g peptone and 10 g dextrose. Stable at room temperature (at least 1–2 years).
4. YMD media: 0.7 % yeast nitrogen base, 2 % dextrose. Take 500 mL of distilled water in a beaker and add 3.5 g of yeast

nitrogen base and 10 g of dextrose. Stable at room temperature (at least 1–2 years).

5. TSB media: 30 g of TSB powder is suspended in 1 L of H_2O.
6. Solid media: solid media was prepared by supplementing liquid media with 1.5 % agar.
7. Transformation solution: 100 mM lithium acetate and 40 % (w/v) PEG 3350. Weigh 4 g of PEG 3350 and transfer into a 15 mL sterile, disposable centrifuge tube. Add sterile distilled water to ~5 mL mark. Vortex thoroughly. Incubate at 37 °C for ~20 min to solubilize the PEG. Vortex the solution to ensure particulates are dissolved. Bring the volume of the solution up to 9 mL using sterile H_2O. Now add 1 mL of 1 M lithium acetate. Thoroughly vortex the solution. Use 600 μL of this solution for each individual transformation reaction.

   Note: Make the transformation solution immediately prior to performing the transformation reaction.
8. Salmon sperm DNA: 50 μL of a 2 mg/mL stock of salmon sperm DNA is required for each individual transformation reaction. This results in a final concentration of ~100 μg of carrier DNA per transformation reaction.
9. Lysis buffer: 2 % Triton X-100, 1 % sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA.
10. Sodium acetate: 3 M sodium acetate pH, 5.2.
11. TE buffer: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.

3 Methods

The workflow for creating a plasmid that can replicate in *S. aureus* using YRC is outlined in Fig. 1.

3.1 Preparation of DNA Fragments for Transformation

1. In silico assembly of the plasmid. An example of de novo plasmid assembly is illustrated in Fig. 2. In this example, the YCC and one DNA fragment of interest is cloned into the pLL39 vector, which is commonly used for genetic complementation of phenotypes in *S. aureus* [8]. As shown in Fig. 2, the fragments to be joined using YRC are: (a) linearized pLL39 plasmid, (b) the YCC, and (c) the DNA fragment of interest.
2. Primer design. To facilitate recombinational cloning, the YCC and DNA fragment(s) of interest are accorded homology to each other using PCR. Oligonucleotides are designed such that they (a) include 30 base pairs of homology to the adjoining fragment (indicated by white boxes in Fig. 2), and (b) contain a priming sequence to amplify the DNA fragments of interest using PCR. The priming sequence is typically another ~29 base

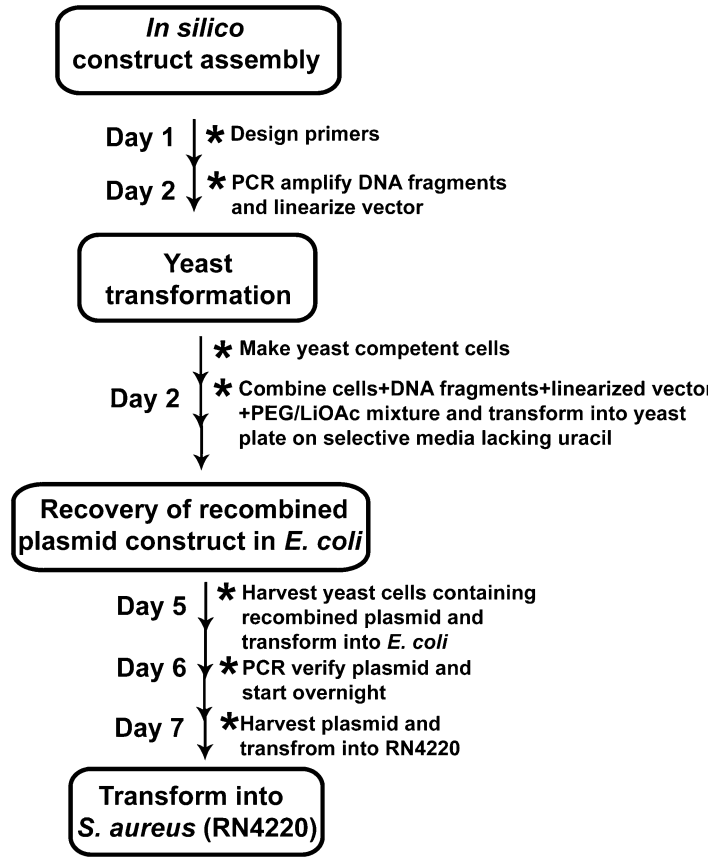

Fig. 1 Workflow for creating a plasmid that can replicate in *S. aureus* using *Y*east *R*ecombinational *C*loning (YCR). First (day 1 and 2), the desired de novo plasmid is assembled in silico, primers are designed to incorporate regions of homology between adjoining fragments, PCR amplicons are obtained, and the vector serving as backbone is linearized by restriction enzyme treatment. Second (day 2), the linearized vector and PCR amplicons are combined and transformed into freshly prepared yeast competent cells. Third (day 5–7), the de novo plasmid is recovered from yeast cells, transformed into *E. coli*, PCR-verified, passaged, and recovered from *E. coli* cells and subsequently transformed into *S. aureus* cells

pairs. Thus, in the illustrated example the forward and reverse primers to amplify the YCC will be as follows: (a) Forward primer—at its 5′ end will contain 30 base pairs of homology to the linearized vector while the 3′ end will contain 30 base pairs of sequence used to amplify the YCC; (b) Reverse primer—at its 5′ end will contain 30 base pairs of homology to the DNA of interest and on the 3′ end will contain 30 base pairs used to amplify the YCC. A PCR reaction, amplifying the YCC, using these forward and reverse primers would then incorporate the YCC with homology on its 5′ end with the linearized vector and on its 3′ end with the DNA sequence of interest (*see* **Note 1**).

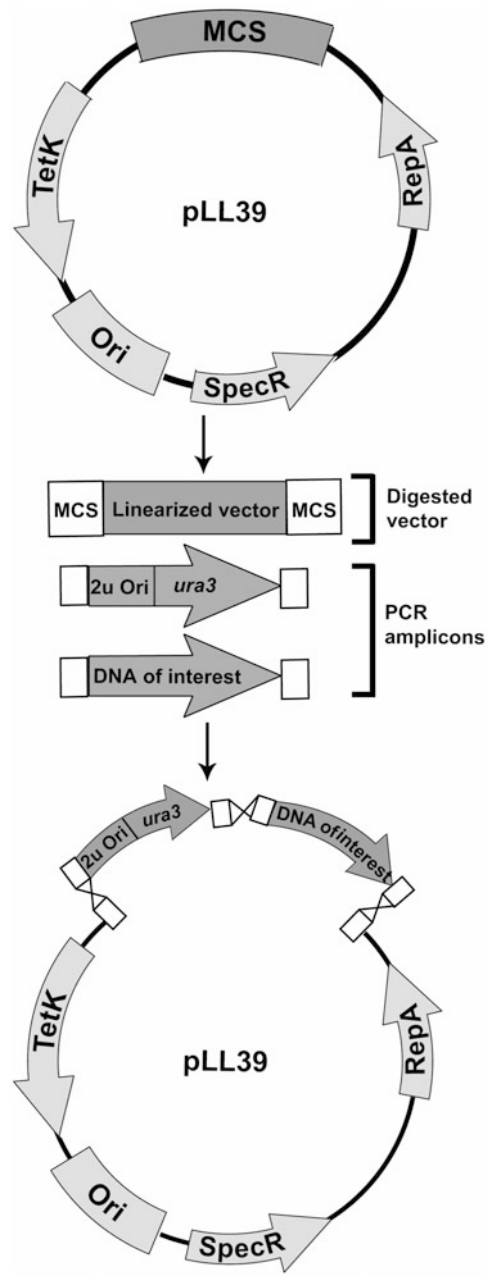

Fig. 2 Schematic representation of de novo plasmid assembly using YCR. The desired starting vector (in this example pLL39) is digested with two separate restriction endonucleases. The digested pLL39 vector is mixed with the PCR amplified fragments including the gene of interest and the yeast cloning cassette (YCC). The fragments are then transformed into yeast for homologous recombination-mediated assembly and subsequent recovery of the de novo plasmid

3. PCR amplification of fragments. Use the oligonucleotides designed in Section 3.1, step 2 to PCR amplify the DNA fragments of interest. 50 μL PCR reactions are recommended. Post amplification, it is necessary to verify that each PCR reaction successfully amplified the DNA fragments of interest. Run 10 μL of each PCR reaction on a 1 % agarose gel along with the appropriate control DNA ladder. Estimate the concentration of the amplicons by comparing to the ladder (*see* **Note 2**).
4. Linearize the vector. The vector is linearized using the desired restriction enzymes. After digestion, the restriction enzymes should be heat-inactivated (*see* **Note 3**).
5. Prepare DNA fragments for transformation into *S. cerevisiae*. Mix ~1000 μg of each DNA fragment and linearized vector in a 1.5-mL microcentrifuge tube. The final volume of ~100 μL is optimal. Bring to final volume using sterile water. The mixture can be frozen or stored at 4 °C until time for transformation.

3.2 Preparation of Transformation-Competent S. cerevisiae Cells

Unless otherwise mentioned all steps are to be performed at room temperature.

1. Recover *S. cerevisiae*. Streak *S. cerevisiae* strain FY2 (*ura3* minus) from a freezer stock onto a YPD agar plate and incubate the plate at 30 °C.
2. Grow an overnight culture by inoculating a single colony of *S. cerevisiae* into 10 mL of YPD media in a shake flask with baffles to improve aeration. Incubate the flask overnight, with shaking, at 30 °C (~15–18 h) (stationary phase >1.5 optical density A_{600}).
3. Generate competent cells. Measure the optical density of the overnight culture and use the culture to inoculate 50 mL of fresh YPD media to a final optical density (A_{600}) of 0.2 (*see* **Note 4**). Grow the cells with shaking at 30 °C for ~3–5 h. When the culture optical density (A_{600}) reaches 0.6–0.8 (~1.5–2 doublings) harvest the cells.
4. Harvest *S. cerevisiae* cells. Transfer cells into a 50 mL conical tube. Pellet cells by centrifugation at 1500 × *g* for 5 min. Discard the supernatant and resuspend the cells in 35 mL of sterile water. Pellet cells by centrifugation. Resuspend the cell pellet in 300 μL of 100 mM lithium acetate (*see* **Note 5**).

3.3 Transformation of DNA Fragments into S. cerevisiae

1. Prepare salmon sperm DNA. Heat salmon sperm DNA at 90 °C for 5 min. Cool the salmon sperm DNA on ice for 5 min. The concentration should be 2 mg/mL and you will need 50 μL per transformation. *This step should be performed* immediately prior to transformation.

2. Prepare samples for *S. cerevisiae* transformation. To the 1.5 mL microcentrifuge tubes containing DNAs from Section 3.1, step 5 add: (a) 50 μL from a 2 mg/mL stock of salmon sperm DNA from Section 3.3, step 1, (b) 600 μL of freshly prepared PEG/lithium acetate mixture, and (c) 22 μL of the yeast cells from Section 3.2, step 4. The total volume should be ~750–800 μL (*see* **Note 6**).
3. Transform *S. cerevisiae*. Incubate cells/DNA mixture from Section 3.3, step 2 at 30 °C for 30 min. We have found that best results occur when a thermomixer is used and the samples are mixed at 300 rpm but, static incubation in a 30 °C water bath will also suffice. Remove samples from 30 °C incubation and rapidly add 70 μL of 100 % DMSO to each transformation reaction. Mix by inverting four to five times. *Do not vortex.* Incubate the samples under *static* conditions at 42 °C for 30 min. Remove your samples from the 42 °C incubation and *immediately* place them on ice for 90 s.
4. Harvest transformed *S. cerevsiae*. Centrifuge the transformation reactions at 17,000 × *g* for 1 min to pellet cells. Discard the supernatant and resuspend the samples in 500 μL of sterile water. *Do not vortex.* Resuspend cells by pipetting. Centrifuge at 16,200 × *g* in a microcentrifuge for 1 min. Discard the supernatant and resuspend the samples in 150 μL water.
5. Recover transformed *S. cerevsiae*. Plate the entire 150 μL of cells onto YMD agar plates. Incubate the samples at 30 °C for 3 days. Single colonies will be visible by 1.5–2 days post transformation, but the cells are incubated for at least 3 days to allow enough biomass to accumulate, facilitating plasmid recovery.

3.4 Recovery of Recombined Plasmid from E. coli

1. Recovery of the assembled plasmid from *S. cerevisiae*. After 3 days incubation colonies should be visible (usually 1000+ colonies) and ready to harvest. Add 1.5 mL of sterile water onto each transformation plate and scrape colonies off the agar plate using a glass microscope slide. Use a pipette to transfer the cells into a 1.5 mL microcentrifuge tube. Centrifuge the samples at 16,200 × *g* for 15 s and discard the supernatant.
2. Lysis of *S. cerevisiae* cells. Add 0.2 mL of lysis buffer, 0.2 mL Tris buffered phenol/chloroform, and ~0.3 g glass beads to the microcentrifuge from Section 3.4, step 1 (*see* **Note 7**). Vortex the tube at maximum setting for 2 min to lyse cells.
3. DNA recovery. Centrifuge at 16,200 × *g* for 10 min. To improve phase separation centrifuge samples at 4 °C (if 4 °C is unavailable, room temperature will suffice). Remove the aqueous layer (top layer; ~250 μL) and place into a fresh 1.5 mL microcentrifuge tube. Use caution as to not to disturb the interface.

4. Ethanol precipitation of DNA. To the aqueous layer add 1:10 volume of sodium acetate (~20 μL, 300 mM final) and 2.5 volumes (~450 μL) of 100 % ethanol. Centrifuge the samples at 16,200 × *g* for 10 min at 4 °C and discard the supernatant.
5. Ethanol wash. At this point a typical extraction will yield a pellet of nucleic acid that is visible to the naked eye. Add 750 μL of 75 % ethanol to the microcentrifuge tube. Be careful not to disturb the pellet when adding ethanol. Remove ethanol by decanting. Minor amount of residual ethanol can be removed using a micropipette.
6. DNA suspension. Allow nucleic acid pellets to air-dry till residual ethanol has evaporated (~30 min at room temperature). Resuspend the pellet in 50 μL of TE buffer.
7. Plasmid recovery in *E. coli*. 1–4 μL of the recovered nucleic acids are electroporated into *E. coli* cells as per manufacturer's instructions (*see* **Note 8**). The electroporated cells are plated on LB agar plates supplemented with the appropriate antibiotic required for plasmid maintenance. Incubate plates at 37 °C overnight (~18 h).
8. Plasmid recovery. Plasmids are recovered out of *E. coli* using standard procedures as described elsewhere [9]. DNA sequence verification is suggested.
9. Plasmid transformation into *S. aureus*. Verified plasmids are recovered from *E. coli* cells and transformed into transformation-competent *S. aureus* strain RN4220 as described elsewhere [10].

4 Notes

1. The oligonucleotides used are typically quite large, but we try to keep them under 59 base pairs to prevent additional costs for synthesis. Constructs may contain a single DNA fragment or multiple DNA fragments to be cloned. We have been successful in combining upwards of nine fragments in addition to the YCC into a plasmid using YRC.
2. There is no need to PCR purify or gel purify the DNA fragments as this does not affect transformation efficiency, but if precise DNA concentrations are desired this step can be performed.
3. The vector does not need to be digested with more than one restriction enzyme. Also, the vector does not need to be gel purified post digestion as this does not affect transformation efficiency.
4. Typically a 1:10 dilution i.e. 5 mL overnight into 45 mL YPD will yield this OD.

5. For best results use the competent cells as soon as possible. However, cells can be stored at room temperature until ready to use. The cells retain competency at room temperature for a couple of hours, with only a minor decrease in efficiency.
6. Reaction mixtures containing only the linearized vector and the YCC fragment are included in a separate transformation reaction and serve as the negative control for the reaction.
7. Seal the microcentrifuge tube by securing the cap carefully. The glass beads stuck to the rim of the tube can prevent a seal forming.
8. *E. coli* cells with a transformation efficiency of $>10^9$ colony forming units per μg pUC19 are required to recover plasmid.

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Splicing by Overlap Extension PCR to Obtain Hybrid DNA Products

Justin A. Thornton

Abstract

Genetic manipulation of bacteria often requires the joining together of more than one DNA segment to form a hybrid DNA molecule. This can be accomplished by PCR followed by restriction endonuclease digestions and ligations. However, this approach can often become laborious and expensive. Here is described a well-established method for using primer design and PCR to obtain hybrid products for use in cloning vectors, mutagenesis protocols, and other applications.

Keywords: Polymerase chain reaction, Gene splicing, Mutagenesis, PCR fusion

1 Introduction

Since the discovery of modern PCR (1, 2), the technique has been used for an extensive array of molecular biology applications. One example is *s*plicing by *o*verlap *e*xtension PCR or SOEing PCR (3, 4). SOEing PCR serves as a simple, yet powerful, method for manipulating DNA sequences without the need for laborious cloning protocols. The technique involves joining together individual segments of amplified DNA using homologous sequences added to the 5′-ends of specific primers. SOEing PCR is dependent on selection of fusion oligonucleotide primers which are a hybrid of the reverse primer for one segment and the forward primer for another segment. Initial DNA segments are amplified by standard PCR using combinations of regular primers and/or the hybrid fusion primers. Ends of PCR segments amplified using the fusion primers will contain a short sequence complementary to the segment to be fused. Following amplification of the individual segments, a final PCR reaction using the outermost primers generates a full length fusion PCR product combining all segments. The complementary ends between segments will serve like "internal primers" to generate a full length template comprised of all segments to be fused. If necessary, a filling reaction can be performed by combining all of the individual segments without primer and cycling with polymerase allowing the overlapping ends to serve

as primers to generate some full length template for the final PCR reaction using the outermost primers. The entire process is simpler and cheaper than performing numerous digestion and ligation reactions.

2 Materials

Dissolve all buffer components in ultrapure water. Use only nuclease-free water for dilution of primers and for setting up PCR reactions. Keep DNA polymerase and PCR reactions on ice at all times prior to beginning PCR amplification run and follow manufacturer's instructions for optimal activity. Oligonucleotide primer sequences can be selected manually or using the software program of your choice.

2.1 PCR Reaction

1. 10× amplification buffer (varies by manufacturer): generally consists of 500 mM KCl, 100 mM Tris–HCl, pH 8.0–8.3, 15–20 mM $MgCl_2$.
2. dNTP mixture: containing approximately 2.5 mM each of dCTP, dATP, dGTP, dTTP. Final concentration of each dNTP in the reaction should be ~200 μM.
3. Stock oligonucleotide primers: dissolve each oligonucleotide primer to in TE (10 mM Tris pH 8.0, 1 mM ETDA) to make a 100 μM stock solution. Dilute from this stock 1:10 (in water or TE) to make a 10 μM working solution.
4. Template DNA: <500 ng (*see* **Note 1**).
5. Proofreading thermo-stable DNA polymerase: TaKaRa Ex Taq polymerase (5 units/μL) performs well in our hands.
6. Nuclease-free water.

2.2 Agarose Gel Electrophoresis

1. 50× Tris-acetate (TAE): 242 g Tris base, 57.1 ml glacial acetic acid, 100 mL 0.5 M EDTA (pH 8.0). Alternatively Tris-borate (TBE) can be used from a 5× stock: 54 g Tris base, 27.5 g boric acid, 20 mL of 0.5 M EDTA (pH 8.0).
2. Molecular biology grade, low-EEO agarose.
3. Ethidium bromide: 1 % solution (*see* **Note 2**).
4. 6× DNA loading buffer: 30 % glycerol, 0.25 % bromophenol blue, and 0.5 % xylene cyanol in distilled water.
5. DNA ladder.

2.3 DNA Isolation and Cloning Kits

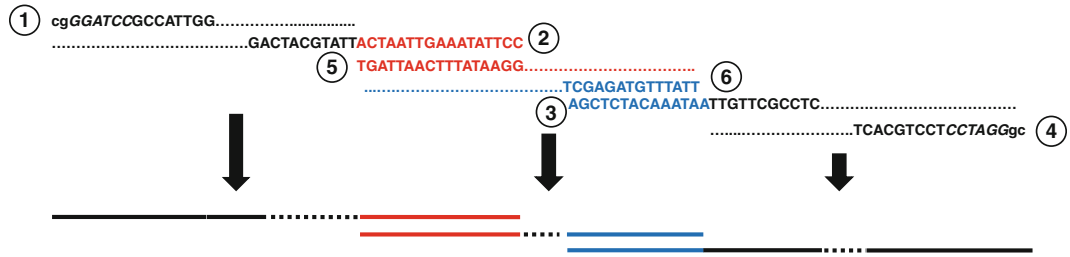
1. DNA Clean & Concentrator-5 (Zymo Research Corporation, Irvine, CA).
2. TOPO-TA Cloning kit (Life Technologies Gaithersburg, MD) or other subcloning vector compatible with polymerase used (determined based on whether polymerase leaves an overhang or blunt end).

3 Methods

SOEing PCR can be used to fuse as many pieces of DNA as desired into a single product. Here we have simply described how to fuse three pieces together.

3.1 Primer Design

1. Design forward and reverse primers for each segment of DNA you wish to amplify. Here we refer to the segments as "upstream flanking," "middle segment," and "downstream flanking." The first two primers, Primers 1 and 2, should serve as forward and reverse primers for your upstream flanking segment. Primers 3 and 4 should serve as forward and reverse primers for your downstream flanking segment and Primers 5 and 6 should amplify your middle segment.
2. Example primer sequences are shown in Fig. 1. Each primer number is located at its 5′-end. BamHI restriction sites have been added to the ends of Primers 1 and 4.
3. Annealing temperatures of all primers should be as similar as possible, prior to adding additional sequences.
4. Primer 2 (reverse primer for upstream segment) will need to have the inverse complement sequence of Primer 5 (forward primer for middle segment) added to its 5′ end. Additionally, Primer 3 will need the inverse complement sequence of primer 6 added to its 5′-end. These modifications will ensure overlap between the sequences during the later SOEing reaction (*see* **Notes 3** and **4**).



Primer Sequences (5´-3´)

Primer 1: cg*GGATCC*GCCATTGG
Primer 2: CCTTATAAAGTTAATCATTATGCATCAG
Primer 3: AGCTCTACAAATAATTGTTCGCCTC
Primer 4: cg*GGATCC*TCCTGCACT
Primer 5: TGATTAACTTTATAAGG
Primer 6: TTATTTGTAGAGCTCATCC

Fig. 1 SOEing PCR diagram with example primer sequences. Primer sequences are numbered consecutively at their 5′-ends. *Red* and *blue letters* indicate overlapping fusion sequences. A BamHI restriction site (shown in *italics*) has been added to Primers 1 and 4 for cloning of the SOEing product

5. Sequences complimentary to the upstream and downstream segments can also be added to the 5′-ends of Primers 5 and 6. However, the SOEing PCR will generally work without the added complimentary sequences and therefore is not shown here (*see* **Note 5**).

3.2 Initial PCR Reactions

1. Set up individual PCR reactions to amplify the upstream, middle, and downstream segments. In a 50 μL reaction volume, add 5 μL of 10× amplification buffer, dNTPs (200 μM each), proofreading Taq DNA polymerase (1.25 units), forward and reverse primers (0.4 μM each), and template DNA (50–100 ng) (*see* **Note 6**).
2. Run a standard 30-cycle PCR program: 1 cycle (4 min at 95 °C), 30 cycles (1 min at 95 °C, 1 min at 53 °C, *X* min at 72 °C where *X* = size in kilobases of product to be amplified), 1 cycle of 5 min at 72 °C (*see* **Note 7**).

3.3 Gel Imaging and PCR Cleanup

Pour a 0.8–1 % agarose gel by melting agarose in TAE buffer and pouring into cast. Immediately add a 10-well gel comb. Place gel into a gel electrophoresis apparatus fill with TAE to cover gel. If TBE is used to cast gel, fill apparatus with TBE instead of TAE.

1. After the gel has solidified, add 1 μL of 6× loading dye to 5 μL of each PCR product, mix well, and load into wells.
2. Apply current (90–100 V) for 35–40 min. Remove gel from apparatus and view on ultraviolet transilluminator.
3. Example PCR products from three individual segments are shown in Fig. 2a. Individual band sizes are 500 bp (UP and DN) and 750 bp (Mid).
4. After confirming amplification of each segment, perform a PCR cleanup using a commercially available kit to remove oligonucleotides and elute using the manufacturer's recommended volume. Assess the concentration of each PCR product by a spectrophotometer.

3.4 SOEing PCR Reaction

1. Add equal concentrations (~100 ng each) of the individual PCR segments to be fused to a PCR reaction mixture containing Primers 1 and 4 (*see* **Note 8**).
2. Set the extension time minutes of the PCR program to equal the sum of all segments in kilobases. For instance, in the example used here the segments total 1.75 kb therefore an extension time of 1.45 min was used.
3. Following PCR, run the SOEing product on a 0.8–1 % agarose gel as described above and check the gel on an UV transilluminator. A successful SOEing PCR should generate a product with a size totaling the sum of the individual segments. Fusion of the segments from Fig. 2a is shown in Fig. 2b.

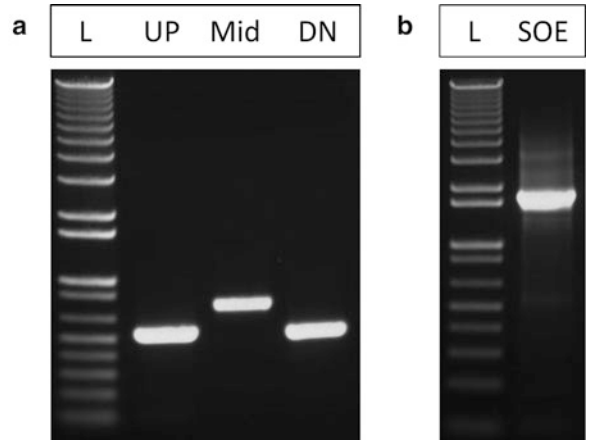


Fig. 2 (**a**) Agarose gel UV image of products amplified using Primers 1 and 2 (UP), Primers 5 and 6 (Mid), and Primers 3 and 4 (DN). "L" indicates DNA ladder. 50 ng of template DNA was used for each reaction and 5 μL of PCR product was loaded into each well. (**b**) Agarose gel UV image of SOEing product following PCR fusion of all three segments from (**a**). Template for the SOEing PCR reaction consisted of a mixture of the three segments (100 ng each) from (**a**)

3.5 Filling Reaction (Optional) (See Note 9)

1. Combine equal concentrations (200 ng each) of the individual segments in a PCR reaction containing all components EXCEPT primers. The overlap sequences of the fusion primers (Primer 2 and 3) will serve as primers and the polymerase will fill the gaps to generate full length template (*see* **Note 10**).
2. Run the standard PCR program as described above allowing an extension time in minutes that equals the length of desired full length product in kilobases.
3. Following the filling PCR reaction, use 2–3 μL of the filling product as template in a SOEing reaction containing Primers 1 and 4. There is no need to perform a PCR cleanup of the filling reaction since it included no primers.
4. Run the SOEing PCR product on a 0.8–1 % agarose gel and visualize on a UV transilluminator.

3.6 SOEing PCR Reaction Isolation and Cloning

1. The SOEing PCR product can be isolated and concentrated using a kit as described for the individual segments (*see* **Note 11**).
2. If more than one band appears on the gel, the annealing temperature may need to be adjusted. Run a gradient PCR to determine optimal conditions for production of a single full-length product.
3. Clone the SOEing PCR product into a cloning vector such as pCR2.1-TOPO per manufacturer's instructions. Confirm and isolate positive clones for freezer stocks. This prevents the need to repeat the SOEing PCR from scratch if the product is needed again in the future (*see* **Note 12**).

4 Notes

1. Typically 20–100 ng of template DNA is sufficient to perform a standard PCR reaction.
2. Ethidium bromide is a mutagen and should be handled with care. DNA gels can be stained following electrophoresis; however, we generally add ethidium bromide to our gels prior to casting. We typically add 2 μL 1 % ethidium bromide per 100 mL of gel.
3. If possible, limit the length of the 3′ ends of primer 2 and primer 3 to approximately 15–20 nucleotides. Additionally, the 5′ additions (inverse complements should be approximately 15–20 nucleotides to help limit an extremely long fusion primer (i.e., final Primers 2 and 3).
4. The additions to the 5′-ends of the primers will drastically increase the overall Tm of the primers. However, this should not be a concern since the entire primer is not adhering to the template.
5. This prevents the need of ordering an additional set of fusion primers for each SOEing reaction you plan to perform. For example, if you are routinely fusing a common antibiotic cassette between segments of DNA, you can use your standard primers to amplify the cassette and will not require a second set of fusion primers.
6. The concentration of template DNA can vary depending on the source. Empirical determination of optimal template concentration is recommended. We typically get strong amplification of DNA segments from 50 to 100 ng of chromosomal DNA. Less may be necessary if amplifying from plasmid templates.
7. All annealing temperatures and extension times should be modified based on the products to be amplified as well as the polymerase used.
8. The optimal ratio of segments for efficient amplification of the SOEing product can sometimes vary. Altering the ratio of the mid segment to the flanking pieces may improve the amplification efficiency and should be determined empirically.
9. SOEing PCR occasionally results in multiple products or products that are not full length. For this reason, it is often beneficial to run a filling reaction prior to or in conjunction with the SOEing PCR reaction. Depending on the primers and sequences to be amplified, the filling reaction will sometimes generate a better full-length template than using the individual gene segments. If neither the standard SOEing PCR nor the filling reaction-as-template PCR leads to full length product,

new primers may need to be designed as they may be incompatible.

10. As described for the SOEing reaction, the optimal ratio of segments may need to be determined empirically if the filling reaction is not successful. Altering the ratio of mid-segment to flanking segments may improve the efficiency.
11. A PCR purification column can be used to isolate the SOEing product only if a single band of the correct size is visualized following agarose gel electrophoresis.
12. TOPO-TA cloning can also be used to isolate the correct SOEing PCR in the event that multiple bands are continually produced. If a band the size of all the combined segments is visualized, the band can be excised and gel purified prior to cloning. It is important to always sequence the TA clones prior to proceeding to ensure the SOEing product is correct.

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Method for Preparation and Electroporation of *S. aureus* and *S. epidermidis*

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Abstract

For bacterial species that are not known to be naturally competent, such as *Staphylococcus aureus* and *Staphylococcus epidermidis*, electroporation is an efficient method for introducing genetic material into the cell. The technique utilizes electrical pulses to transiently permeabilize bacterial cell membranes, which allows for the passage of plasmid DNA across the membranes. Here, we describe methods for preparing electrocompetent *S. aureus* and *S. epidermidis* cells and outline a procedure for electroporation of the prepared competent cells.

Keywords: Electroporation, Competent cells, Electrocompetent, Transformation

1 Introduction

Genetic manipulation of bacterial strains in the laboratory necessitates the ability to introduce specific genetic material at will. Because neither *S. aureus* nor *S. epidermidis* are known to be naturally competent, the most efficient method for introducing plasmid DNA into these bacteria is through electroporation (1). Although other methods such as protoplast transformation, protoplast fusion, and chemically induced competence were explored in the 1970s and 1980s (2–5), these techniques are no longer used as they are time-consuming and generally unsuccessful. Electroporation offers a quick and highly efficient alternative. Phage transduction can also be used to move plasmids between *S. aureus* strains, but transformation is required for the initial introduction of foreign DNA into *S. aureus*.

Although electroporation is one of the most commonly used techniques in molecular genetics, much of its theoretical basis remains a matter of debate (6–8). An applied electric pulse produces an electric field across the cell membrane that alters the transmembrane potential of the cell in a position-specific manner. Due to the directional flow of current, the membrane becomes hyperpolarized on the side facing the anode of the electroporator, and depolarized on the side facing the cathode (8). When the field

strength is high enough, the areas of the membrane directly facing the electrodes become electropermeabilized due to the transmembrane potential difference reaching a critical value (9, 10). Although many models attribute electropermeabilization to pore formation, pores have never actually been observed in cells that subsequently recovered from the pulse (8, 11). An alternative theory is that the membrane simply becomes destabilized by the increase in potential difference, leading to formation of transient permeable domains but not necessarily defined pores (8). Either way, it is clear that the electric field increases membrane permeability in localized areas to allow for the transport of small molecules across the membrane. Negatively charged DNA is directed towards the cathode-facing portion of the cell membrane by electrophoresis from the applied electric field (12). There, the DNA interacts with the electropermeabilized membrane and forms aggregates (13). The subsequent steps, which include membrane recovery and DNA migration into the cytoplasm, are still not well understood and require further characterization (8).

Typically, preparation of electrocompetent cells involves a series of washes in water and/or non-ionic solutions to remove all salts from the original culture media. Low ion content in mixtures of cells and DNA is desirable for electrophoresis because large ion concentrations can result in arcing, or "shorting" of the electroporation cuvette; this produces an electric discharge that vastly decreases cellular viability and typically indicates an unsuccessful electroporation event. Other important determinants of transformation efficiency in *S. aureus* and *S. epidermidis* include growth medium, cell density, cuvette size, and electric pulse parameters (1, 14–16). Here, we describe culture conditions designed to result in optimal transformation efficiency, including a medium, B2, which was previously optimized for preparation of *S. aureus* electrocompetent cells (14). We outline a series of wash steps to create electrocompetent *S. aureus* and *S. epidermidis* cells, and then describe an electroporation protocol optimized for transforming these bacteria.

2 Materials

2.1 Competent Cell Preparation

1. B2 broth: 10 g/l casamino acids, 25 g/l yeast extract, 1 g/l K_2HPO_4, 5 g/l D-glucose, and 25 g/l NaCl. Dissolve in water and adjust pH to 7.5 with NaOH. Sterilize by autoclaving or filter sterilization (*see* **Note 1**).
2. Water: Use sterile double distilled H_2O (ddH_2O).
3. Glycerol: Prepare a 10 % solution in ddH_2O and sterilize.
4. Glass culture tubes (for 5 ml overnight cultures).

5. Glass Erlenmeyer flask, 250 ml capacity (cover with aluminum foil and autoclave prior to use).
6. Incubator at 37 °C containing a shaker.
7. Conical centrifuge tube, 50 ml.
8. Centrifuge.

2.2 Electroporation

1. Electroporation cuvettes with 2 mm gap.
2. Electroporation apparatus (Gene Pulser, MicroPulser, Xcell, etc.).
3. Microcentrifuge tubes, 1.5 ml.
4. Tryptic soy agar plates containing appropriate antibiotic.

3 Methods

3.1 S. aureus Competent Cells

1. Grow up an overnight culture of the desired *S. aureus* recipient strain in B2 Broth for approximately 16 h.
2. Dilute the overnight culture 1:100 into 50 ml of fresh B2 broth in a sterile 250 ml Erlenmeyer flask. Grow at 37 °C to an approximate OD_{660} of 0.8–1.
3. Transfer the culture to a 50 ml conical centrifuge tube. Centrifuge at 5,000 × *g* force at room temperature (RT) for 15 min. Decant and discard the supernatant.
4. Resuspend cells in 50 ml of RT, sterile ddH_2O. Centrifuge at 5,000 × *g* force, RT, for 15 min. Decant and discard the supernatant.
5. Repeat step 4 two additional times.
6. Resuspend cells in 10 ml of RT, sterile 10 % glycerol. Centrifuge at 5,000 × *g* at RT for 15 min. Decant and discard the supernatant.
7. Repeat step 6 one additional time.
8. Resuspend cells in 5 ml of RT, sterile 10 % glycerol. Centrifuge at 5,000 × *g* at RT for 15 min. Decant and discard the supernatant.
9. Resuspend cells in 5 ml RT 10 % glycerol and incubate at RT for 15 min.
10. Centrifuge at 5,000 × *g* for 15 min.
11. Decant all but 1 ml of the supernatant. Resuspend the pellet in the remaining 1 ml (*see* **Note 2**).
12. Divide into 70 μl aliquots in 1.5 ml-microcentrifuge tubes and continue with electroporation (*see* **Note 3**).

3.2 S. epidermidis Competent Cells

1. Grow up an overnight culture of the desired *S. epidermidis* recipient strain in B2 Broth for approximately 16 h.
2. Dilute the overnight culture 1:100 into 50 ml of fresh B2 broth in a sterile 250 ml Erlenmeyer flask. Grow at 37 °C to an approximate OD_{660} of 0.8–1.
3. Transfer the culture to a 50 ml conical centrifuge tube. Centrifuge at 5,000 × *g* force at 4 °C for 15 min. Decant and discard the supernatant.
4. Resuspend cells in 50 ml of ice cold sterile 10 % glycerol. Centrifuge at 5,000 × *g* at 4 °C for 15 min. Decant and discard the supernatant.
5. Repeat step 4 three more times using decreasing volumes of ice cold 10 % glycerol. Resuspend cells in 1/2 original culture volume (25 ml 10 % glycerol for 50 ml culture volume), then 1/20 original culture volume (2.5 ml 10 % glycerol for 50 ml culture volume), and finally 1/50 original culture volume (1 ml 10 % glycerol for 50 ml original culture volume).
6. Aliquot into 70 μl samples in 1.5 ml-microcentrifuge tubes and continue with electroporation.

3.3 Electroporation

1. Add plasmid DNA to the electrocompetent cell suspension (typically 1 μg of plasmid DNA) (*see* **Note 4**). Mix in DNA by gently swirling pipette. For *S. epidermidis*, incubate the plasmid and DNA mixture at room temperature for 30 min.
2. Transfer cells and DNA to a 2 mm pulse cuvette (*see* **Notes 5** and **6**).
3. For *S. aureus*, set the waveform to exponential decay and pulse at 1.8 kV, 600 Ω, 10 μF. Look for $t_c = 4.5–5$ (*see* **Note 7**). For *S. epidermidis*, set the waveform to exponential decay and pulse at 2.0 kV 100 Ω, 25 μF.
4. Immediately add 500 μl of B2 broth to the cuvette to resuspend cells and then transfer to a 1.5 ml microcentrifuge tube.
5. Shake for 1 h for *S. aureus* or 4 h for *S. epidermidis* at 37 °C (or permissive temperature for temperature-sensitive plasmids).
6. Plate 1 and 100 μl of cells on Tryptic soy agar (TSA) plates containing the appropriate antibiotic (*see* **Note 8**). Centrifuge the remainder of cells for 1 min, then resuspend the pellet in 100 μl of the supernatant and plate this as well.
7. Incubate overnight at 37 °C (or permissive temperature for the selected plasmid) or until colonies appear.

4 Notes

1. B2 broth can be sterilized by autoclaving, but we recommend running no more than a 30-min sterilization cycle followed by prompt removal of the media from the autoclave to prevent excessive caramelization of the glucose contained within the media.
2. Typically, electroporation success increases with the viscosity of the final competent cell suspension. This can be achieved by removing as much supernatant as possible (depending on the number of aliquots desired) following the final centrifugation steps.
3. Competent cell aliquots can be stored at −80 °C. However, fresh competent cells ensure the highest likelihood of success.
4. The majority of wild-type *S. aureus* strains cannot take up foreign DNA, including *E. coli* propagated plasmids, due to two restriction pathways (17). The SauUSI type IV restriction modification (RM) system recognizes and digests cytosine methylated DNA (18). The SauI type 1 RM system of *S. aureus* includes a restriction component *hsdR*, and two copies each of the modification component *hsdM*, and the specificity component *hsdS* (19). HsdM catalyzes the transfer of a methyl group to adenine residues of specific sequences determined by HsdS; HsdR cleaves DNA that is unmethylated at HsdS-determined target sequences (also, see review chapter entitled Restriction Modification Systems as Barriers).

   There are two strategies for bypassing these restriction barriers for introduction of plasmid DNA into *S. aureus*. The first and most efficient strategy is to passage *E. coli* propagated DNA through *S. aureus* strain RN4220 (20). This strain was generated via mutagenesis of the parent strain 8325-4 and can accept foreign DNA because it contains nonsense mutations in both *hsdR* and *sauUSI* (18, 21). RN4220 retains the methylation function of the SauI system, and can thus appropriately methylate DNA for subsequent uptake by other S. aureus strains containing functional RM systems. Alternatively, *S. aureus* can directly take up DNA from a strain of *E. coli*, DC10B, in which the *dcm* methylation system has been deleted (21). This is less efficient than passage through RN4220 because the donor DNA still lacks appropriate adenine methylation by the SauI system. Therefore, our lab generally uses passage through RN4220 before introducing new plasmids into other *S. aureus* strains.
5. Chill cuvettes prior to electroporation (we store them at 4 °C and then transfer to an ice bucket) to reduce the chance of arcing.

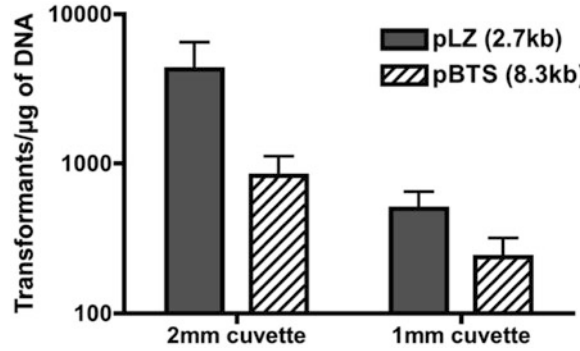

Fig. 1 Plasmid size and cuvette gap width determine transformation efficiency. Using the protocol described in this chapter, *S. aureus* strain RN4220 was transformed with 1 μg of either pBTS (8.3 kb) or pLZ (2.7 kb) plasmid DNA prepped from *E. coli* strain DH10B. Cells were plated on TSA containing 100 μg/ml spectinomycin. For each plasmid, transformation efficiency was compared when using 1 mm versus 2 mm gap cuvettes. $n = 3$, error bars = SE

6. 2 mm gap cuvettes provide nearly a log higher efficiency when electroporating *S. aureus*. Furthermore, the size of the plasmid DNA is inversely proportional to the electroporation efficiency (*see* Fig. 1).
7. The Time Constant t_c refers to the amount of time (msec) required to dissipate 63 % of the maximal charge during electroporation. It is a function of resistance and capacitance of the system ($t_c = R \times C$ in msec). Anything that would affect the resistance of the sample will alter the t_c. For instance, increasing sample volume decreases resistance and therefore lowers the t_c.
8. Refer to the table below for appropriate antibiotic concentrations.

Antibiotic	Stock concentration (mg/ml)	Working concentration (μg/ml)
Chloramphenicol	10 (100 % ethanol)	10
Erythromycin	5 (100 % ethanol)	5
Tetracycline	2.5 (70 % ethanol)	2.5
Kanamycin	50 (water)	50
Spectinomycin	100 (water)	100

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Rapid Isolation of DNA from *Staphylococcus*

Kelsey L. Krausz and Jeffrey L. Bose

Abstract

Many methods exist to extract DNA from bacteria. Indeed, there is no shortage of kits available from manufacturers that allow for isolation of highly purified DNA. However, for many applications samples do not need to be extremely pure (i.e., free of contaminating proteins or RNA). Furthermore, for quick genetic screening, it is often useful to have a rapid and inexpensive option for DNA isolation from small samples. For these occasions, the method found in this chapter provides a cost-efficient, yet rapid, isolation of DNA.

Keywords: DNA isolation, DNA extraction, DNA purification, Phenol–chloroform, *Staphylococcus* DNA

1 Introduction

Phenol–chloroform extractions have been used for decades in all types of organisms as a means of isolating nucleic acids. Indeed, when combined with isopropanol and ethanol, very pure nucleic acid preparations can be produced. Today, this procedure serves as a predecessor for the many commercial kits available for DNA and/or RNA extraction. Simply put, this technique combines a phenol–chloroform mix with an aqueous sample (e.g., a cell extract) and allows for the separation of molecules between the resulting organic and aqueous phases. Once mixed, the phases are separated by centrifugation, resulting in two distinct phases with the organic phase below the aqueous phase. Furthermore, there is a whitish interphase between them containing proteins and other compounds that should not be disturbed. A variety of factors can contribute to the partitioning of nucleic acids, including salt and pH. For this procedure, a high pH is necessary (*see* **Note 1**).

The procedure itself is useful for a variety of applications. While many kits are available for the isolation of large quantities of pure DNA, this is not always necessary. For example, when screening for genetic mutations, i.e., deletions or antibiotic cassette insertions, a large number of colonies may have to be screened by polymerase chain reaction (PCR) for the desired mutation and large quantities of pure DNA are not required. These experiments may become costly when using commercial isolation kits. As another example,

one may want to amplify a particular gene from a plasmid or chromosome from multiple strains of *S. aureus*. Again, in this scenario, isolation of large quantities of pure DNA may not be desired if these samples are only destined for a single use. Indeed, this method yields plenty of DNA for multiple PCR reactions and would satisfy many needs. Importantly, this is an adapted method published by the McGavin lab (1). For easy reference, a diagram of the procedure is provided as Fig. 1.

2 Materials

Prepare all solutions with purified deionized water and store at room temperature. Dispose of all reagents and materials according to proper waste disposal regulations. Since phenol and chloroform are volatile and hazardous, tubes containing the phenol–chloroform mixture should be only opened in a fume hood.

2.1 Extraction Components

1. Lysis Buffer: 150 mM NaCl, 25 mM Tris–HCl, pH 8.0, 50 mM Glucose, 10 mM EDTA, pH 8.0.
2. Lysostaphin (10 mg/ml).
3. 3 % Sodium dodecyl sulfate (SDS).
4. Phenol–Chloroform–Isoamyl alcohol (25:24:1), pH 8.0 (*see* **Note 1**).
5. TE buffer: 10 mM Tris–HCl (pH 7.5), 1 mM EDTA.
6. Water bath set to 37 °C.
7. Heat block set to 95 °C.
8. Microcentrifuge.
9. Vortex.

3 Methods

3.1 Preliminary Bacterial Preparations

1. Take part of a single colony and streak onto a new prepared plate of media (*see* **Note 2**).
2. Use rest of colony for further processing (*see* **Note 3**).

3.2 DNA Extraction

1. In a sterile 1.5-ml centrifuge tube, pipette 100 µl of the Lysis Buffer and add enough of the remaining colony to make buffer slightly turbid.
2. Add 2 µl of lysostaphin.
3. Incubate in a 37 °C water bath for 30 min.
4. Add 3 µl of 3 % SDS.
5. Incubate at 95 °C for 10 min (*see* **Note 4**).

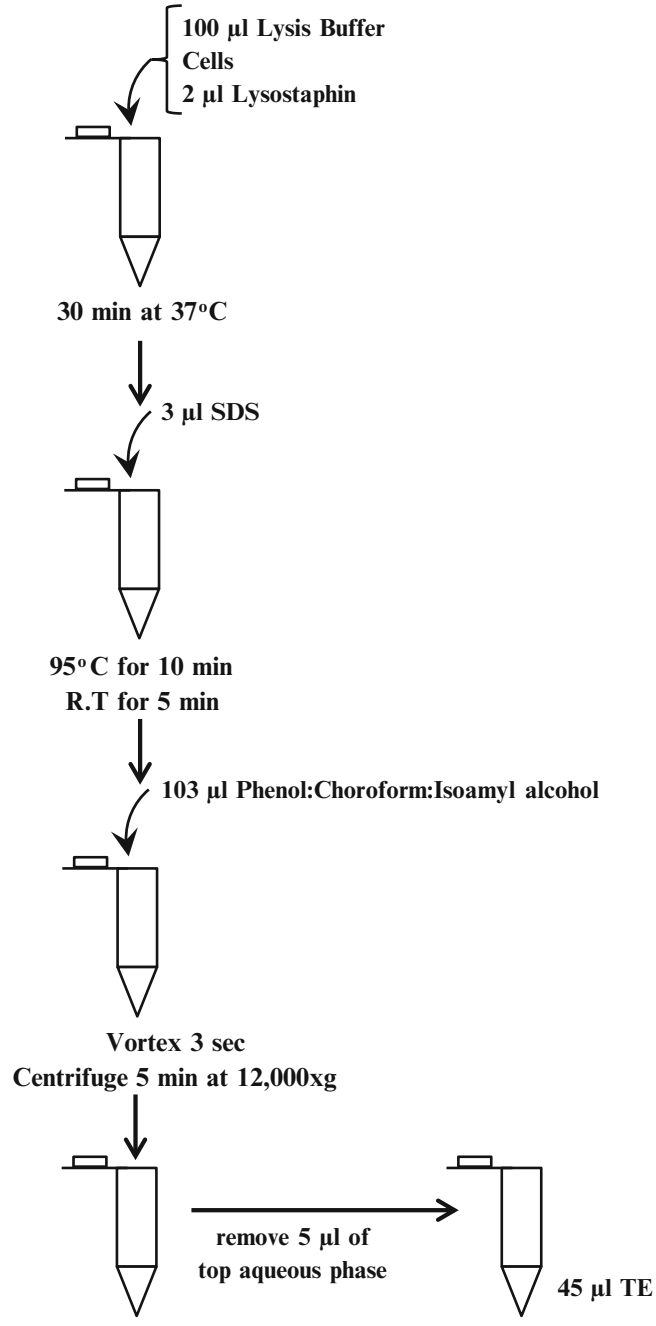

Fig. 1 Schematic of protocol. Note that opening tubes containing phenol–chloroform mixture should be performed in a fume hood

6. Cool at room temperature for approximately 5 min.
7. Add 103 µl of Phenol–Chloroform–Isoamyl alcohol.
8. Vortex on high for 3 s.

9. Centrifuge at 12,000 × *g* for 5 min.
10. Pipette 5 μl of top aqueous layer to a new 1.5-ml centrifuge tube (*see* **Note 5**).
11. Add 45 μl of TE buffer.
12. Use sample for further analysis (*see* **Note 6**).

4 Notes

1. Using the proper pH is important for nucleic acid partitioning. At lower pH (4–6), DNA remains in the organic phase while RNA is found in the aqueous phase. However, at higher pH (7.5–8.0), both RNA and DNA partition to the aqueous phase. While isoamyl alcohol is not required for this procedure, it serves as an antifoaming agent.
2. Re-streaking part of the colony on a fresh plate provides a source of those cells after the genetic screening is done.
3. While the procedure here isolates DNA from a colony, we have also had success when using cells from overnight cultures. In this case, centrifuge the samples to form a pellet and then resuspend the pellet in Lysis Buffer.
4. After this step, the sample should be mostly clear.
5. Be careful to not remove part of the organic/aqueous phase interphase. If this is disturbed, use a new tip and remove sample from aqueous phase alone.
6. For PCR analysis from plasmid and chromosomes sources, 0.75 μl is generally sufficient in a 25 μl reaction. The amount needed can be adjusted based on the turbidity in step 1.

Acknowledgement

We would like to thank Martin McGavin at Western University in Ontario for first publishing this method and for his permission to publish this adapted protocol.

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Bacteriophage Transduction in *Staphylococcus aureus*: Broth-Based Method

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Abstract

The ability to move DNA between *Staphylococcus* strains is essential for the genetic manipulation of this bacterium. Often in the Staphylococci, this is accomplished through transduction using generalized transducing phage and can be performed in different ways and therefore the presence of two transduction procedures in this book. The following protocol is a relatively easy-to-perform, broth-based procedure that we have used extensively to move both plasmids and chromosomal fragments between strains of *Staphylococcus aureus*.

Keywords: Transduction, Bacteriophage, phi11, phi80α

1 Introduction

In order to genetically manipulate a bacterium, one must first be able to move DNA into that bacterium. This can be accomplished in several ways, including: transformation of free DNA, conjugation between cells, electroporation, and bacteriophage transduction. Working with clinical strains of *Staphylococcus* is complicated by the fact that these bacteria are not naturally competent and can be difficult to electroporate with DNA isolated from more genetically amendable bacteria such as *E. coli*, the host we most often use to generate plasmid constructs. This hurdle was largely overcome with the generation of strain RN4220 (1) which, due to mutations in restriction modification systems, can readily uptake DNA isolated from *E. coli* by electroporation. However, that DNA must be transferred to a strain of interest and is often accomplished by bacteriophage transduction.

Several factors contribute to the success or failure of transductions. First, a generalized transducing phage must be chosen. Unlike specialized transducing phage, which are more restrictive in the genes they transfer, generalized transduction is less discriminate and occurs when a phage mispacks the phage head with bacterial DNA. The goal for this method is to obtain phage particles packed only with bacterial DNA of interest from the donor cell.

Another factor that affects the outcome of transductions is the length of DNA to be moved to the recipient cell. Bacteriophage can only pack a certain amount of DNA into the capsid, and therefore, there is a limit to which a specific phage can transfer. For example, two commonly used transducing phage, φ11 and φ80α, have genomes of 43.6 kb and 43.9 kb, respectively (2, 3). Often, this is not a major concern, since typically phage transduction is used to move plasmids of less than 15 kb or marked chromosome mutations between strains. However, this does mean that when moving chromosome mutations, up to ~45 kb of DNA around the mutation will likely be deposited into the recipient strain and any amount of that 45 kb may end up incorporated into the chromosome. This could lead to unexpected changes or mutations due to sequence variations between strains. Finally, another contributing factor is the relative phage-resistance of either the donor or recipient strains. The sensitivity of a particular strain of *S. aureus* can vary by several orders of magnitude and therefore it is important to understand the individual phage resistances of the strains being used (*see* Section 4 below and Fig. 1).

Taking the above items into consideration, this chapter provides a step-by-step workflow to (1) propagate phage to produce a phage lysate, (2) quantify the number of phage isolated, and (3) transduce donor DNA into recipient cells of interest. Included in this workflow are a variety of Notes to provide tips and considerations to maximize the successful outcome of the procedure.

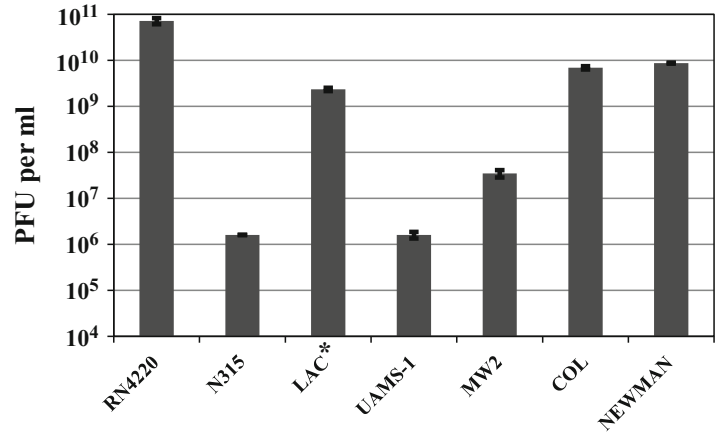

Fig. 1 A φ11 prep previously determined to contain 2.7×10^{10} pfu per ml using RN4220 was used to make serial dilutions in phage buffer and then titer determined on different *S. aureus* strains. As the data demonstrates, the results differ greatly between strains and highlight the different sensitivities of these strains to phage. * We used AH1263 which is a derivative of LAC provided by Dr. Alexander Horswill at the University of Iowa. Data is the average ($n = 3$) with standard error of the mean

Using this method, we have been able to relatively easily move both plasmids and chromosomal alleles between many different strains of *Staphylococcus aureus*.

2 Materials

Prepare all solutions with purified deionized water and store at room temperature. Dispose of all reagents and materials according to proper waste disposal regulations.

1. Phage Buffer: to 477.5 ml of water, add 6.47 g of β-glycerol phosphate disodium salt, 60 mg $MgSO_4$, 2.4 g NaCl, and 0.5 g gelatin. Autoclave to sterilize and then add 22.5 ml of sterile 10 mg/ml $CaCl_2$.
2. $CaCl_2$ (10 mg/ml) stock.
3. Prepared bacteriophage stock (*see* **Note 1**).
4. 30 and 37 °C shaking incubators.
5. 50 °C water bath.
6. 0.45 μm filter unit.
7. Donor and recipient strains.
8. Culture of *S. aureus* RN4220.
9. Tryptic Soy Broth (TSB).
10. Tryptic Soy Agar (TSA).
11. 0.5 % agar in TSB (soft agar).
12. Appropriate antibiotics.

3 Methods

3.1 Producing Phage Prep

1. Inoculate 3 ml of TSB containing appropriate antibiotics with the donor strain. Grow overnight at 37 °C (30 °C for temperature-sensitive strains) with shaking (250 rpm).
2. Dilute the donor *S. aureus* 1:100 in 25 ml TSB in a 125-ml flask and incubate at 30 °C for 1.5 h.
3. Add 1 ml of fresh 10 mg/ml $CaCl_2$ (*see* **Note 2**) and 10 μl of 10^{10} bacteriophage (*see* **Note 3**).
4. Incubate flask at room temperature for 30 min statically and then rotate slowly (*see* **Note 4**) at 30 °C for 5–6 h until cells are lysed (*see* **Note 5**).
5. Filter phage preparation solution thru a 0.45 μm filter (*see* **Note 6**).
6. Place preparation at 4 °C.

3.2 Phage Titering

1. Microwave soft agar and place in a 50 °C water bath.
2. Aliquot 4.5 ml of soft agar into four tubes (two tubes per dilution) and place tubes at 50 °C.
3. Make a dilution series of the phage prep to 1×10^{-8} and 1×10^{-9} dilutions (these are usually sufficient) in Phage Buffer.
4. To each 4.5 ml tube of soft agar, add 0.2 ml of 10 mg/ml $CaCl_2$, 0.2 ml *S. aureus* RN4220, and 0.1 ml of appropriate phage dilution.
5. Vortex slowly to minimize bubble production.
6. Pour contents of tubes onto a pre-warmed TSA plate and allow to solidify.
7. Incubate plates at 37 °C overnight or until plaques develop.

3.3 Transduction

1. Dilute an overnight culture of recipient *S. aureus* 1:100 in 25 ml TSB in a 250-ml flask.
2. Incubate at 37 °C with shaking (250 rpm) for 1 h, then transfer contents of the flask to a 50-ml conical tube.
3. Centrifuge at 4,500 × *g* for 10 min to pellet the cells.
4. Discard supernatant and resuspend pellet in 0.5 ml TSB.
5. Add 40 μl of 10 mg/ml $CaCl_2$ (*see* **Note 2**) and the appropriate amount of bacteriophage to give an MOI of 0.1 (*see* **Note 7**).
6. Incubate conical tube at room temperature for 10 min and then at 30 °C for 35 min without shaking.
7. Add an additional 2.5 ml TSB (do not vortex) and centrifuge at 4,500 × *g* for 10 min to pellet the cells.
8. Discard supernatant and resuspend pellet in 5 ml TSB.
9. Incubate with shaking (250 rpm) for 1.5 h at 37 °C (or 30 °C for temperature sensitive strains).
10. Repeat steps 3 and 4.
11. Plate 0.1 ml per plate on selective media.
12. Incubate plate at 37 °C (or 30 °C for temperature sensitive strains) for 24–48 h.

4 Notes

1. This note describes how to make a stock of bacteriophage from a lysogenized strain. First, dilute a fresh overnight lysogenized *S. aureus* culture 1:100 in 25 ml TSB and grow at 37 °C until mid-exponential phase. Next, induce the bacteriophage with either 0.5 μg/ml mitomycin C or 1,200 ergs/mm^2 per min UV irradiation (4, 5) and rotate slowly at 30 °C for 5–6 h or

until lysis occurs. Next, filter-sterilize using a 0.45 μm filter and store at 4 °C until use. Finally, titer the phage using the protocol in Section 3.2.

2. Generally, 1 ml of 10 mg/ml calcium chloride is sufficient. However, we have had great success getting better lysis on phage-resistant strains when increasing the amount of $CaCl_2$ to 1 ml of 20 mg/ml. This can also be done for the transduction procedure (Section 3.3, step 5).
3. This amount of phage generally works well for phage-sensitive strains, but may need to be increased for phage-resistant strains (*see* **Note 5**).
4. The term "slowly" is used relatively since the amount of agitation is going to vary from machine to machine. We typically perform this step at 70 rpm in a shaking incubator with a 1 in. orbit. In addition, we have done this on a Belly Dancer placed in a 30 °C non-shaking incubator.
5. The time until the culture clears can change due to a number of factors. First, the amount of phage added will alter the amount of time to lysis, with larger number of added phage decreasing time until the culture clears. In addition, the sensitivity of a particular strain to the phage also impacts this time with phage-resistant strains taking longer to completely lyse. For phage-resistant strains, we will often add higher levels of phage or more $CaCl_2$ to improve lysis (sometimes after several hours of slow shaking). At times, some cultures never completely lyse out. Even when this occurs, we have been successful using these filtered lysates to transduce both plasmids and chromosome alleles. Longer incubations may be needed to maximize lysis and when necessary we have placed the culture at 4 °C statically overnight following the 5–6 h at 30 °C.
6. It is important to use a 0.45 μm filter for filtration in order to minimize the possibility of unlysed cells contaminating the filtrate. A more typical 0.2 μm filter is not used in order to maximize the isolation of phage. For reference, φ11 heads are 50 nm in diameter and have a tail of 156 nm long (6), which equals 0.206 μm, close to the size of a 0.2 μm filter.
7. *M*ultiplicity *o*f *i*nfection (MOI) = # phage/# bacteria. While an MOI of 0.1 is suggested within the method, this needs to be determined empirically for each strain or application performed. For example, while an MOI of 0.1 may be sufficient for transferring a multi-copy plasmid, a higher MOI may be needed for moving chromosome fragments since, due to there being only one copy per cell, there will likely be fewer phage with the desired DNA. Therefore, for a given MOI, there will be fewer resulting positive transductants when moving chromosomal fragments than plasmids. In addition, the phage

sensitivity of *Staphylococcus* isolates varies greatly (Fig. 1), and a higher MOI will be needed for phage-resistant strains. However, it is important to keep the MOI as low as possible because if too many phage are added, there is a possibility of the strain becoming lysogenized by the phage. To determine if this has occurred, resulting transductants should be tested for phage sensitivity. This is done simply by titering the phage on the transductants. For example, if a diluted phage prep forms equal number of plaques on the parent and transductant strains, then it is not lysogenized. However, if the transductant strain produces fewer plaques, then it is likely lysogenized. Alternatively, lysogeny may be observed when streaking a strain for isolated colonies. In this case, there will often be gaps or plaques that form in the heavy streak due to the lysogenized phage entering a lytic cycle in a percentage of the cells.

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Bacteriophage Transduction in *Staphylococcus aureus*

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Abstract

The genetic manipulation of *Staphylococcus aureus* for molecular experimentation is a valuable tool for assessing gene function and virulence. Genetic variability between strains coupled with difficult laboratory techniques for strain construction is a frequent roadblock in *S. aureus* research. Bacteriophage transduction greatly increases the speed and ease of *S. aureus* studies by allowing movement of chromosomal markers and plasmids between strains. This technique enables the *S. aureus* research community to focus investigations on clinically relevant isolates.

Keywords: *Staphylococcus aureus*, Bacteriophage, Transduction, Bacteriophage 80α, Bacteriophage Φ11

1 Introduction

Norton Zinder and Joshua Lederberg discovered transduction in 1951 (1). In transduction, DNA is transferred from one bacterium to another via bacterial viruses known as bacteriophage. The life cycle of bacteriophage reproduction uses the bacterial cell replicational, transcriptional, and translation machinery to make new virions. This process is a relatively low fidelity event during which pieces of bacterial chromosome (or a plasmid) are accidentally packaged into the bacteriophage capsid. The bacteriophage then undergoes a lytic cycle, which creates a bloom of new particles upon lysis of the host cell (2, 3). By taking advantage of the bacteriophage's accidental DNA packaging researchers are able to manipulate recipient strains by selecting for the movement of antibiotic resistance markers or other genetic determinants.

Generalized transduction is the process by which any bacterial gene may be transferred to another bacterium via a bacteriophage. This is in contrast to specialized transduction, which is restricted on the DNA the bacteriophage can move. Generalized transduction occurs by a headful packaging mechanism, followed by infection of the recipient bacterial host and recombination onto the chromosome. The use of transduction in molecular microbiology labs relies on lytic bacteriophages, as opposed to a lysogenic cycle where the bacteriophage DNA integrates into a specific site in the host

chromosome and remains dormant. During the lytic cycle of infection, the virus takes control of the bacterial cell's machinery to replicate its own DNA and produce more viral particles. In headful packaging, the bacteriophage fill their capsid with viral genetic material, but low frequency accidental packing of bacterial plasmid or chromosomal DNA into the viral capsid allows generalized transduction to occur. Following a second round of lytic infection, the bacteriophages infect recipient bacteria and inject the foreign DNA (viral and bacterial) into the cells. In this transduction event, the transferred bacterial DNA can integrate into the recipient bacterium's genome through homologous recombination or recircularize into a replicating plasmid (3). The final result is the movement of bacterial genetic information from one strain to another.

In nature, transduction is especially important, as it is a mechanism through which antibiotic-resistance genes are exchanged between bacteria (2, 4). Horizontal gene transfer allows for advantageous traits to be sampled by a bacterium within its own unique genetic makeup. In addition, bacteriophage are also responsible for the mobilization of *Staphylococcus aureus* pathogenicity islands (SaPIs), which encode major toxin genes, such as the toxic shock syndrome toxin 1 and other superantigens (2). SaPIs are discrete chromosomal DNA segments that have been acquired by horizontal transfer and are widespread in *S. aureus* genomes (5). The vast majority of *S. aureus* phages known so far are double-stranded DNA phages belonging to the *Siphoviridae* family of the *Caudovirales* order (6). In general, they are temperate phages detected as prophage inserted in the chromosome, some of them being lytic due to mutations in the lysogeny functions (7). According to the morphological classification, staphylococcal *Siphoviridae* are composed of an icosahedral capsid and a non-contractile tail ended by a base-plate structure (8).

Early uses of bacteriophage included typing of clinical isolates in the early 1960s (9–11). In the laboratory, transduction has been adapted as a method for transferring genetic material in order to manipulate *S. aureus*. Here we outline a straightforward method for generalized transduction of chromosomal markers or plasmids in *S. aureus* using either bacteriophage 80α (12) or bacteriophage Φ11 (13).

2 Materials

1. 13 × 100 mm tryptic soy agar (TSA)/brain heart infusion (BHI) slants.
2. Tryptic soy broth (TSB) + 5 mM $CaCl_2$.
3. Petri plates.

4. 15 mL conical tubes.
5. TSA (1.5 % agar) + 5 mM $CaCl_2$.
6. TSA (1.5 % agar) + 500 mg/L NaCitrate + antibiotic of choice.
7. Soft agar TSA (0.5 % agar) + 5 mM $CaCl_2$.
8. 0.5 M $CaCl_2$.
9. 0.02 M NaCitrate.
10. Antibiotic of choice; typical antibiotics and concentrations include erythromycin 10 μg/mL, chloramphenicol 10 μg/mL, trimethoprim 10 μg/mL, tetracycline 2–10 μg/mL, kanamycin 50 μg/mL.
11. Centrifuge.
12. Incubators—Static and shaking.
13. Water bath at 50 °C.

3 Methods

3.1 Bacteriophage Propagation

Transduction of plasmid and chromosomal markers of interest requires a phage titer of approximately 10^{10} pfu/mL. As phage titers gradually decrease during storage at 4 °C, it is appropriate to propagate phage to acquire a high titer (10^{10}) before the transducing lysate is generated.

1. Grow *S. aureus* propagation strain on 13 × 100 mm TSA slant overnight at 37 °C. Ensure that a plasmid-free strain of *S. aureus* is used that is susceptible to the phage of choice. Strain RN4220 is recommended as it is both a good recipient and propagation strain for either phage 80α or Φ11 and allows for optimal phage titers (10^{10} PFU—Plaque Forming Units; (*see* **Note 1**)).
2. Resuspend *S. aureus* propagation strain in 1 mL TSB + 5 mM $CaCl_2$ (*see* **Note 2**).
3. Add 4 mL TSA soft agar to ten conical tubes (15 mL). Hold in 50 °C water bath to prevent agar from solidifying.
4. Serially dilute bacteriophage stock tenfold to 10^{-10} in TSB + 5 mM $CaCl_2$.
5. Combine 10 μl *S. aureus* cells and 100 μl bacteriophage dilution in soft agar. Gently mix (do not vortex) and pour onto TSA + 5 mM $CaCl_2$ plates. Repeat for all ten bacteriophage dilutions. Fresh TSA plates work best to prevent soft agar from excess drying during phage propagation.

6. Incubate overnight (plates right side up) at 37 °C. Do not invert plates to ensure soft agar is maintained on the agar surface.

3.2 Harvest Bacteriophage and Titer Determination

1. Select up to three plates for bacteriophage harvest. Optimal plates will show near confluent lysis and minimal bacterial growth (Ideally the 10^{-3}–10^{-5} plates, but this depends upon the original titer of the bacteriophage stock).
2. Add 3 mL TSB to plates. Harvest bacteriophage by breaking up and scraping off soft agar with a plate spreader. Transfer resulting agar/TSB mixture to a 50 mL conical tube.
3. Disrupt agar as much as possible by gently pipetting up and down. Avoid bubbles, vortexing, and sonication as they mechanically sheer bacteriophage tails. This step facilitates the release of bacteriophage particles from agar encasement.
4. Centrifuge for 10 min at 10,000 × *g*.
5. Filter supernatant through 0.22 μm filter.
6. Store bacteriophage at 4 °C.
7. The titer of the resulting bacteriophage lysate should be determined by repeating the experiment outlined in Section 3.1; optimal bacteriophage titer should be approximately 10^{10} pfu/mL. In some cases, when the titer of the original bacteriophage stock is low, multiple propagation experiments may be required to acquire the desired titer of 10^{10} pfu/mL.

3.3 Preparation of Transducing Lysate

1. Repeat bacteriophage propagation and harvest protocol (Sections 3.1 and 3.2) using *S. aureus* strain of interest (either plasmid or chromosomal marker). Note that overnight growth may require 30 °C if using temperature sensitive plasmid (i.e., $pE194_{ts}$-derived). 10^{10} pfu/mL of the transducing lysate should be achieved to ensure an appropriate transduction frequency (~10^{-8}).

3.4 Transduction

1. Grow the strain to be transduction recipient overnight on a 13 × 100 mm TSA slant.
2. Resuspend recipient strain in 1 mL TSB + 5 mM $CaCl_2$.
3. Add 500 μL of the recipient strain suspension to a 50 mL tube.
4. Add 1.5 mL TSB + 5 mM $CaCl_2$.
5. Add 500 μL bacteriophage lysate (10^{10} pfu/mL) to tube (*see* **Note 3**).
6. Shake at 225 RPM for exactly 20 min at 37 °C (30 °C if transducing a temperature-sensitive plasmid).
7. Add 1 mL cold (4 °C) 0.02 M NaCitrate.
8. Centrifuge at 3,000 × *g*, 4 °C for 10 min.

9. Resuspend pellet in 1 mL cold (4 °C) 0.02 M NaCitrate.
10. Plate ten aliquots (100 μL/plate) of transduced cells to TSA + 500 mg/L NaCitrate + antibiotic plates.
11. Incubate at 37 °C (30 °C if transducing a temperature-sensitive plasmid).
12. Pick single colonies to streak for isolation on the TSA + 500 mg/L NaCitrate + antibiotic plates (*see* **Note 4**).
13. Confirm movement of plasmid/chromosomal marker by standard methods including: plasmid analysis, PCR, or Southern blot.

4 Notes

1. Determine if strain of choice is phage susceptible by first streaking the strain on TSA containing 5 mM $CaCl_2$. 10 μl of phage stock (10^{10} pfu/mL) is then spotted on the plate in the first quadrant and allowed to dry. The plate is then incubated at 37 °C for 24 h; an area of clearing (lysis) will be evident in susceptible strains.
2. Calcium chloride ($CaCl_2$) is added to the media to facilitate bacteriophage attachment to *S. aureus*. NaCitrate chelates the calcium, arresting the bacteriophage infectious cycle and preventing reinfection.
3. As a negative control to test for contaminated lysate, perform the transduction experiments using all components except the bacteriophage.
4. NaCitrate is required in these plates to chelate residual calcium in the TSA. Although individual transductant colonies are picked, bacteriophage titer on the plate is high enough that phage can be transferred to subsequent TSA plates resulting in partial lysis of colony growth if NaCitrate is not added.

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Batch Transduction of Transposon Mutant Libraries for Rapid Phenotype Screening in *Staphylococcus*

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Abstract

In the gram-positive pathogen *Staphylococcus aureus*, transposon mutagenesis is a useful method of screening large numbers of mutants for a given phenotype. However, constructing a transposon mutant library can take several months of work and validation in the laboratory. In this chapter, we describe a method for batch transduction of existing transposon mutations into new genetic backgrounds. Transduction in *S. aureus* is accomplished quickly and easily in most commonly used laboratory strains. The method described herein utilizes transduction to facilitate the rapid creation of new libraries and quick screening in strains containing phenotypic reporter constructs.

Keywords: Transposon mutagenesis, Transduction, Staphylococci, Mutant, *Staphylococcus aureus*

1 Introduction

Transposon mutant libraries containing mutations in large numbers of nonessential genes may be screened to discover new players in various processes. The methods for transposon mutagenesis in *Staphylococcus aureus* have been well described [1–4] and detail the production of new libraries. While this is an extremely useful research strategy, the identification of transposon insertions is laborious and expensive. In addition, in order to have confidence that all likely mutants have been screened, it is often prudent to screen a tenfold coverage of the genome. For example, with *S. aureus* having approximately 2500 genes, one would want to screen about 25,000 transposition mutants to ensure a likely coverage of all possible nonessential genes. However, access to an existing library can allow for more rapid screening to be accomplished by transduction of existing library mutations into a new genetic background rather than creating a library de novo. For example, existing mutations may be transferred into a strain featuring a downstream phenotypic reporter of gene expression, such as a promoter of interest driving expression of fluorescence produced by *gfp* or β-galactosidase activity encoded by *lacZ*. Transduction in *S. aureus* can be easily and quickly accomplished in most strains used in laboratory studies,

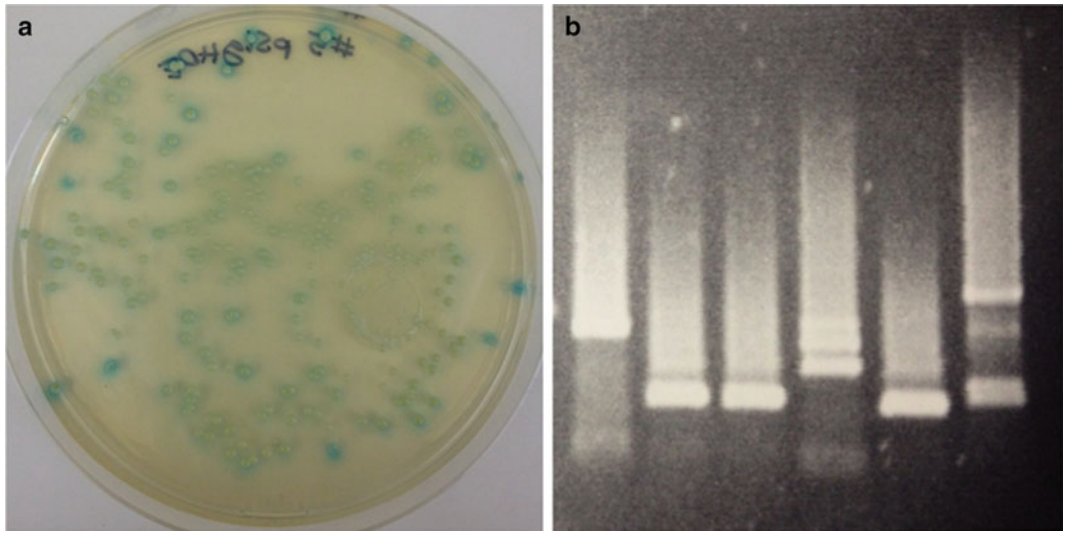

Fig. 1 (**a**) Representative plate showing screening of colonies resulting from the batch transduction of the existing NTML into a β-galactosidase reporter strain. *Blue colonies* were selected for further analysis. (**b**) The transposon insertion site of several colonies was determined by inverse PCR to validate the relatively equal transfer of transposon mutations by our batch transduction method

making it possible to screen a library of thousands of mutations in a short period of time. Importantly, while *S. aureus* is the focus here, this technique is possible for libraries in other staphylococci.

In this chapter, a novel technique will be described by which mutations from the Nebraska Transposon Mutant Library (NTML) [5] were transferred into a strain containing a chromosomal *lacZ* reporter construct for screening purposes. The end goal was to identify novel regulators of a promoter of interest without the need to generate a new transposon library of insertions. This was accomplished by transduction of the NTML mutations *en masse* into the chromosomally encoded reporter strain. The resulting transductants were plated on media containing X-gal and screened for increased β-galactosidase activity, indicative of increased promoter activity. Thus, it would be expected that colonies with blue color received a transposon insertion in a repressor of a transcriptional reporter (Fig. 1a). While this is a single example, the method described here may be modified for a variety of purposes and methods of screening.

In order to mobilize the entire library of mutations into a desired recipient strain, transducing bacteriophage such as 80α or Φ11 must first be propagated on groups of transposon mutants (*see* **Note 1**). In our laboratory, we accomplished this by utilizing groups of 96 mutants as they were in storage in 96-well plates. Each of the 21 groups of mutants was inoculated onto a single rectangular agar plate using a sterilized 96-prong replicator. Bacteriophage ϕ11 was propagated on each individual plate as described

below for a total of 21 unique lysates, each representing 96 mutants. This scheme was chosen to maximize the possibility of all transposon mutants being screened. All 1952 mutants could have been combined into a single transducing phage preparation; however, this may have decreased complete library coverage due to the frequencies of phage infection, improper packaging of chromosomal fragments into the phage, subsequent phage attachment to recipient cells, and recombination. Twenty-one transductions were performed to transfer the library mutations into the desired reporter strain. Using this method, we were able to screen nearly 2000 mutants contained in the NTML and identify new regulators of interest in a matter of a few weeks. It should be noted that the NTML only contains mutants in coding sequences and therefore screening using the NTML does not account for regulatory function of noncoding sequences such as regulatory RNAs. This procedure saved significant time over traditional transposon screening and allowed for the screening of almost all nonessential genes.

Although a variety of methods may be used for *S. aureus* phage propagation and transduction [6, 7], we have used the soft agar-based method here. The reason for this is that because the mutants of the transposon library were inoculated individually onto agar plates using the 96-prong replicator, each should be represented fairly equally in the phage lysate. If each mutant was inoculated into broth and allowed to incubate for an extended period of time, it is likely that individual mutants would outcompete the others and thus be overrepresented in the phage lysate. Additionally, in our method, the transduction mixture is incubated for only 20 minutes, thus minimizing the potential for overgrowth of individual mutants in the population. After batch transduction, randomness of transduced mutations was confirmed using inverse PCR to identify the transposon insertion site of several mutants (Fig. 1b).

2 Materials

All media and solutions should be prepared with deionized, distilled water and autoclaved or filtered.

1. Existing transposon mutant library stored in 96-well plates.
2. Recipient strain harboring phenotypic reporter (*see* **Note 2**).
3. Prepared phage lysate (ϕ11).
4. Brain heart infusion (BHI) agar slants.
5. Rectangular tryptic soy agar (TSA) plates.
6. Tryptic soy broth (TSB).
7. Tryptic soy broth +5 mM calcium chloride (prepared fresh).
8. 20 mM sodium citrate solution.

9. Soft agar (TSB containing 0.5 % agar).
10. TSA plates containing 5 mM calcium chloride (prepared fresh).
11. TSA plates containing 15 mM sodium citrate + selective antibiotics (*see* **Note 3**).
12. 37 °C stationary incubator.
13. 37 °C shaking incubator.
14. 50 °C water bath.
15. 0.45 μm filter.
16. Sterile 10–20 ml syringe.
17. Sterile 5 ml snap-cap tube.
18. Sterile 15 ml polypropylene conical tubes.
19. Empty Petri dishes.
20. Centrifuge.
21. 96-prong replicator.
22. Appropriate antibiotics.

3 Methods

3.1 Propagate Bacteriophage on Transposon Mutant Library

1. Using the flame-sterilized 96-prong replicator, inoculate the existing transposon mutant library onto rectangular TSA plates.
2. Grow for 24 h at 37 °C.
3. Store liquefied soft agar (0.5 % TSA) in 50 °C water bath until ready for use (*see* **Note 4**).
4. After transposon mutants have grown, carefully pipet 5 ml TSB onto each plate, and then scrape colonies off the agar into a suspension.
5. Pipet each suspension into an individual 5 ml snap-cap conical tube, ensuring that clumps of cells have been fully resuspended in the medium.
6. Repeat **step 5** for each plate of transposon mutants.
7. Prepare a serial dilution to 10^{-8} of phage ϕ11 lysate in TSB (*see* **Note 5**).
8. In a 5 ml snap-cap tube:
   (a) Pipet 10 μl of the library cell suspension onto the side.
   (b) Add 4 ml warm soft agar.
   (c) Add 100 μl of the corresponding dilution of phage.
9. Mix by inverting a few times, and then dump the soft agar mixture onto a fresh TSA plate containing 5 mM calcium chloride.
10. Repeat **steps 8** and **9** for each phage dilution.
11. Incubate plates, upright, overnight at 37 °C.

3.2 Harvest Bacteriophage Propagated on Transposon Mutant Library

1. After about 24 h, plates containing dilutions 10^{-1} to about 10^{-4} should be completely lysed with minimal bacterial overgrowth. Select 2–4 plates with near-confluent lysis to harvest.
2. Pipet 3 ml TSB onto each plate to be harvested.
3. Using a flame-sterilized glass spreader or scoopula, gently scrape the soft agar off the top of the plate. This portion contains the bacteriophage along with lysed host cells.
4. Transfer soft agar/TSB mixture to a screw-cap 15 ml conical tube using a pipet with the end of the tip cut off, pipetting up and down to break up agar and facilitate the release of the phage from the agar. Avoid bubbles and do not vortex, as this will shear phage tails and hamper its activity.
5. Centrifuge the soft agar mixture for 10 min at 5900 × *g*.
6. Following centrifugation, the agar and lysed cells should form a thick layer at the bottom of the tube. Carefully separate the cleared supernatant and pass through a 0.45 μm filter into a new 15 ml screw-cap conical tube for storage.
7. Store bacteriophage at 4 °C until ready to perform transduction (*see* **Note 6**).

3.3 Transduction of Mutations from Transposon Mutant Library into Desired Recipient Strain

1. Grow the desired recipient strain overnight at 37 °C on a BHI slant.
2. The following day, pipet 1 ml TSB + 5 mM calcium chloride onto the slant and scrape off cells into a suspension.
3. Transfer the suspension to a microcentrifuge tube.
4. Pipet 1.5 ml TSB + 5 mM calcium chloride into a 15 ml screw-cap conical tube.
5. Add 500 μl cell suspension along with 500 μl of the phage lysate prepared on the transposon mutant library in previous steps.
6. Incubate the transduction mixture for 20 min at 37 °C with shaking at 250 rpm.
7. Add 1 ml cold (4 °C) 20 mM sodium citrate solution to each transduction tube.
8. Pellet the cells by centrifuging at 5900 × *g* for 10 min.
9. Suspend the pellet in 1 ml cold 20 mM sodium citrate.
10. Spread plate 100 μl of the suspension on each of the 10 plates containing TSA + 15 mM sodium citrate, selective antibiotics, and additives for screening (if appropriate) (*see* **Note 7**).
11. Incubate transduction plates at 37 °C for 24–48 h.
12. Use appropriate screening methods for the reporter used to determine mutants of interest.
13. Identify the location of transposon insertion in mutants of interest by inverse PCR as previously described (*see* **Note 8**).

4 Notes

1. It should be noted that phage packages specific lengths of DNA into the phage head during assembly. Often, this can encompass 20–30,000 base pairs. Recombination can occur between homologous DNA anywhere within this region. If a chromosomally encoded reporter is used, it is possible for transposon mutations close to the reporter to yield false-negative results due to disruption or replacement of the reporter.
2. For our studies, we used β-galactosidase activity for screening as it is easily visible on an agar plate (as opposed to fluorescence, for example). It is also worth considering when you expect your downstream marker to be expressed; it may not work well to use antibiotic resistance as your phenotype of choice if the reporter gene driving its expression is only expressed during a certain part of the growth cycle.
3. Plates for selecting transductants should contain sodium citrate (to chelate calcium and thus stop phage adsorption to the recipient cell membrane) as well as antibiotics to select for the transposon library mutations. *Bursa aurealis*, for example, contains an erythromycin resistance cassette.
4. If the soft agar has been stored at room temperature and solidified, it may be re-heated on a hot plate or in the microwave at low power. It is not advisable to re-heat soft agar more than once or twice, as it becomes more concentrated each time and is more difficult to harvest the phage.
5. For our studies, we used bacteriophage ϕ11. However, depending on the lysogeny of the strain being used, other phage such as 80α would also work well.
6. At this point, a library of phage lysates encompassing the entire transposon mutant library being utilized will have been generated. This library may be stored for several months and used again, although phage titers will diminish over time.
7. As we used β-galactosidase activity for screening our library, we included X-gal at a final concentration of 40 μg/ml in our media. Any screening protocol used should be optimized before performing batch transduction as described in this chapter.
8. Once mutants with a phenotype of interest have been selected, they must be identified and confirmed as previously described using inverse PCR [2, 5].

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Conjugative Transfer in *Staphylococcus aureus*

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Abstract

The acquisition of plasmids has led to a significant increase in antimicrobial resistance within the staphylococci. In order to study these plasmids effectively, one must be able move the plasmid DNA into genetically clean backgrounds. While the smaller staphylococcal class I (1–5 kb) and class II (10–30 kb) plasmids are readily transferred using bacteriophage transduction or electroporation, these methods are inefficient at moving the larger class III (30–60 kb) plasmids. This review describes methods to transfer class III plasmids via conjugative mobilization.

Keywords: Conjugation, Plasmid, Staphylococci

1 Introduction

Resistance to multiple classes of antibiotics observed in staphylococci can be attributed to the acquisition of plasmid-encoded antimicrobial resistance genes acquired via horizontal gene transfer. Staphylococcal plasmids, first discovered in the early 1960s, are characterized into three general classes: (1) Class I plasmids are high-copy number plasmids approximately 1–5 kilobases (kb) in size, replicate via rolling circle replication, and usually encode a single antibiotic or heavy metal resistance gene. Examples of class I plasmids include pT181 which is 4.4 kb in size and has a copy number of 22, pC194 which is 2.9 kb and has a copy number of 15, and pE194 which is 3.7 kb and has a copy number of 55 [1]; (2) Class II plasmids have low copy numbers, are usually 10–30 kb in size, replicate via theta replication, and encode inducible resistance to β-lactam and macrolide antibiotics, along with resistance to an assortment of heavy metals. pI524 (31.8 kb) and pI258 (28.2 kb) are examples of class II plasmids, each having a low copy number of 5 [1]; (3) Class III plasmids are large 30–60 kb plasmids with low copy numbers, replicate via theta replication, encode multiple antibiotic resistance genes, and are usually capable of conjugative transfer [2, 3]. pGO1 is a typical class III plasmid that is 52 kb in size and has a low copy number [1].

To determine the resistance properties and other characteristics of staphylococcal plasmids carried by clinical isolates, one must be

able to transfer them into genetically clean (i.e. strain containing no plasmids or antibiotic resistance) backgrounds, such as RN450 (also called 8325-4) [4]. Plasmid DNA is most commonly introduced into *Staphylococcus aureus* by electroporation or bacteriophage transduction. Typically, these methods are efficient at moving small plasmids (class I and II plasmids) between staphylococcal strains. However, transfer and subsequent investigation of larger class III plasmids is not possible using electroporation (due to size) and, in many cases, transduction of these plasmids is also not efficient due to size restriction; typical staphylococcal transducing phage such as φ11 or 80α can efficiently transduce approximately 35–40 kb. In many cases, conjugative plasmids transferred via transduction have significant deletions. Thus, transfer of larger plasmids, such as class III plasmids, requires use of conjugative transfer systems. Staphylococcal conjugation, first described in 1983 by Forbes and Schaberg [5], and confirmed by McDonnel et al. [6] was initially studied due to its linkage with gentamicin resistance (Gm^r). Class III plasmids encoding Gm^r are commonly found in *Staphylococcus epidermidis* isolates, and were responsible for the spread of Gm^r in *S. aureus* in the early 1980s in the United States [7–11].

Bacterial conjugation is a mechanism of horizontal gene transfer in which plasmid DNA is transferred between bacterial cells via cell-to-cell contact. Staphylococcal conjugative plasmids encode the conjugative transfer region, *tra*, allowing for self-transmission among strains [12]. These self-transmissible plasmids can be transferred between nonlysogenic strains of *S. aureus* at low frequencies in broth and more efficiently by filter mating on an agar medium [8, 13]. Comparative analysis of conjugative plasmids from both *S. aureus* and *S. epidermidis* have demonstrated that many of these plasmids are closely related and encode similar 12–15 kb regions involved in plasmid transfer [13]. Conjugative plasmids also have the ability to mobilize smaller antibiotic resistance plasmids that are not self-transmissible. In fact, conjugative mobilization is an efficient method to introduce plasmid DNA into *S. epidermidis* where electroporation and transduction are not as efficient [8, 13, 14]. In summation, while electroporation and bacteriophage-mediated transduction are the most common ways to transfer plasmid DNA in *S. aureus*, conjugative transfer is the most practical method to transfer large (40–60 kb) antibiotic resistance plasmids from *S. aureus* isolates into strains with genetically clean backgrounds for subsequent study. The conjugative transfer experiment listed below can be viewed as a control experiment and includes RN450/pGO1 [12, 15–18] as the donor and RN450 NR (RN450 resistant to both novobiocin and rifampin) as the recipient.

2 Materials

1. Tryptic soy agar (TSA).
2. Donor *S. aureus* strain (here, RN450 containing pGO1).
3. Recipient *S. aureus* strain (here, RN450 NR).
4. Sterile 0.9 % NaCl.
5. Nitrocellulose membrane filters (0.22-μm pore size, 13 mm diameter).
6. Spectrophotometer.
7. 1.5 mL microcentrifuge tubes.
8. Tryptic soy agar (TSA; four plates each) plates supplemented with:
   (a) Gentamicin (5 μg/mL)
   (b) Novobiocin (1 μg/mL) and Rifampin (10 μg/mL)

3 Methods

1. Grow the donor (*S. aureus* RN450/pGO1) and recipient strain (*S. aureus* RN450 NR) overnight on a TSA plate at 37 °C (*see* **Note 1**).
2. Scrape overnight cultures and resuspend in sterile 0.9 % NaCl to an OD_{600} of 1.0.
3. Pellet 1 mL volumes of the donor and recipient strains in microcentrifuge tubes (*see* **Note 2**).
4. Resuspend pellets in 100 μL of sterile 0.9 % NaCl.
5. Pipette both cell suspensions onto a 0.22-μm nitrocellulose filter membrane and place inverted onto a TSA plate.
6. Incubate overnight at 37 °C (*see* **Note 3**).
7. The following day, remove the filter with sterile forceps and suspend the filter in 1 mL sterile 0.9 % NaCl or TSB (tryptic soy broth) and vortex to remove cells from membrane.
8. Make serial dilutions and plate onto TSA plates containing appropriate selective antibiotics (*see* **Note 4**).
   (a) Plate 10^{-5} through 10^{-8} (final dilutions) on TSA containing gentamicin for donor CFU.
   (b) Plate 10^{-5} through 10^{-8} (final dilutions) on TSA containing rifampin and novobiocin for recipient CFU.
   (c) Plate 10^{-1} through 10^{-4} (final dilutions) on TSA containing gentamicin, rifampin, and novobiocin for transconjugant CFU.

4 Notes

1. One common method to generate antibiotic counter selection in staphylococcal genetics is to make a recipient strain resistant to novobiocin and rifampin. This is easily performed by streaking your recipient strain of choice (~10^{10} cells) on a TSA plate containing 10 μg/mL of rifampin. Following incubation at 37 °C for 48 h, single colonies are picked and struck again onto a TSA plate containing 10 μg/mL of rifampin to ensure rapid and luxuriant growth. To generate rifampin- and novobiocin-resistant cells, approximately 10^{10} cells of the rifampin-resistant population of the recipient strain is struck onto a TSA plate containing 1 μg/mL of novobiocin and incubated at 37 °C for 48 h. Single rifampin- and novobiocin-resistant colonies are then picked and confirmed on TSA containing rifampin (10 μg/mL) and novobiocin (1 μg/mL). Rifampin and novobiocin resistance is generated via point mutations in either RNA polymerase β-subunit (*rpoB*) or gyrase B subunit (*gyrB*), respectively [19, 20]
2. The recipient and donor should have approximately the same number of viable cells for optimal conjugation frequency (~10^9 CFU)
3. Alternatively, the conjugation experiment can be performed using a Swinnex® filter device with a 0.45 μm nitrocellulose membrane. Note that the increased pore size allows for more efficient concentration of the staphylococci on the membrane (otherwise, the device becomes clogged too easily). Use an OD_{600} of ~0.5 for both the donor and recipient strain if a Swinnex® filter device is used to ensure that the filter does not become clogged.
4. The donor and recipient subpopulation in the conjugation experiment should be similar (~10^9 CFU/mL). It is also important to know these populations to calculate the transconjugation frequency; the transconjugation frequency of pGO1 is ~10^{-5}.

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Allelic Exchange

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Abstract

Methods used to understand the function of a gene/protein are one of the hallmarks of modern molecular genetics. The ability to genetically manipulate bacteria has become a fundamental tool in studying these organisms and while basic cloning has become a routine task in molecular biology laboratories, generating directed mutations can be a daunting task. This chapter describes the method of allelic exchange in *Staphylococcus aureus* using temperature-sensitive plasmids that have successfully produced a variety of chromosomal mutations, including in-frame deletions, insertion of antibiotic-resistance cassettes, and even single-nucleotide point mutations.

Keywords: Allelic exchange, Mutation, Homologous recombination, Cloning

1 Introduction

Chromosomal mutations made via allelic exchange have proven to be an essential technique to understanding basic physiological and pathogenic strategies used by bacteria. Allelic exchange is defined as the replacement of a specific region of DNA by homologous recombination. These replacements can take many forms, with the most common being replacement with an antibiotic-resistance cassette or complete deletion of a region of the chromosome. Successful allelic exchange requires adequate homologous DNA (*see* **Note 1**) and makes use of the recombination machinery of the cell to mediate the recombination events. The methods outlined below will describe allelic exchange using temperature sensitive shuttle vectors in *Staphylococcus aureus* (Table 1). The plasmids used for recombination are shuttle vectors that have an *Escherichia coli* origin of replication to aid in the cloning, along with a temperature-sensitive *S. aureus* origin from pE194. The temperature sensitive origin of replication replicates at the permissive temperature of 30 °C, but not at higher temperatures. This allows for temperature shifts to be used for selection of integration and recombination events. These vectors also include Staphylococcal elements for selection and/or screening along with the nucleotides encoding the desired nucleotide changes. There are many considerations to weigh when designing the recombination vector. These

Table 1
Suggested plasmids

Plasmid	Characteristics		Source
	S. aureus	*E. coli*	
pCL10	$oriV^{TS}$, Chl^r	$oriV^{HC}$, Amp^r	(1)
pCL52.2	$oriV^{TS}$, Tet^r	$oriV^{LC}$, Spt^r	(1)
pJB38	$oriV^{TS}$, Chl^r, Atet	$oriV^{HC}$, Amp^r	(2)
pKOR1	$oriV^{TS}$, Chl^r, Atet	$oriV^{HC}$, Amp^r, Chl^r	(3)

$oriV^{TS}$ Temperature-sensitive origin, *$oriV^{LC}$* low-copy origin, *$oriV^{HC}$* high-copy origin, *Atet* anhydrotetracycline-inducible counterselection, *Amp^r* resistance to ampicillin, *Chl^r* chloramphenicol, *Spt^r* spectinomycin, and *Tet^r* tetracycline

include the type of mutation you wish to make, such as point mutations, in-frame deletions, marked-deletions, and insertions for complementation. One must also have an understanding of the effects the mutation will have on expression of neighboring genes.

2 Materials

1. Tryptic soy broth (TSB).
2. Tryptic soy agar (TSA).
3. Selective antibiotic.
4. 44 °C stationary incubator.
5. 30 and 37 °C shaking and stationary incubator.
6. Appropriate plasmid (see Table 1).
7. DNA polymerase for PCR.
8. Restriction endonuclease(s).
9. DNA ligase.
10. Allelic exchange plasmid in strain of interest (*see* **Note 2**).

3 Methods

The process of allelic exchange begins with the introduction of the recombinant plasmid into the strain of interest. To encourage the first step of recombination, the cultures are subjected to a high temperature (44 °C) in the presence of an antibiotic that will maintain selection for the plasmid. This temperature is not permissive for replication of the plasmid, enriching for colonies in which the plasmid has recombined into the chromosome using a

mechanism comparable to Campbell-type recombination (4). The colonies in which the plasmid has recombined into the chromosome are often referred to as single recombinants (Fig. 1, steps 1 and 2). Selected single recombinants are then subcultured for multiple days at 30 °C to encourage the second recombination event and loss of the plasmid. Following this second recombination event, the cells are referred to as double recombinants. Importantly, the second recombination event can result in the restoration of the original wild-type allele or the desired allelic exchange mutation depending on where the recombination event occurs (Fig. 1).

1. Day 1: In the late afternoon, streak strain (not for isolated colonies, but a single streak over the entire plate) with allelic exchange plasmid onto selective media from freezer stock.
2. Day 2: The next morning, differentiate large colonies from small colonies (Fig. 2). Large colonies are likely to be the single

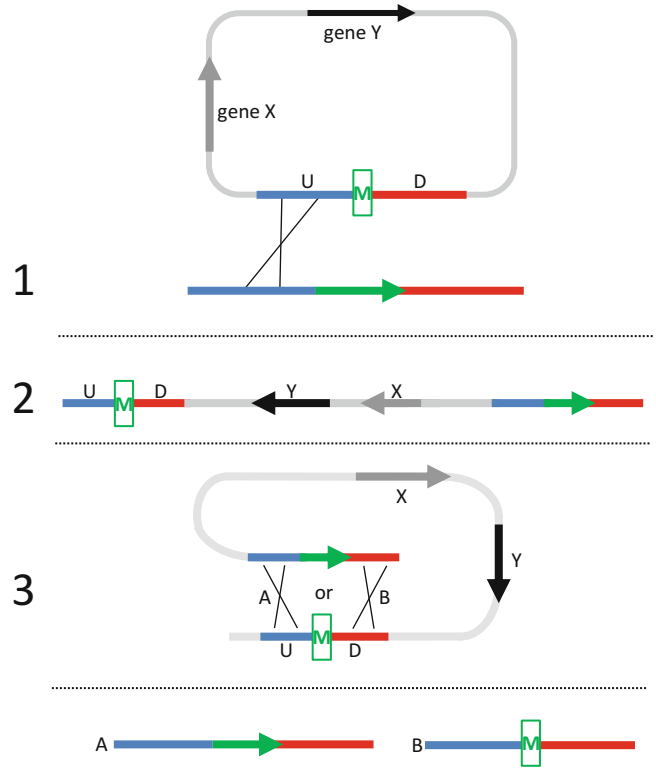

Fig. 1 Allelic exchange. Homologous recombination (*1*) occurs between the DNA in the upstream regions (*blue*), or the downstream regions (*red*, not shown), of the target gene and the recombinant plasmid resulting in the integration of the plasmid into the chromosome (*2*). A second recombination event (*3*) can either occur between the upstream regions or the downstream regions resulting in the regeneration of the wild-type allele (A) or the formation of the mutant allelic exchange construct (B), respectively

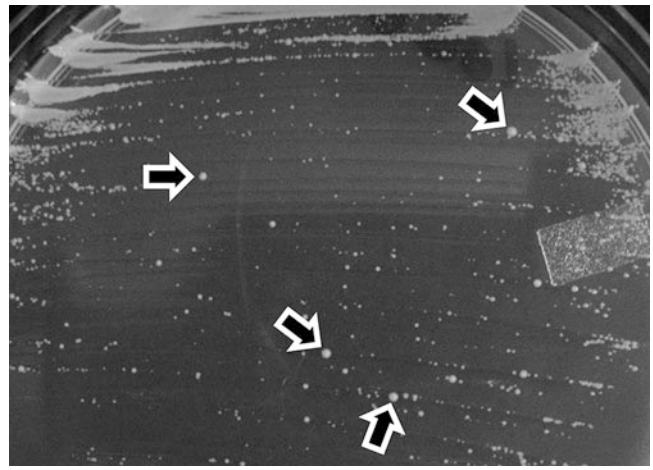

Fig. 2 Picture of a representative colonies following incubation at the non-permissive temperature of 44 °C. Note (*arrows*) the colonies that are larger than most are likely the desired single recombinants

recombinants. Select 2–4 large colonies and restreak (for isolated colonies) on select antibiotics and grow overnight at 44 °C (*see* **Note 3**).

3. Day 3: Inoculate a single colony into TSB and incubate overnight at 30 °C, shaking at 250 rpm.
4. Day 4: Subculture the bacteria by inoculating a fresh tube of media with a 1:1,000 dilution of the overnight culture. Incubate at 30 °C, shaking at 250 rpm.
5. Day 5: Subculture the bacteria by inoculating a fresh tube of media with a 1:1,000 dilution of the overnight culture. Incubate at 30 °C, shaking at 250 rpm.
6. Day 6: Subculture the bacteria by inoculating a fresh tube of media with a 1:1,000 dilution of the overnight culture. Incubate at 30 °C, shaking at 250 rpm. In addition, serial dilute the bacteria to 10^{-7} and 10^{-8} and plate on TSA. Grow plates at 37 °C (*see* **Note 4**).
7. Day 7: Repeat steps of day 6. In addition, replica-patch colonies from Day 6 dilution plating onto TSA and TSA with antibiotic corresponding to the resistance encoded by the recombinant plasmid. Incubate plates at 37 °C.
8. Day 8: Check plates for double recombinants. The double recombinants will grow on TSA, but not on TSA with antibiotic because they will have lost the plasmid. Confirm mutation by PCR (see chapter entitled Rapid Isolation of DNA for Staphylococci). If PCR does confirm the mutation, restreak colony for freezer stocks. If PCR does not confirm the mutation, replica-patch colonies from Day 7 dilution plating onto TSA and TSA with selective antibiotic. Incubate plates at 37 °C.

9. Day 9: This step is only necessary if there are no antibiotic sensitive colonies on Day 8 or if only wild-type colonies are present. If necessary, repeat steps from Day 8, with the exception of replica patching.
10. Confirm mutations as outlined by **Note 5**.

4 Notes

1. Allelic exchange requires homologous DNA to initiate the recombination process, and thus, the length of the DNA fragments used has a role in dictating the efficiency of recombination. It is recommended to use between 500 and 1,000 bp of homologous DNA flanking the site of the mutation to increase the frequency of mutations. Thus, when designing a plasmid for gene deletion, 500–1,000 bp upstream and downstream fragments are suggested. For point mutations, a 2 kb fragment centered on the mutation site is recommended.
2. Generating recombinant plasmids for various allelic exchange mutations

   Deletion constructs. There are multiple techniques available for generating deletion constructs. A simple method of PCR amplification of the flanking DNA and the replacement of a desired sequence of DNA with a restriction endonuclease recognition site is presented here. This method can be used to delete any size of DNA fragment, but the example only deletes a single gene. As noted above, 500–1,000 bp of DNA upstream and downstream of the deletion site is optimal for recombination events, so the first step in generating a deletion construct is to design primers that will amplify approximately 500–1,000 bp upstream and downstream of the deletion site. The primers will amplify the upstream fragment flanked by restriction endonuclease recognition sites for two different restriction endonucleases (designated R1 and R2). Conversely, the downstream PCR-amplified DNA fragment will be flanked by the R2 restriction endonuclease recognition site as well as a third restriction endonuclease recognition site (designated R3). If it is desired to delete the entire open reading frame (ORF), the start codon for the deleted ORF should be directly upstream of the R2 site on the upstream fragment and the stop codon directly downstream of the R2 site on the downstream fragment. This will allow the start and stop codon to remain in the same translation reading frame. The upstream and downstream fragments can then be cloned sequentially into the allelic exchange plasmid (Table 1).

   Marked deletion constructs. This is a similar procedure as the deletion constructs. To have a marked deletion, insert an

antibiotic cassette into the R2 restriction site created by the deletion construct.

Point mutation constructs. In some instances, rather than deleting an entire gene, it is useful to alter single amino acids to alter the function of the protein. Construction of point mutation allelic exchange plasmids begins with cloning the site of the mutation, flanked by approximately 1-kb (to promote recombination) on both sides into a small *E. coli v*ector such as pCR-Blunt (Invitrogen). Once the newly designed plasmid insert is sequenced, the next step is to amplify the entire plasmid with 5′-phosphorylated primers that encode the desired nucleotide change. More specifically, the primers are designed around the targeted site and face in opposite directions such that DNA replication progresses away from the mutation site and around the plasmid. One primer is designed to encode the desired nucleotide change near the 5′ end and the other primer designed to the complementary strand of DNA flanking the mutation (Fig. 3, option A). Importantly, the primers must contain the 5′ phosphate group (a standard modification offered by most venders) because T4 ligase requires a free 5′ phosphate for the subsequent ligation step. Once successful PCR amplification is confirmed by agarose gel electrophoresis, the PCR-product is self-ligated with T4 ligase, resulting in a circularized plasmid. After ligation, there are two different plasmids present, the original template plasmid, which does not encode the point mutation and the newly synthesized plasmid containing the point mutation. To remove the template (non-mutated) plasmid, the plasmids are digested with DpnI, which only recognizes methylated DNA (only the template plasmid is methylated). The DpnI treated sample is transformed into *E. coli,* and the isolated plasmids are verified by sequencing. The fragment containing the plasmid can then be moved to an allelic exchange vector. Alternatively, a mutation can be designed into two opposite-facing primers (Fig. 3, option B) that also amplify around the plasmid. Amplification continues around the entire plasmid, leaving a small unphosphorylated nick. Following PCR amplification, the mixture is DpnI-treated, and transformed into *E. coli*. The nick is then filled by the cell's native enzymes and therefore this approach does not require phosphorylated primers or ligation.

3. As a precaution, it is suggested that freezer stocks of individual single recombinants are made by growing up a colony in TSB with antibiotic at 44 °C until turbid. If the first round of allelic exchange does not result in a mutated allele, the single recombinants that were frozen can be used to inoculate a TSB culture for the first day of subculturing. This prevents having to restreak for single recombinants.

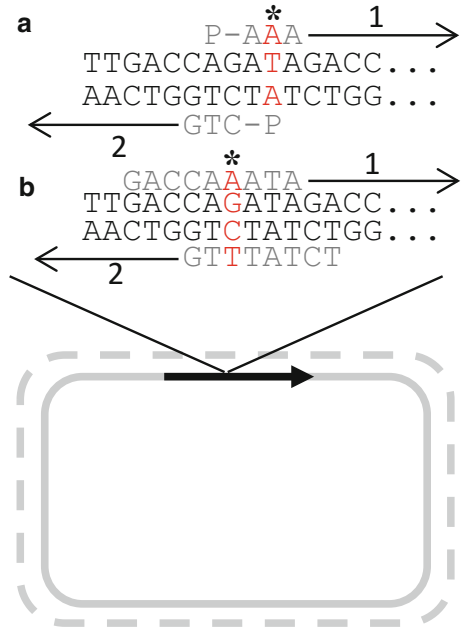

Fig. 3 Generation of point mutations. In *option A*, 5′ phosphorylated primers (1 and 2) are designed directly adjacent to each other but in opposite orientations such that when using a plasmid as the template they amplify the entire plasmid. Primer 1 encodes the desired nucleotide change ATA → AAA (noted by *asterisk*). Following self-ligation, the newly created plasmids are digested with DpnI, an enzyme specific for methylated DNA, to remove any remaining template plasmid. In *option B*, two overlapping primers (1 and 2) each contain a nucleotide change that would result in an AGA → AAA mutation (noted by *asterisk*), resulting in the amplification of the entire plasmid. DpnI treatment removes remaining template plasmid. The primers are not phosphorylated and do not require ligation prior to transformation

4. Some of the allelic exchange plasmids such as pKORI and pJB38 allow for the use of counterselection, to increase the likelihood of selecting colonies that have lost the plasmid. These plasmids encode an anhydrotetracycline-inducible promoter that makes an antisense *secY* transcript, which inhibits growth. When using these plasmids, on the first day of dilution plating (day 6), plate the overnights at a 10^{-7} final dilution on TSA supplemented with 100 ng/ml anhydrotetracycline. The following day, replica-patch the colonies onto TSA and TSA supplemented with antibiotic. The counterselection is helpful in determining potential double recombinants that have lost the plasmid, but is not perfect, and some large colonies (much like those in Fig. 2) may still have the plasmid present.
5. To screen deletion mutants, choose primers located outside of the region of potential recombination and PCR-amplify. The PCR product from the mutant should be smaller (by the

amount of DNA deleted) than the wild-type strain. Once you have confirmed the presence of the mutation, it is always important to sequence the entire region involved in mutagenesis to ensure that no unintended mutations were introduced during the recombination process. Screening point mutations can be a little more laborious. In an ideal scenario, the nucleotide change also alters a cut site in the DNA. For example, the nucleotides to be altered are part of a SalI recognition site, and once altered, the SalI recognition site is destroyed. To screen for the point mutant, PCR-amplify the region, and digest the PCR product with SalI. If the mutation is present, the PCR product will not cut with SalI, whereas the wild-type sequence will. In many cases, the nucleotides that are mutated do not result in an altered restriction endonuclease recognition site, and therefore there is no quick screen for the point mutation. In this scenario, PCR-amplify the region of recombination, and sequence a few of the potential mutants. Once the point mutation is confirmed, sequence the entire region involved in mutagenesis. It is always important to screen multiple double recombinants for the presence of the desired mutation. In theory, there should be 50 % wild-type and 50 % mutant alleles after screening due to the randomness of recombination, but this is not always the case. In some instances all of the alleles will be wild-type (non-mutated) or mutant. This result suggests that the second recombination event occurred in the early days of subculturing with an expansion of double recombinant clones. If the first day of patching results in a large amount of double recombinants that once screened prove to be wild-type, return to the single recombinant freezer stocks that were made (*see* **Note 3**) and repeat the subculture steps.

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Creation of Staphylococcal Mutant Libraries Using Transposon Tn917

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Abstract

Non-directed mutagenesis of the staphylococcal genome is a global approach that can be used to identify the genetic basis for phenotypes of interest as well as for identifying regulators of gene expression. One such approach that has been widely used in the study of *S. aureus* and *S. epidermidis* is transposon Tn917 mutagenesis to generate random libraries of mutants. This chapter describes the use of plasmid pLTV1 (containing Tn917-lac) to generate Tn917 transposon mutants. Through the use of temperature manipulation and antibiotic selection, staphylococcal strains harboring this plasmid can be effectively mutagenized to create random libraries amenable to subsequent phenotypic screening and identification of transposon insertion sites.

Keywords: Untargeted mutagenesis, Transposon, Tn917, Random mutagenesis

1 Introduction

Site-directed mutagenesis is a powerful tool to make precise and intended mutations, and is an essential step in characterizing bacteria. However, at times, a non-directed approach is needed to answer a specific question, or a more global approach is desired. For example, it may be important to identify what mutations affect mannitol fermentation, which can be viewed using indicator media. Or, using a reporter of gene expression, what mutations would alter the expression of a particular gene? Questions such as these cannot be accomplished using site-directed mutagenesis since the desired mutations are not known. Therefore, an unbiased approach to finding important mutations is needed. While there are several approaches to doing this, this chapter describes the use of Tn917 to make random mutations via transposon-mediated mutagenesis.

Transposons are mobile genetic elements with the ability to transpose or "hop" into the chromosome. The simplest of transposons can consist of a gene encoding the transposase (the enzyme which facilitates transposition) flanked by a pair of homologous DNA sequences that may be inverted repeats. Transposases allow

for two types of transposition: (1) a "replicative" mechanism whereby a copy of the transposon inserts into distal site of the chromosome, leaving the original copy in place; and (2) a "cut and paste" mechanism in which the transposon leaves the current site and integrates into a different location. Some transposons are very specific as to which sites they insert into, while other transposons hop randomly and are useful for mutagenesis studies. While transposons are naturally occurring and often contain advantageous genes such as those encoding antibiotic resistance, they have also been manipulated or made synthetically to be a practical tool for molecular biology. As a molecular tool for making mutant libraries, a transposon must have several important properties, including random-but-stable insertion into the chromosome, little preference for coding and noncoding sequences, and carriage of a selectable marker such as antibiotic resistance.

Tn917 was first described as a being a mobile element carrying erythromycin resistance in *Enterococcus faecalis* (then *Streptococcus faecalis*) (1, 2). This transposon contains three genes, *ermB*, *tnpR*, and *tnpA*, encoding an erythromycin ribosome methylase enzyme, resolvase, and transposase (3, 4), which are flanked by a 38 bp imperfect inverted repeat. An updated, corrected sequence of the entire transposon was subsequently submitted directly to GenBank by Flannagan and Clewell in 2002 (accession #M11180.2). To increase the utility of Tn917, the Tn917-lac variant was generated, containing the *E. coli lacZ* gene which can be used to measure the promoter activity of disrupted genes (5). While Tn917 does insert randomly, it does appears to have some preference to two locations within the staphylococcal genome (6). Two methods to control transposition of Tn917 have been utilized. First, transposition can be induced by growth in the presence of sub-inhibitory erythromycin (2). Secondly, Tn917 has been placed on various plasmids containing a gram-positive temperature-sensitive origin of replication, whereby plasmid replication can be controlled by growth at a permissive (30 °C) or non-permissive (43 °C) temperature (7, 8). Exposure to the non-permissive temperature for plasmid replication will lead to plasmid loss and, in the presence of antibiotic selection, only cells that have undergone Tn917 transposition will survive. Due to its ability to undergo transposition and generate relatively random and stable insertions, Tn917 has been widely used as an untargeted mutagenesis tool in a variety of gram-positive bacteria, including *Staphylococcus epidermidis* (7, 9–13), *S. aureus* (14–20), various *Bacillus* species (21, 22), *Listeria monocytogenes* (8), and *Streptococcus mutans* (23).

In this chapter, we provide a simple method for performing random transposon mutagenesis using Tn917-lac harbored on the plasmid pLTV1, created by Camilli et al. (8). This plasmid contains the pE194Ts temperature-sensitive replicon (24), a tetracycline resistance gene (*tet*) (25), and Tn917-lac (5). To facilitate

subsequent rescue-cloning and mapping of the transposon genomic insertions, the transposable Tn917-lac region of this plasmid has been engineered to contain a ColEl-derived replicon with the pBR322 β-lactamase gene (26), M13mpl9 polylinker (27), and *S. aureus* pC194-derived chloramphenicol acetyltransferase gene (19). Under the selective pressures of high temperature (non-permissive for plasmid replication) and erythromycin, Tn917-lac will transpose into the host cell chromosome, leaving behind pE194Ts and the *tet* resistance gene. In this manner transposon mutants can be identified with relative ease based on their resistance to erythromycin and sensitivity to tetracycline. The mutagenesis method presented in this chapter is based on previously published protocols used to create pLTV1-based transposon mutant libraries in both *S. epidermidis* (13) and *S. aureus* (20). Although not covered in this chapter, identification of transposon insertion sites can be subsequently performed as described elsewhere (13, 28).

2 Materials

1. Frozen (−80 °C) glycerol stock of *S. aureus* or *S. epidermidis* strain of interest containing plasmid pLTV1.
2. Sterile tryptic soy broth (TSB) containing 5 μg/ml tetracycline (TSB-Tet) (*see* **Note 1**).
3. Sterile TSB containing 10 μg/ml erythromycin (TSB-Erm) (*see* **Note 1**).
4. Sterile 50 ml culture tubes.
5. Tryptic soy agar (TSA) plates containing 10 μg/ml erythromycin (TSA-Erm).
6. TSA plates containing 5 μg/ml tetracycline (TSA-Tet).
7. Sterile enrichment media containing 10 μg/ml erythromycin (if selecting for specific phenotypes) (*see* **Note 2**).
8. 30 °C static incubator.
9. 30 °C shaking incubator.
10. 37 °C static incubator.
11. 37 °C shaking incubator.
12. 43 °C static incubator.
13. 43 °C shaking incubator.
14. Sterile tubes for preparing serial dilutions.
15. Sterile PBS or TSB for preparing serial dilutions.
16. Vortex mixer.
17. Sterile 50 % (vol/vol) glycerol.

18. Sterile cryogenic vials.
19. −80 °C freezer for storage of transposon library.

3 Methods

1. Streak *S. aureus* or *S. epidermidis* (containing plasmid pLTV1) from a frozen (−80 °C) glycerol stock onto TSA-Tet. Grow at 30 °C (permissive temperature for plasmid replication) for 24 h.
2. Pick a single isolated colony from the plate in step 1 and inoculate 10 ml TSB-Tet in a sterile 50 ml culture tube. Grow this culture for 24 h in at 30 °C (permissive temperature) and 250 RPM (*see* **Note 3**).
3. Dilute the culture from step 2 1:1,000 into 10 ml TSB-Erm in a sterile 50 ml culture tube (or in 10 ml enrichment media-containing Erm) and grow for 24 h at 43 °C (non-permissive temperature for plasmid replication) and 250 RPM (*see* **Note 2**).
4. Withdraw a 100 μl sample of the overnight culture from step 3, and prepare tenfold serial dilutions in sterile PBS or TSB (no antibiotic). Plate the entire dilution range on both TSA-Erm and TSA-Tet. Grow overnight at 37 °C. A significant reduction in tetracycline-resistant colonies observed on the serial dilution plates indicates that the majority of the clones in the library represent "true" transposon mutants (*see* **Note 4**).
5. Prepare replicate glycerol stock solutions of each overnight culture from step 3 by adding 0.5 ml of overnight culture to an equal volume of sterile 50 % glycerol in a cryogenic vial. Vortex on high speed to mix, and store library stocks at −80 °C.
6. Screen transposon mutant library for phenotypes of interest (*see* **Note 5**).

4 Notes

1. The recommended antibiotic concentrations for *S. aureus* growth are 5 μg/ml tetracycline and 10 μg/ml erythromycin. For *S. epidermidis*, 12.5 μg/ml tetracycline and 1 μg/ml erythromycin are the recommended concentrations for growth (13).
2. If interested in screening for specific phenotypes, such as deficiency in biofilm growth, an enrichment step can be included when growing the staphylococcal pLTV1 strain at the non-permissive temperature. For example, the transposon library could be enriched for biofilm deficient mutants by several

rounds of subculturing of the upper, planktonic phase of cultures grown statically in media promoting biofilm growth [TSB containing 0.75 % (wt/vol) glucose and 3.5 % (wt/vol) NaCl] at the non-permissive temperature in the presence of erythromycin. This strategy has been previously employed using Tn917-lac mutagenesis of *S. epidermidis* by pLTV1 (13).

3. It is recommended that several (up to 10) libraries be generated in parallel, in order to produce a greater number of independently derived mutants. This will help avoid potential over-representation of clonally derived isolates in the library that might otherwise be obtained from a single, large-scale mutagenesis experiment (13).
4. This step is to ensure that significant loss of tetracycline-resistance is observed, which indicates that true transposition has occurred in the majority of the recovered clones. It has been previously reported that this mutagenesis method results in about a 5 % retention rate of Tet-resistance (13). Potential reasons for this include clones that retained pLTV1 due to its loss of temperature sensitivity, or due to integration of the pLTV1 plasmid into the chromosome by homologous recombination (13).
5. When potential mutants are identified in subsequent phenotypic screens, loss of tetracycline resistance should be confirmed for each individual mutant of interest. Furthermore, the transposon mutation should be backcrossed into the wild-type staphylococcal strain by chromosomal phage transduction, in order to verify that the observed phenotype is due to the transposon mutation itself and not an unrelated second-site mutation in the genome. These quality-control checks should be completed prior to rescue-cloning (8, 13) and/or direct sequencing to identify the insertion site (28).

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Generation of a Transposon Mutant Library in *Staphylococcus aureus* and *Staphylococcus epidermidis* Using *bursa aurealis*

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Abstract

Transposon mutagenesis is a genetic process that involves the random insertion of transposons into a genome resulting in the disruption of function of the genes in which they insert. Identification of the insertion sites through DNA sequencing allows for the identification of the genes disrupted and the creation of "libraries" containing a collection of mutants in which a large number of the nonessential genes have been disrupted. These mutant libraries have been a great resource for investigators to understand the various biological functions of individual genes, including those involved in metabolism, antibiotic susceptibility, and pathogenesis. Here, we describe the detailed methodologies for constructing a sequence defined transposon mutant library in both *Staphylococcus aureus* and *S. epidermidis* using the mariner-based transposon, *bursa aurealis*.

Keywords: *Bursa aurealis*, Transposon, *Staphylococcus aureus*, *Staphylococcus epidermidis*

1 Introduction

Transposons have been exploited as tools to study gene function in a wide variety of bacteria, including pathogens like *Staphylococcus aureus* and *S. epidermidis*. Their ability to randomly insert into genome sequences make them ideal for the generation and analysis of gene function. However, their random insertion makes them impractical for the targeted mutagenesis of a gene of interest without the development of elaborate screening strategies. To overcome this limitation, a "brute force" strategy, involving the identification of the insertion sites of thousands of mutants by DNA sequencing, was developed in *Escherichia coli*, allowing for defined mutants to be arrayed in a microtiter plate format for the rapid retrieval of specific mutants on demand (1, 2). This "Keio Collection" has proved to be a valuable resource for the research community and has greatly streamlined the genetic analysis of this organism. Since the creation of this library, several other

sequence-defined transposon insertion libraries in other bacterial species have also been developed (1).

In the staphylococci, Tn917 and Tn551 have historically been the primary transposons used to conduct genetic analyses of these important pathogens, leading to great insight into their metabolic and pathogenic potential (3). Although these tools have been the mainstay of genetic research in these organisms, the fact that they exhibited significant bias to two regions in the chromosome (4) makes them impractical for the generation of sequence-defined libraries similar to those produced in other organisms. However, this limitation was overcome by the development of a mariner-based transposon system, designated *bursa aurealis*, in *S. aureus*, which was shown to exhibit much less insertional bias, and a random sequence-defined transposon mutant library in *S. aureus* was finally generated (4).

To date, two sequence-defined libraries have been constructed in *S. aureus* using the *bursa aurealis* system; one in strain Newman (4, 5) and the other in the USA300-derivative JE2 (6, 7). Both of these libraries have been very instrumental in conducting phenotypic screens, querying the genome for factors involved in nonessential pathways, virulence in models of infection and rapid dissection of the metabolic pathways (4, 7–9). The Nebraska Transposon Mutant Library (NTML) in *S. aureus* JE2 is a accessible to investigators (8) and contains a total of 1,952 mutants, representing approximately 90 % of the nonessential genes in the *S. aureus* genome. This chapter describes a detailed protocol to generate *bursa aurealis* insertion mutants in both *S. aureus* and *S. epidermidis* 1457 (4, 10).

2 Materials

Prepare all reagents using ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature (unless otherwise indicated).

2.1 Components Required for Heat Shock and Patching

1. Ultrapure water.
2. Tryptic soy agar (TSA).
3. Incubator and heat block set at 45.0 °C for *S. aureus* JE2 and 46.5 °C for *S. epidermidis* 1457.
4. Antibiotic stocks: chloramphenicol (10 mg/ml), erythromycin (2.5 mg/ml), and tetracycline (5 mg/ml).
5. 1.5 ml microcentrifuge tubes.

2.2 Components for Genomic DNA Isolation

1. Tryptic soy broth (TSB).
2. Erythromycin (2.5 mg/ml).

3. Shaking incubator set at 37 °C.
4. Fifty percent glycerol.
5. Colony Picker (V&P Scientific, Inc.) or toothpicks.
6. 1 ml 96 deep well polypropylene plate.
7. 2 ml 96 deep well plate.
8. Wizard Genomic DNA purification Kit (Promega, Madison, WI) or similar product.
9. 50 mM EDTA.
10. Lysostaphin (AMBI Products LLC, Lawrence, NY).
11. Tris–EDTA Buffer—[For 100 ml; 1 ml of 1 M Tris–HCl (pH 8.0), 0.5 ml of 0.5 M EDTA (pH 8.0), and 98.5 ml water].
12. 70 % ethyl alcohol.
13. Isopropyl alcohol (isopropanol).

2.3 Molecular Genetic Components to Confirm Transposon Insertion Site

1. 96-well PCR plates.
2. Semi-skirted 96-well PCR plates.
3. AciI restriction enzyme plus appropriate enzyme buffer.
4. Ligation master mix: 2.5 ml 10 × T4 ligase buffer (Monserate Biotechnology Group), 0.5 ml Dilution buffer (Monserate Biotechnology Group), 0.5 ml T4 ligase (Monserate Biotechnology Group); 1.5 μl nuclease-free water.
5. Taq polymerase (Monserate Biotechnology Group, San Diego, CA).
6. Forward primer (Buster) 5′-GCTTTTTCTAAATGTTTTTT AAGTAAATCAAGTACC-3′ (5).
7. Reverse primer (Martin ermR) 5′-AAACTGATTTTTAGTAAA CAGTTGACGATATTC-3′ (5).
8. Thermocycler.
9. ExoSAP-IT (GE healthcare Life Sciences).
10. 1 % agarose gel.

3 Methods

Staphylococcus aureus JE2 or *S. epidermidis* 1457 carrying pFA545 (encodes *bursa aurealis* transposase; (6, 7)) is transduced with phage φ11 or φ71, respectively, (11) that has been propagated on JE2 or 1457 carrying pBursa (encoding the *bursa aurealis*; (6, 7)). Following incubation at 30 °C (due to temperature sensitivity of both pBursa and pFA545), individual chloramphenicol (encoded by pBursa) and tetracycline (encoded by pFA545) resistant colonies are then subsequently heat-shocked using the following protocol (*see* **Note 1**).

3.1 Heat Shock to Cure Plasmids pBursa and pFA545 and Detect the Transposition Event

1. Aliquot 1 ml of sterile ultrapure water into ten 1.5-ml microcentrifuge tubes.
2. Place tubes in a heat block at 45.0 °C for *S. aureus* or 46.5 °C for *S. epidermidis* for at least 1 h.
3. With a sterile cotton swab, pick 1–2 transductants per tube and gently resuspend the colonies in the water to an OD_{600} value between 2 and 4 (*see* **Note 2**).
4. Plate 200 μl of the sample onto pre-warmed (45.0 °C for *S. aureus* or 46.5 °C for *S. epidermidis*) TSA plates containing erythromycin (25 mg/ml for *S. aureus;* 2.5 mg/ml for *S. epidermidis*) and incubate at 45.0 °C for *S. aureus* or 46.5 °C for *S. epidermidis* for 2 days (*see* **Note 3**).

3.2 Patching for Agar-Based Selection of Mutants

1. Following 2 days incubation at 45.0 °C for *S. aureus* or 46.5 °C for *S. epidermidis*, pick only those colonies that are distinct and large on a TSA plate containing the respective concentrations of erythromycin.
2. Patch the colonies obtained from heat shock experiment onto three TSA plates each with a different selection marker (erythromycin 2.5 μg/ml, chloramphenicol 10 μg/ml, and tetracycline 5 μg/ml) and incubate at 46.5 °C.
3. Pick only those colonies that are resistant to erythromycin but susceptible to chloramphenicol and tetracycline. These antibiotic phenotypes suggest that the isolate has lost both pBursa and pFA545 but *bursa aurealis* (encoding erythromycin resistance) has transposed randomly into the chromosome (*see* **Note 4**).

3.3 Identification of Transposon Insertion Sites by Inverse PCR and DNA Sequencing

3.3.1 Isolation of Genomic DNA by Modified Promega Wizard Genomic Prep Protocol

Day 1:

1. Fill the 1 ml, 96-well plate with 400 μl TSB with erythromycin (2.5 μg/ml).
2. Using the colony picker or toothpicks, inoculate the probable *bursa aurealis* mutants (erythromycin resistant; chloramphenicol and tetracycline susceptible) into the wells containing TSB with erythromycin (2.5 μg/ml) and shake vigorously (250 rpm) at 37 °C overnight.

Day 2:

1. Centrifuge the 96-well plate to pellet the cells (3,000 × *g* for 10 min).
2. Discard supernatant and resuspend pellets in 110 μl of 50 mM EDTA.
3. Add 10 μl of a 10-mg/ml solution of lysostaphin and mix vigorously until an evenly distributed cell suspension is obtained.

4. Incubate at 37 °C for 90 min (mixture should become gelatinous and translucent).
5. Add 600 μl Promega Nuclei Lysis Buffer and incubate the plate at 80 °C for 10 min
6. Cool to room temperature and then add 200 μl of Promega Protein precipitation solution. Vortex vigorously for 2 min and then place on ice for 10 min.
7. Centrifuge at 3,000 × *g* for 10 min.
8. Transfer supernatant (without disturbing pellet) into a 2-ml 96-well plate containing 600 μl ice cold isopropanol. Mix well by inverting the mixture until all components are evenly distributed.
9. Centrifuge at 3,000 × *g* for 10 min to collect precipitated DNA.
10. Discard supernatant, being careful not to disrupt the DNA pellet. Add 600 μl ice-cold 70 % ethanol and invert five to ten times to wash the pellet. Centrifuge at 3,000 × *g* for 10 min.
11. Discard ethanol wash and dry the pellet by leaving the plate open for 15–20 min until all ethanol is evaporated.
12. Rehydrate the DNA pellet in 100 μl TE buffer and incubate the plate at 65 °C for 1 h.
13. Store genomic DNA at −20 °C until further use.

3.3.2 Acil Digest of Genomic DNA and Ligation

1. Digests are performed in 20 μl total volume in a 96-well PCR plate. Add 17 μl genomic DNA, 2 μl 10× AciI restriction enzyme buffer, and 1 μl AciI restriction enzyme.
2. Incubate at 37 °C for 2 h and subsequently heat inactivate AciI at 65 °C for 20 min.
3. Aliquot 5 μl of ligation master mix into each reaction and mix (total volume 25 μl).
4. Incubate overnight at 4 °C.

3.3.3 Inverse PCR Protocol and Purification of Amplified DNA

Inverse polymerase chain reaction is used for the identification of the *bursa aurealis* insertion site within the genome using primers that anneal to two different regions on the transposon. After AciI digestion and ligation with T4 ligase, the genome is a collection of circular DNA fragments of various sizes. Some of these circular fragments will contain *bursa aurealis*, thereby supplying a known sequence, allowing primers to bind and amplify the entire circumference of the circular DNA molecule. PCR fragments are then sequenced to identify the *bursa aurealis*–genome junction site.

1. Add the following components together for the PCR reaction to amplify the *bursa aurealis*–genome junction site. 5 μl DNA digested with AciI and ligated with T4 DNA ligase,

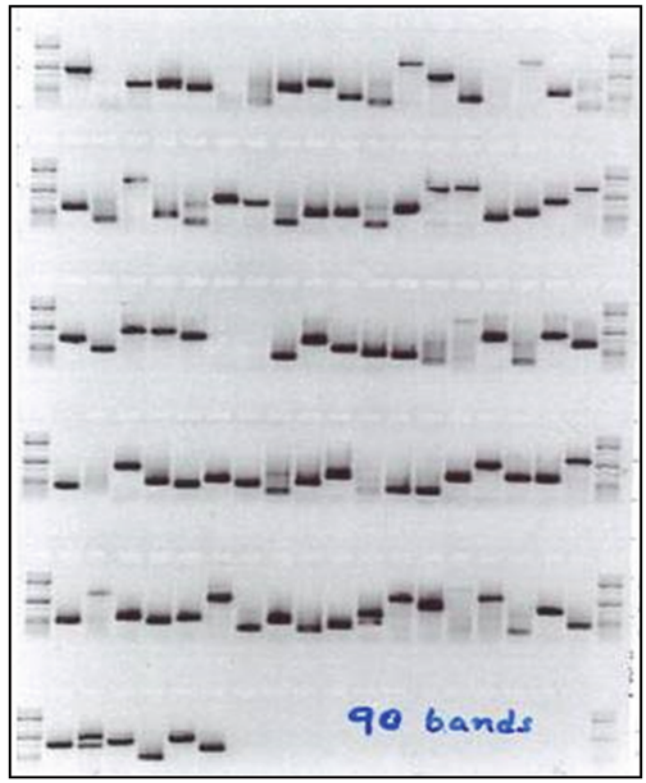

Fig. 1 Image of a 1 % agarose gel loaded with PCR products following *bursa aurealis* mutagenesis protocol. Note the random size of the PCR fragments demonstrating random insertion of *bursa aurealis* into the *S. aureus* chromosome

5 μl 10× Taq polymerase buffer, 1 μl Taq polymerase, 1 μl 10 mM forward primer (Buster), 1 μl 10 mM reverse primer (martin ermR), 1 μl dNTPs, 1 μl 50 mM $MgCl_2$, 35 μl nuclease-free water.

2. Perform the amplification with 40 cycles of 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 3 min.
3. 10 μl of subsequent PCR reaction is analyzed on a 1 % agarose gel. If multiple *bursa aurealis*–genome junctions are being assessed at the same time, amplicon sizes should be variable due to randomness of *bursa aurealis* insertion (Fig. 1).

3.3.4 Purification of Inverse PCR Products for Sequencing

1. Once confirmation of random insertions through the banding pattern on the gel is obtained, aliquot 6 μl of the PCR product into the 96-well sequencing plates (semi-skirted). Add 2 μl of EXOSAP-IT, mix to each sample and incubate at 37 °C for 15 min and then 80 °C for 15 min.

2. Use the buster primer to obtain DNA sequence data of the *bursa aurealis*–genome junction site.
3. After high quality DNA sequence files are obtained in chromatogram format, use FinchTV (A brilliant trace viewer from Geospiza, Inc.) to identify the insertion site of the transposon by BLAST (7) (*see* **Note 5**).

4 Notes

1. To guard against transposition of *bursa aurealis* and subsequent competition between unique staphylococcal mutants within a freezer stock carrying both pBursa and pFA545, pBursa and pFA545 strain stocks are kept separate. Therefore, once pBursa is transduced into a staphylococcal strain carrying pFA545, a heat shock is performed with individual colonies to isolate unique *bursa aurealis* mutants from each colony. In addition, for unknown reasons, more transposition events are detected following heat shock when transductant colonies (carrying both pBursa and pFA545) are allowed to sit at 4 °C for 5–7 days.
2. *S. epidermidis* 1457 colonies express high levels of PIA (Polysaccharide intercellular adhesin), which makes it difficult for the cells to go into suspension. However, this seems to have no effect on transposition efficiency. This issue is not observed in *S. aureus.*
3. Transposition frequency of *bursa aurealis* in *S. epidermidis* 1457 occurs at ~10^{-6}; therefore, 100–200 colonies should be present following incubation at 46.5 °C on TSA containing 2.5 μg/ml erythromycin.
4. In our experience, approximately 90 % of colonies are both chloramphenicol and tetracycline susceptible demonstrating adequate curing of the temperature sensitive plasmids pBursa and pFA545.
5. This note explains in a stepwise fashion how to identify the insertion site.
   (a) To start the query of a particular sequence, manually look for a substring sequence of CCTGTTA which marks the end site of transposon.
   (b) Select 100–200 nucleotides downstream of the Transposon end site CCTGTTA and subject it to a Nucleotide BLAST. However, do not go past the first AciI site.
   (c) Upon receiving BLAST results, scroll down to find the reference genome that is most closely related to the strain in which you are conducting transposon mutagenesis and

select it; typically you would want to choose the genome that is most closely related to the strain you are modifying. For example, in *S. aureus* JE2 this would be strain USA300 FPR3757 and for *S. epidermidis* this would be strain RP62A or ATCC12228.

(d) The following information is obtained, which is tabulated in an Excel sheet to calculate the transposon insertion sites and orientation:

- Transposon insertion site coordinate.
- The gene or the intergenic region in which the transposon has inserted.
- The orientation of the gene if inserted into a gene.
- The start and end coordinates of the gene if inserted into a gene.
- The orientation of transposon whether it is on the sense or antisense strand of the genome using the information provided by BLAST as Plus/Plus for sense and Plus/Minus for antisense.
- If the gene and the transposon are in the same orientation, then one can utilize the green fluorescent protein (*gfp*) located on *bursa aurealis* as a transcriptional fusion.

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Chemical and UV Mutagenesis

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Abstract

The ability to create mutations is an important step towards understanding bacterial physiology and virulence. While targeted approaches are invaluable, the ability to produce genome-wide random mutations can lead to crucial discoveries. Transposon mutagenesis is a useful approach, but many interesting mutations can be missed by these insertions that interrupt coding and noncoding sequences due to the integration of an entire transposon. Chemical mutagenesis and UV-based random mutagenesis are alternate approaches to isolate mutations of interest with the potential of only single nucleotide changes. Once a standard method, difficulty in identifying mutation sites had decreased the popularity of this technique. However, thanks to the recent emergence of economical whole-genome sequencing, this approach to making mutations can once again become a viable option. Therefore, this chapter provides an overview protocol for random mutagenesis using UV light or DNA-damaging chemicals.

Keywords: Mutation, Chemical mutagenesis, Nitrosoguanidine, UV mutagenesis, Random mutagenesis

1 Introduction

The ability to generate mutants is an essential tool for understanding gene regulation, protein function, and in turn, bacterial physiology and virulence. Common strategies to generate mutants in *S. aureus* include the use of antibiotic cassettes in targeted mutagenesis, or the creation of random mutant libraries via transposon mutagenesis. However, many mutations that may provide tremendous insight cannot be identified by using DNA insertions. These may include single nucleotide changes to identify amino acids critical to protein function or single base pair changes that affect expression at either the transcriptional or translational level. When this is desired at a whole-genome level, an alternative approach to insertion mutagenesis is needed.

Before the exploitation of transposons as a molecular biology tool, chemical and UV mutagenesis were commonly used to generate mutations with a selectable or easily screened trait. For example, if a bacterium was an auxotroph for a particular amino acid, one could perform random mutagenesis for mutants that could now grow in the absence of that particular amino acid. While random

Table 1
Common chemical agents for mutagenesis

Chemical	Mutation	Select reference in *S. aureus*
Acriflavine	Intercalation of DNA leading to deletions and frameshifts	(4, 7)
Ethyl methanesulfonate (EMS)	Guanine alkylation leading to point mutations	(6)
Diethyl sulfate	Ethylates DNA leading to point mutations	(3)
N-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)	Alkylation leading to point mutations	(5, 8)

mutagenesis using transposons can identify such mutations, transposons almost always cause loss-of-function mutations, due to their insertion into DNA and disruption of coding- and noncoding-sequences. By comparison, both chemical and UV mutagenesis have the potential to generate gain-of-function mutations that can yield tremendous insight. For example, a single base-pair change in an enzyme may alter specificity for its substrate or make it constitutively active. Such a result could not be identified using mutagenesis methods that insert DNA into the chromosome.

Chemical mutagenesis can be performed using a variety of chemicals (Table 1) that cause DNA damage, leading to single nucleotide changes and/or deletions. Similarly, UV mutagenesis exposes the cells to a UV light source and leads to DNA damage both directly and through the generation of reactive oxygen species (1). Since these mutations are chemical- or photo-based, mutations are generally random, but some may have hotspots close to the replication origin (2). These techniques also provide a relatively easy method of generating mutants for those researchers with little to no molecular biology or genetic manipulation experience. For *Staphylococcus aureus*, these methods have been used for a variety of tests including resistance to antibiotics (3–8). However, perhaps the most well-known use of UV and chemical mutagenesis of *S. aureus* was the creation of RN4220 (9), which most labs still use as an essential intermediate strain during genetic manipulation of clinical isolates.

The primary reason that chemical and UV mutagenesis lost popularity is likely due to the task of identifying the specific genetic mutation generated, since they are not easily recoverable compared to mutations generated by transposons. However, recent technology advances have made chemical and UV mutagenesis a viable option again. Not many years ago, whole-genome sequencing was

extremely laborious and expensive. However, with current sequencing technology, whole bacterial genomes are now sequenced in days at very affordable prices. This will surely only improve in the coming years. In this modern genomics era, random mutagenesis using chemical or UV light can be performed to isolate mutations of interest, and combined with whole-genome sequencing, can identify single nucleotide changes with relative ease. Therefore, this once dated procedure may once again become a viable option for many research labs.

2 Materials

All materials should be made using ultrapure water and waste should be disposed of properly based on university guidelines. Care should be taken whenever handling chemical mutagens as they are dangerous to humans.

1. Strain to be mutagenized.
2. Mutagen (chemical or UV source (*see* **Note 1** and Table 1)).
3. Phosphate buffered saline (PBS) or other buffer.
4. Selective or screening media (*see* **Note 2**).
5. Tryptic soy broth (TSB).

3 Methods

While there are multiple options available for mutagens, the protocol here is written with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) as an example. Therefore, concentrations and exposure times will need to be adjusted for each mutagen used.

1. Dilute an overnight culture of strain to be mutagenized to an OD_{600} of 0.1 in 12.5 ml of TSB.
2. Incubate at 37 °C with shaking at 250 rpm until mid-exponential phase (approximately $OD_{600} = 1.0$).
3. Harvest 1 ml of cells by centrifugation, decant supernatant, and wash cells twice with PBS.
4. Resuspend the cell pellet in 1 ml of PBS and add 50–500 μg/ml MNNG and incubate at 37 °C for 30–60 min (*see* **Note 3**).
5. Wash cells to remove MNNG.
6. Plate on selective or screening media and incubate at 37 °C overnight or until colonies form.
7. Identify mutants of interest and determine mutations using whole-genome sequencing (*see* **Note 4**).

4 Notes

1. A variety of mutagens are available for *S. aureus*. Chemical mutagens include: 0.014 μg/ml diethyl sulfate (3, 10), 50–500 μg/ml *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (5, 8, 11), 5-50 μg/ml acriflavine (4, 7). Of note, these concentrations should be a guide and actual amounts determined empirically for each bacteria, condition, and exposure time (*see* **Note 3**). For UV mutagenesis, the amount of energy exposed will depend on wavelength and power source. As a reference point, at 254 nm, an estimated power of 8.5 W/m^2 should be used as a starting point (12).
2. To be successful, there must be an easy way to screen or select for the desired mutation. This could be the ability to grow in the presence of a chemical that inhibits the growth of wild-type cells, allowing for easy selection. Alternatively, a screen for the expression of a particular reporter such as GFP and flow cytometry coupled with cell sorting could be used as a screening process.
3. It is important to determine the right combination of concentration and exposure time for mutagenesis. If the time is too short, mutation frequency will be low, while long incubation times will kill the cells. As a general rule, the dose and exposure should be such that it achieves 50 % killing of the bacteria. Furthermore, testing the mutation frequency of a given dose and time can be used to determine the right exposure. In this case, perform mutagenesis on RN4220 and plate on media containing rifampicin; this is an easy screen to give some idea as to the efficiency of procedure.
4. There are several current technologies (Illumina, Oxford Nanopore, and Life Technologies, to name a few) that produce high-throughput machines for rapid whole-genome sequencing. This technology changes very rapidly and therefore is not discussed here, except to say that a quick Internet search can uncover a variety of companies/university core facilities that provide this service on a per charge basis.

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Pulse Field Gel Electrophoresis

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Abstract

Pulse Field Gel Electrophoresis (PFGE) is a powerful genotyping technique used for the separation of large DNA molecules (entire genomic DNA) after digesting it with unique restriction enzymes and applying to a gel matrix under the electric field that periodically changes direction. PFGE is a variation of agarose gel electrophoresis that permits analysis of bacterial DNA fragments over an order of magnitude larger than that with conventional restriction enzyme analysis. It provides a good representation of the entire bacterial chromosome in a single gel with a highly reproducible restriction profile, providing clearly distinct and well-resolved DNA fragments.

Keywords: Pulse field gel electrophoresis, Restriction enzyme, Genomic DNA, Genotyping technique

1 Introduction

Genotyping of microorganisms is very important in evaluating the global evolution of the pathogens and studying their genetic relatedness to determine their point source during epidemiological investigations (1, 2). A variety of genotyping methods exists for *Staphylococcus aureus*. Each has strengths and weaknesses. These methods include pulse field gel electrophoresis (PFGE), surface protein A typing (*spa*-typing), multi locus sequence typing (MLST), SCC mec typing, plasmid profile analysis, restriction fragment length polymorphism (RFLP), RFLP-Southern blot, Rep-PCR typing, Multilocus VNTR (Variable Number Tandem Repeat) analysis (MLVA), and whole-genome DNA sequence typing (3–9).

S. aureus is one of the most important causes of life-threatening bacterial infections in the industrialized world causing infections both in the hospitals and the community. *S. aureus* is the second most common overall cause of healthcare-associated infections reported to the National Healthcare Safety Network, the most common cause of surgical site infections (10), the leading cause of infections involving heart valves and cardiac devices (11, 12), and a leading cause of bacteremia (13, 14) and endocarditis (15, 16). Additionally, *S. aureus* routinely becomes resistant to many of the currently available antibiotic therapies. Recently, the Centers for

Disease Control and Prevention estimated that 80,461 invasive MRSA infections and 11,285 related deaths occurred in 2011 in the USA, even more than HIV/AIDS (17). Thus, a reproducible and highly discriminatory genotyping technique to rapidly differentiate and type these isolates is needed to prevent the illness and costs associated with these infections. In addition, the availability of a sensitive genotyping method for staphylococcal isolates is essential in understanding the epidemiological evolution and outbreak of several antibiotic resistant strains including MRSA.

Among the various DNA-based methods available for genotyping *S. aureus* and other bacterial pathogens, PFGE is often considered as a gold standard due to its discriminatory power, reproducibility, and ease of execution, data interpretation, cost, and availability (18). PFGE is a powerful genotyping technique used for the separation of large DNA molecules (entire genomic DNA) after digesting with unique restriction enzyme. First developed by Schwartz et al. (19) in yeast, PFGE is reported to be very sensitive, highly reproducible with a very good discriminatory power in genotyping of *S. aureus* isolates (20). PFGE involves the isolation of the intact chromosomal DNA by lysing bacterial cells embedded in an agarose plug to avoid the mechanical shearing of DNA molecules during the extraction (21). This is followed by digestion of the chromosomal DNA within the agarose plug by a rare cutting restriction enzyme to produce $\geq$12 high-molecular weight DNA fragments. Finally, the digested DNA samples (10–800 kb) are subjected to separation by alternating the electric field between spatially distinct pairs of electrodes (Fig. 1). This will facilitate megabase (mb) size DNAs to reorient and migrate at different speeds through the gel pores towards the anode in a size dependent manner. The time required for reorientation is also inversely proportional to the size of DNA fragment. Overall, this process will achieve a good resolution of large DNA fragments in the agarose gel (19, 22). The obtained gel images will then be normalized and patterns of the DNA fragments will be analyzed by BioNumerics Software following the criteria to interpret PFGE patterns developed by Tenover et al. (23). These patterns serve a virtual barcode (Fig. 2), which "types" the strains and allows for the determination whether isolates are closely related.

2 Materials

Prepare all the reagents using ultrapure deionized water and analytical grade reagents. Follow all waste disposal regulations when disposing of waste materials.

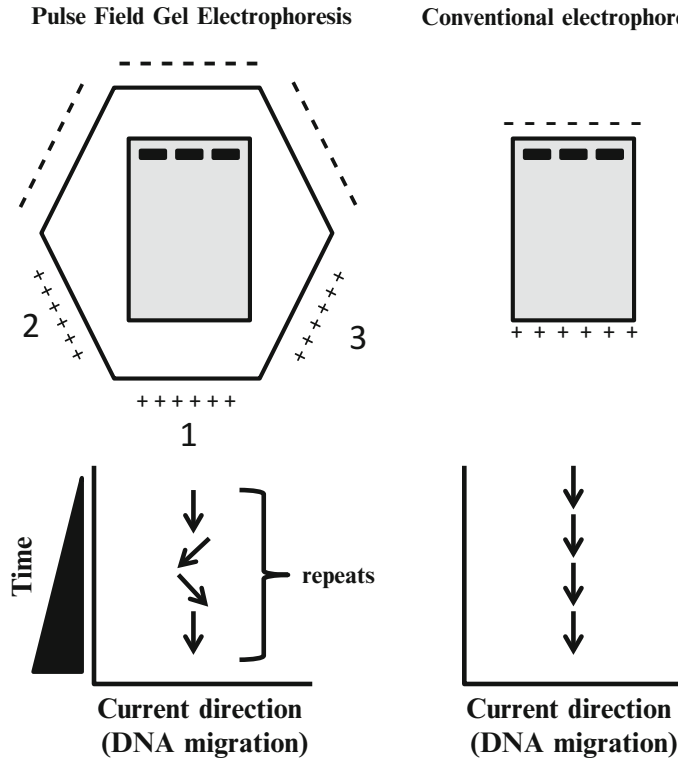

Fig. 1 An example comparison of PFGE versus conventional electrophoresis. In both cases, the gel (*grey rectangle*) is placed in a buffer inside a gel rig with anodes (+) and cathodes (−) (*top diagrams*). In the case of PFGE, the direction of current cycles between 1, 2, and 3. As depicted in the *bottom graphs*, unlike conventional electrophoresis where current only runs in a single direction, PFGE cycles between several directions, allowing for separation of large molecular weight DNA

2.1 Preparation of Bacterial Cells

1. Trypticase soy agar (TSA) plates.
2. 37 °C shaking incubator.
3. Turbidity meter or spectrophotometer for preparation of cell suspensions.
4. Microcentrifuge to pellet cell suspensions.
5. Vortex mixer.

2.2 Preparation of Agarose Plugs

1. 55–60 °C stationary water bath.
2. 37 °C stationary water bath.
3. SeaKem Gold agarose (Bio-Rad #161-3109).
4. *TE buffer*: 10 mM Tris, 1 mM EDTA, pH 8.0. Mix 20 ml of 1 M Tris, pH 8.0 with 4 ml of 0.5 M EDTA, pH 8.0 and add water to make a final volume of 2,000 ml in a graduated

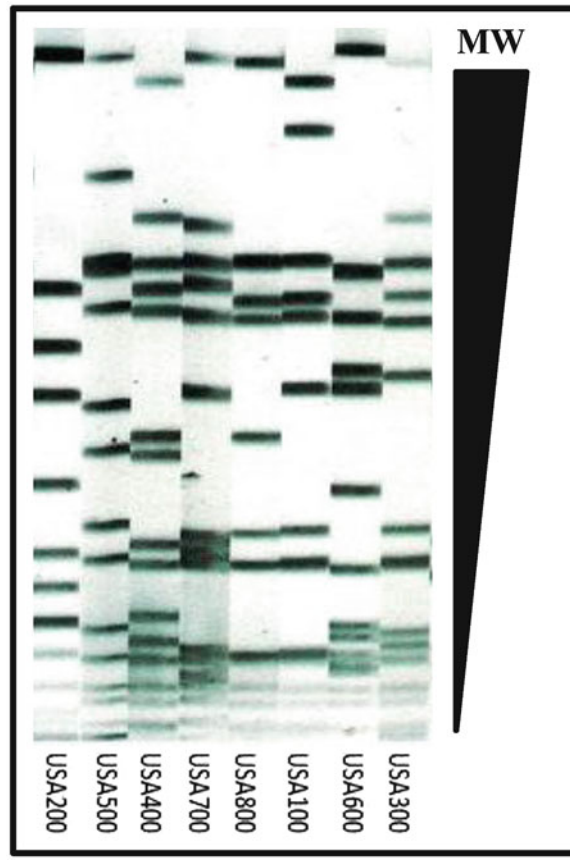

Fig. 2 A representative processed gel showing the different banding patterns of eight USA types. Image is a negative image of a processed gel with higher molecular weight (MW) DNA towards *top* of the image

cylinder. Transfer to two 1,000 ml glass screw-top bottles and autoclave. Store at room temperature for up to 6 months.

5. Clean beaker/container for "working" TE buffer.
6. 250 ml screw-capped Erlenmeyer flask.
7. Microwave oven.
8. PFGE plug mold (Bio-Rad, Hercules, CA).
9. Lysostaphin enzyme. Prepare a 1 mg/ml suspension in 20 mM sodium acetate (pH 4.5), aliquot, and freeze at −20 °C for up to 6 months.
10. Stainless steel spatulas.
11. Other basic lab supplies.

2.3 Plug Lysis

1. 55–60 and 37 °C stationary water bath.
2. *EC Lysis buffer*: 6 mM Tris, 100 mM EDTA, pH 8.0, 1 M NaCl, 0.5 % Brij-58, 0.2 % Deoxycholate, 0.5 % Sarcosyl.

Mix 5.4 ml of 1 M Tris, pH 8.0; 180 ml of 5 M NaCl; 180 ml of 0.5 M EDTA, pH 8.0; 4.5 g of Brij-58 (Polyoxyethylene 20 Cetyl Ether); 1.8 g of sodium deoxycholate and 4.5 g of sodium lauroyl sarcosinate with 500 ml of water in a glass beaker using magnetic stir bar and low heat. Once completely dissolved, add water to make a final volume of 900 ml. Transfer to a screw-top bottle and autoclave. Store at room temperature for up to 6 months.

3. Tubes to hold plug and buffer.
4. Spatula to remove plugs from mold.
5. 5 ml pipettes.

2.4 Plug Washing

1. TE buffer (*see* above).
2. Spatula or equivalent to hold plug in tube.
3. 5 ml pipettes.
4. Orbital rocker, rotator, or equivalent at room temperature.

2.5 Restriction Enzyme Digestion

1. Pretested *Salmonella* serotype Braenderup strain H9812 plugs (outlined in Section 4).
2. Microcentrifuge tubes.
3. 5 ml pipettes.
4. Standard Pipetmans.
5. Restriction endonuclease SmaI and XbaI with packaged 10× restriction buffer and 100× bovine serum albumin (BSA).
6. Commercial enzyme buffer appropriate for enzyme.
7. Sterile, Type I water.
8. Sterile plastic tubes (5–15 ml) for preparing buffer-water and enzyme-buffer-water mixtures.
9. Spatula or equivalent to hold plug in tube.
10. 5 ml pipettes.
11. Cutting dish (sterile disposable petri dish or equivalent).
12. Sharp scalpel or razor blade for cutting agarose plugs.
13. Bucket of ice or −20 °C insulated storage box.
14. Orbital rocker, rotator, or equivalent at room temperature.

2.6 Preparing and Running the Gel

1. 10× TBE buffer.
2. 55–60 °C stationary water bath.
3. Sterile distilled water, pre-warmed to 55 °C.
4. SeaKem Gold agarose (Bio-Rad # 161-3109).
5. Gel-casting platform and accessories.
6. Appropriate comb and comb holder.

7. Gel leveling bubble or equivalent.
8. 1.8 % SeaKem Gold agarose gel (For sealing wells).
9. CHEF-DR II system (Bio-Rad) for running pulsed-field gels.
10. Spatula, Kimwipes, and related basic lab supplies.

2.7 Staining and Documentation

1. Ethidium bromide solution, 10 mg/ml (AMRESCO #X328 or equivalent).
2. Containers (Covered glass dishes) to stain and destain gels.
3. Distilled water, 2 l.
4. Gel Doc 2000 (Bio-Rad) or equivalent gel documentation apparatus

2.8 Data Analysis

1. BioNumerics software version 4.0 (Applied Maths, Belgium).

3 Methods

3.1 Overview

Day 0: Streak plates.

Day 1: Make plugs.

Day 2: Wash plugs.

Day 3: Restriction digest and electrophoresis.

Day 4: Stain, take photograph, analyze in Bionumerics.

3.2 Day 0: Streak Plates

1. Streak *Staphylococcus aureus* isolates onto TSA plates and incubate at 37 °C for 18–24 h.

3.3 Day 1: Make Plugs

1. Turn on 37 and 55 °C water baths, and prepare two boxes of ice.
2. Label a 15 ml conical tube, a 5 ml polystyrene round-bottom tube, and a 1.5 ml microcentrifuge tube for each sample.
3. Add 2 ml sterile water to each 5 ml polystyrene round-bottom tube.
4. Add 3 ml EC lysis buffer to each 15 ml conical tube.
5. Use a sterile swab to collect cells from plate and suspend them in water in the 5 ml polystyrene round-bottom tube. Vortex briefly. Place the tube in the turbidity meter. Aim for a reading of 0.80–0.89. If the reading is too high, add more sterile water (in 1.0 ml increments); if the reading is too low, add more cells. Store the tubes on ice.
6. Transfer 200 μl of suspended cells to a 1.5 ml microcentrifuge tube. Centrifuge at 13,000 rpm for 6 min.
7. While samples are being centrifuged, prepare the gram-positive agarose (Gram negative—Salmonella agarose described separately in Section 4).

8. Combine 0.9 g SeaKem Gold agarose and 50 ml TE buffer in a 200 ml screw-top flask. Screw lid on tightly and microwave for 1 min 50 s. Gradually loosen the lid and swirl agarose. If not completely melted, retighten the lid and continue microwaving for 25 s intervals, loosening lid and swirling after each time, until agarose has thoroughly melted. Place the flask in the 55 °C water bath to equilibrate for ≥30 min (*see* **Note 1**).
9. When centrifugation is complete, use a SAMCO disposable sterile pipette to aspirate the entire supernatant from microcentrifuge tubes and discard. The pellet should be 2–3 mm in diameter (*see* **Note 2**).
10. Add 300 µl TE buffer to each microcentrifuge tube and vortex to resuspend the cells. Place in 37 °C water bath for 10 min.
11. For each isolate, label two wells on the plug mold. Remove the microcentrifuge tubes from the 37 °C water bath.
12. The following must be done one tube at a time: add 3 µl Lysostaphin (1 mg/ml) and 300 µl 55 °C agarose (made in step 8) to the microcentrifuge tube. Quickly but gently mix with pipettor ten times. Using a SAMCO disposable sterile pipette, fill two of the plug mold wells, overfilling slightly to produce a rounded top.
13. Repeat step 12 for each sample, and then allow plugs to harden for 10–15 min at room temperature (or 5 min in the 8 °C refrigerator).
14. When plugs are hardened, use the snap-off tool provided with each mold (or a spatula cleaned with ethanol) to push the plugs (two per sample) into the 15 ml conical tubes containing ~3 ml of EC lysis buffer. Make sure the plugs are fully immersed in the buffer.
15. Incubate tubes in 37 °C water bath for ≥4 h (preferably overnight).

3.4 Day 2: Wash Plugs

1. Pour off EC lysis buffer into glass beaker, holding cap close to rim to prevent plugs from escaping. Check beaker for escaped plugs.
2. Add 5 ml TE buffer to each tube, ensuring that all plugs are immersed. Place securely capped tubes horizontally in glass tray on rocker table for 60 min at room temperature. Set the speed of the rocker table to ~60 rpm.
3. Remove buffer as described in step 1. Repeat wash two more times for a total of three washes. The last wash may be done overnight. This can be stored refrigerated until all reagents are prepared for the enzyme digestion.

3.5 Day 3, Part 1: Restriction Digest

1. Turn on 37 and 55 °C water baths and prepare one box of ice. Remove 10× multicore, SmaI, XbaI, and BSA from freezer and allow to thaw at room temperature. Centrifuge for 1 min, then place on ice.
2. Label a 1.5 ml microcentrifuge tube for each sample, plus one staphylococcal control (NCTC 8325—reference standard for data normalization) and four Salmonella size standards.
3. In a 15 ml conical tube, prepare the restriction buffer:

10× multicore stock	420 µl
Sterile water	3,780 µl

   This makes enough for 21 samples. Adjust the volumes accordingly based on the number of samples.
4. Add 200 µl of restriction buffer to each tube.
5. Clean a petri dish, spatula, and scalpel with ethanol. Place a plug in the petri dish and, using the scalpel and spatula, cut two 2–3 mm slices (Five slices for Salmonella). Place these slices in the corresponding microcentrifuge tube, and return what is left of the plug to its original tube. Repeat for each sample, cleaning the dish, spatula, and scalpel every time. Store the slices and remaining plugs at 8 °C.
6. Allow the staphylococcal samples to equilibrate at room temperature for 30–45 min, and the Salmonella samples (preparation described later) in a 37 °C water bath for 30–45 min.
7. Keeping reagents and prep tube on ice at all times, prepare restriction enzyme for staphylococcal samples by mixing (in a 15 ml conical tube):

10× multicore stock	360 µl
SmaI (10 U/µl)	54 µl
Acetylated BSA	36 µl
Sterile water	3,150 µl

   Invert tube, vortex, and then return to ice. This makes enough for 18 samples. Adjust the volume accordingly based on the number of samples.
8. Keeping reagents and prep tube on ice at all times, prepare restriction enzyme for Salmonella samples by mixing (in a 1.5 ml microcentrifuge tube):

10× multicore stock	100 µl
XbaI (10 U/µl)	20 µl
Acetylated BSA	10 µl
Sterile water	869 µl

Keep on ice at all times. Invert tube, vortex, and then return to ice. This makes enough for five samples. Again, adjust the volume accordingly based on the number of samples.

9. Remove buffer from the plugs using disposable SAMCO pipettes.
10. Add 200 µl of the appropriate restriction enzyme/buffer mix to each tube (SmaI for staphylococcal samples, XbaI for Salmonella samples).
11. Incubate for 3–4 h at room temperature for staphylococcal samples, and in 37 °C water bath for Salmonella samples.

3.6 Day 3, Part 2: Electrophoresis

1. Make 2,200 ml of 0.5× TBE by combining: 110 ml 10× TBE in 2,090 ml purified water in a 4 l plastic beaker with a large magnetic stir bar. Set on magnetic stirrer to mix while completing the next steps.
2. To make the 1 % agarose gel, combine: 1.05 g SeaKem Gold agarose with 100 ml 0.5× TBE in a 200 ml screw-top flask, then dissolve and equilibrate as in Section 3.3, step 8.
3. Assemble the leveling table, gel casting stand, and combs. Clean thoroughly with alcohol and Kimwipes to remove lint. Use 360° level to ensure table is levelled.
4. Pour the remaining 0.5× TBE into the PFGE system. Turn on (in order) the command system, pump, and chiller. Set temperature to 14 °C.
5. When the plug slices have completed their incubation period, place them on the comb according to the layout on the log sheet. Scoop a slice out of its microcentrifuge tube with a spatula, and blot the slice with a Kimwipes. Slide the slice onto the end of a comb "tooth." Be sure to clean spatula with ethanol between tubes. Allow the slices to set on the comb for 10 min.
6. Set the comb upright in the casting stand, with the slices facing forward. Look closely to be sure that slices are uniformly positioned, and wait for a few moments to be sure that they have not and will not slide off the comb.
7. Gently pour the 55 °C agarose into the casting tray, saving a small amount (1–2 ml) to fill in the wells after the comb is

removed. Keep this agarose at 55 °C while the gel solidifies at room temperature for 1 h.

8. When the gel is solid, remove the comb and fill the holes with the saved agarose. Let it to set for 5–10 min.
9. Remove the gel with the black bottom tray from the casting stand and clean the black tray of all residues. Place in the electrophoresis cell and start the cycle:
   - Press "Volts" and set to 5.6 (for system on right) or 5.8 (system on left).
   - Set time for 21 h (right) or 21.5 h (left).
   - Press "Block" and "Volt" together, and set at 5.0.
   - Press "Volts" and "Run Time" together, and set at 40.0.
   - Press "Start."

   Follow the manufacturer's instruction depending on the available system.

3.7 Day 4, Part 1: Stain Gel

1. Add 30 μl of 1 mg/ml ethidium bromide to 300 ml purified water in a glass tray (Or equivalent amount based on the tray size).
2. Slide gel off its black backing and into the tray. Incubate at room temperature for 45 min.
3. Decolorize in 1 l of purified water for 90 min.

3.8 Day 4, Part 2: Photograph Gel (Picture Can Be Captured in Any Equivalent Way)

1. Slide gel back on to the black backing to remove it from water and transfer it to the UV transilluminator.
2. Slide gel off black backing and on to the transilluminator table. Position the camera and hood over the table and turn on the UV light and camera.
3. Set the image type to RAW and the exposure time to 15 s.
4. Zoom in to frame the gel and take two pictures.

3.9 Processing PFGE Images Using BioNumerics

1. Open the .TIF image file of the gel in BioNumerics Software (Applied Maths) by clicking on "Add new experiment file."
2. Follow the instructions to process the TIFF image using the software mainly through the following four steps:
   (a) Convert a TIFF to gel strips.
   (b) Define curves.
   (c) Normalize the gel.
   (d) Find gel bands.
3. For cluster analysis, select the isolates to be compared, click the "Calculate Cluster Analysis" and follow the instructions.

4 Preparation of *Salmonella* PFGE Plugs

Salmonella plugs should be used as standards in each gel. Thus plugs of standard strain *Salmonella* serotype Braenderup strain H9812 needs to be made and pretested. Once the plugs are made and have passed as controls on plug preparation, they can be stored and used in each gel as a test for enzyme efficacy. Prepare all the reagents using ultrapure deionized water and analytical grade reagents. Follow all waste disposal regulations when disposing of waste materials.

4.1 Day 0: Streak Plates

1. Streak *Salmonella* serotype Braenderup strain H9812 onto TSA plates and incubate at 37 °C for 18–24 h.

4.2 Day 1: Make Plugs

1. Turn on 54 °C shaker incubator and water bath (55 °C); Prepare ice in a Styrofoam box.
2. To prepare 1 % SeaKim Gold (1 % SDS agarose gel for *Salmonella plugs*), mix 0.5 g SeaKim Gold agarose and 47.5 ml TE buffer in 200 ml screw-top flask, then dissolve in Section 3.3, step 8. Place the flask in the 55 °C water bath to equilibrate for 5 min before adding SDS.
3. Add 2.5 ml of 20 % SDS (preheated to 55 °C), mix well, and keep in the 55 °C water bath until ready to use.
4. Prepare *Cell Suspension Buffer*: 100 mM Tris, 100 mM EDTA, pH 8.0. Mix 10 ml of 1 M Tris, pH 8.0 with 20 ml of 0.5 M EDTA, pH 8.0 and add water to make a final volume of 100 ml in a graduated cylinder. Transfer to a screw-top bottle and autoclave. Store at room temperature for up to 6 months.
5. Transfer 2 ml of Cell suspension buffer to labeled 5 ml polystyrene round-bottom tubes, and keep tubes with the buffer on ice.
6. Use a sterile swab to collect cells from the TSA plate and suspend them in the Cell suspension buffer. Vortex briefly. Place the tube in the turbidity meter. Aim for a reading of 0.48–0.52. If the reading is too high, add more buffer (in 500 µl increments); if the reading is too low, add more cells. Store the tubes on ice.

4.3 Casting Plugs

1. Label wells of PFGE plug molds (40 plug molds or as little as 20 plug molds).
2. Transfer 400 µl of cell suspension with strain in Cell suspension buffer to labeled 1.5 ml microcentrifuge tubes (>15 tubes).
3. Add 20 µl of Proteinase K (20 mg/ml) to each microcentrifuge tube and mix gently with pipette tip, one or two tubes at a time.

4. Add 400 μl of melted 1 % SDS agarose from above to microcentrifuge tube containing cell suspension (Brought to the room temperature) and mix gently with fine tip transfer pipette. If cell suspensions are cold, place tubes containing cell suspensions in 37 °C water bath for a few minutes to warm.
5. Fill plug molds by immediately dispensing the agarose mix into appropriate well(s) of reusable plug mold. Do not allow bubbles to form. Two plugs of each sample can be made from these amounts of cell suspension and agarose. Allow plugs to solidify at room temperature for 10–15 min. They can also be placed in the refrigerator (4 °C) for 5 min.

4.4 Lysis of Cells in Agarose Plugs

1. Label 50 ml polypropylene screw-cap tubes with culture numbers (ten tubes).
2. Prepare *Cell Lysis Buffer*: 50 mM Tris, 50 mM EDTA, pH 8.0, 1 % Sarcosyl. Mix 25 ml of 1 M Tris, pH 8.0; 50 ml of 0.5 M EDTA, pH 8.0 and 5 g of Sodium Lauroyl Sarcosinate and add water to make a final volume of 500 ml in a graduated cylinder. Transfer to a 1 l screw-top bottle and warm to 50–60 °C for 30–60 min or leave at room temperature for 2 h to completely dissolve Sodium Lauroyl Sarcosinate. Autoclave for 20 min and store at room temperature for up to 6 months.
3. Prepare Cell lysis buffer/Proteinase K mix by adding 250 μl of Proteinase K solution (20 mg/ml) to 50 ml of Cell lysis buffer.
4. Add 5 ml of Cell lysis buffer/Proteinase K mix to each tube labeled above (ten Orange screw-cap tubes from above).
5. Push out plugs (3–4 plugs) into each tube making sure that the plugs are immersed under buffer.
6. Place tubes in rack and incubate in 54 °C shaker incubator for 1.5–2 h with vigorous shaking at 150–175 rpm.

4.5 Washing of Agarose Plugs

1. Preheat sterile purified water (10 tubes × 10–15 ml × 2 times = 2–300 ml) to 50 °C for washing plugs.
2. Remove tubes from shaker incubator and pour off Cell lysis buffer/Proteinase K solution.
3. Add 10–15 ml preheated sterile purified water to each tube and shake tubes vigorously (150–175 rpm) in 50 °C shaker incubator for 10–15 min. Pour off water from plugs. Repeat the wash process one more time.
4. While the plug is being washed with water, preheat sterile TE buffer (10 tubes × 10–15 ml × 4 times = 4–600 ml) to 50 °C for washing plugs.
5. Add 10–15 ml of preheated (50 °C) TE buffer to each tube and shake tubes in 50 °C shaker incubator for 10–15 min. Pour off

the TE buffer from plugs and repeat the wash process for a total of four times.

6. Store plugs in microcentrifuge tubes with TE buffer at 4 °C (2–4 plugs per tube) until needed.

5 Notes

1. The purpose of screw capped flask is to reduce the amount of evaporation. If the volume is diminished during agar melting, readjust the volume to the original level by adding TE buffer.
2. Pellet size is crucial, if too small, add more suspension and recentrifuge; if too large, resuspend the pellet, remove some of the suspension, and recentrifuge.

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RNA-Sequencing of *Staphylococcus aureus* Messenger RNA

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Abstract

RNA-sequencing (RNA-seq) is a technique that employs next-generation DNA-sequencing technology to simultaneously sequence all of the RNA transcripts in a cell. It can provide valuable insights into transcript and operon structure, and is rapidly replacing expression microarrays as the technique of choice for determining global gene expression profiles in bacteria. Herein we outline the procedures involved in performing RNA-seq with samples of RNA from *Staphylococcus aureus*. We draw particular attention to key aspects of sample preparation, such as RNA integrity and removal of ribosomal RNA, and provide details of critical steps in downstream data analysis.

Keywords: RNA-seq, RNA-sequencing, RPKM, rRNA reduction, RNA enrichment, *S. aureus*

1 Introduction

The introduction of next-generation DNA sequencing (NGS) has facilitated the development of techniques and applications for high-throughput genetic analysis that were previously not possible. Perhaps the most beneficial application of NGS in microbial research has been the introduction of RNA-sequencing (RNA-seq). In this technique total RNA from the bacterial cell is purified and sequenced using NGS technology. The resulting reads, once aligned to a reference genome, can be used to quantitate gene expression, determine transcript and operon structure, and identify noncoding RNA species, in essence giving a "snapshot" of global RNA expression within the cell. An overview of the RNA-seq experimental procedure is presented in Fig. 1. RNA samples are isolated and contaminating genomic DNA removed using an RNase-free DNase (Fig. 1a–b). RNA samples are then enriched, a process whereby ribosomal RNA (rRNA) is removed (Fig. 1b–c). Following enrichment the RNA-sequencing process begins. Each NGS platform has specific RNA-seq protocols that are used to process enriched RNA samples. In most cases the enriched RNA is fragmented before being reverse-transcribed into cDNA and sequenced (Fig. 1d). The sequencing reads are then aligned to a reference genome and analyzed to determine gene expression values (Fig. 1e).

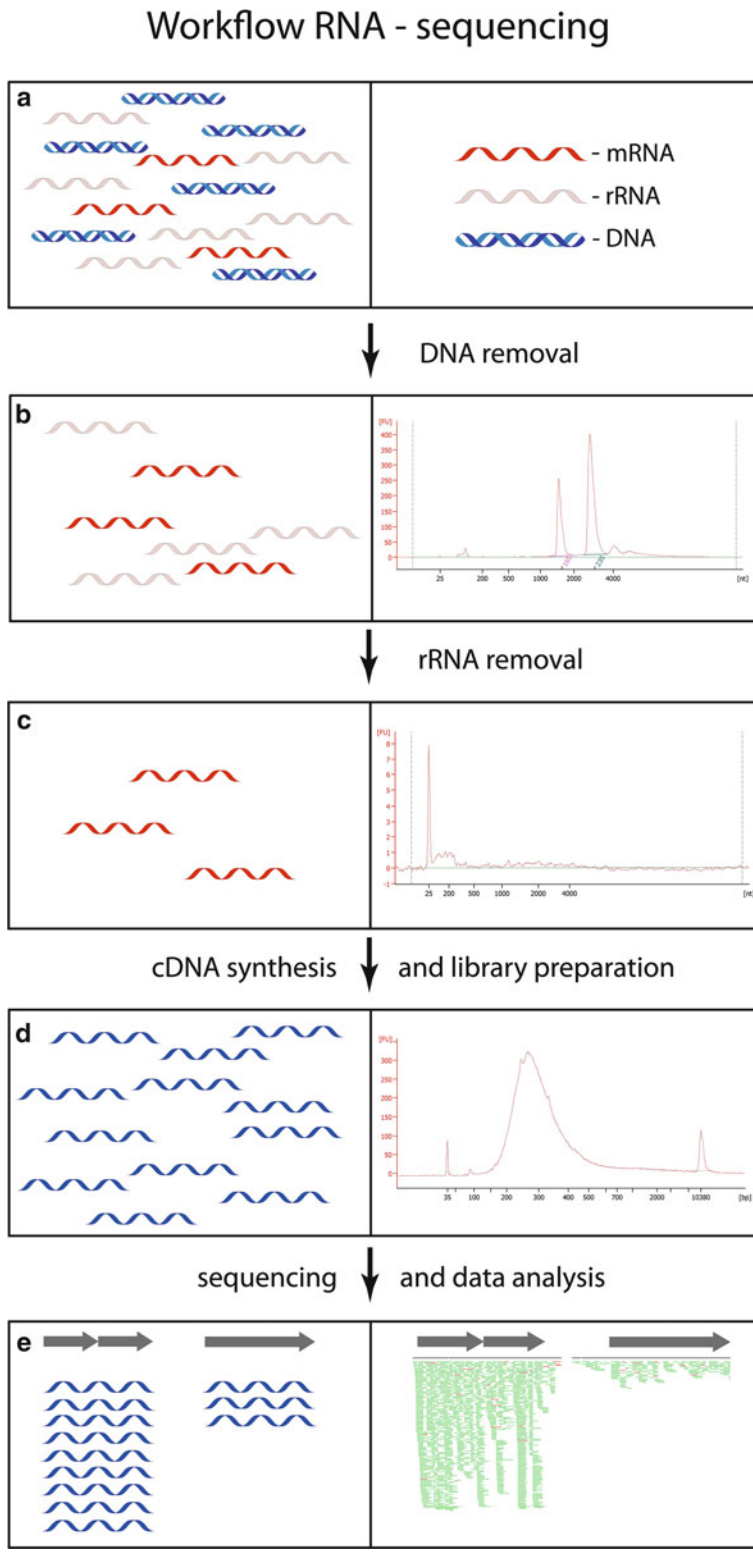

Fig. 1 Schematic overview of RNA-sequencing. (**a**) Total RNA purified from *S. aureus* cells contains messenger RNA (mRNA), ribosomal RNA (rRNA) as well as small amounts of genomic DNA that are co-purified with the RNA.

Each NGS platform has its own set of unique protocols for processing enriched RNA. Platform-specific, detailed protocols (as well as instructional videos) are freely available online, and therefore, there is little value in reiterating them in detail herein. In addition, many of these procedures are now available as services at core facilities, removing the need for individual labs to perform these steps themselves. As such, it is our intention to focus this protocol on critical aspects of sample preparation that must be carefully performed to generate samples of sufficient quality for RNA-seq. We provide detailed protocols for RNA isolation, DNase treatment, enrichment, and quality evaluation. In addition we include important points for RNA-seq data handling which facilitate accurate downstream analysis.

2 Materials

2.1 RNA Isolation

1. RNeasy Mini Kit (Qiagen), Buffer RLT must be prepared fresh each day. Prepare a sufficient quantity of buffer RLT by adding 10 μl of β-mercaptoethanol to 1 ml buffer RLT provided in the Qiagen RNeasy kit.
2. Nuclease-free 1.5 ml tubes.
3. TE buffer: 10 mM Tris pH 8.0, 1 mM EDTA.
4. Lysing matrix B 2 ml tubes (MP Biomedical).
5. Mini-beadbeater.
6. Ethanol (100 %).
7. Nuclease-free water.
8. RNaseZap (Life Technologies).

2.2 DNase Treatment of Purified RNA

1. Turbo DNA-free kit (Ambion).
2. Agilent 2100 Bioanalyzer or 2200 TapeStation.
3. Agilent RNA 6000 Nano kit.

2.3 RNA Enrichment

1. ERCC RNA spike in mix (Life Technologies).
2. Ribo-Zero Magnetic Kit for Gram-Positive Bacteria (Epicentre).

Fig. 1 (continued) (**b**) Contaminating DNA is removed using an RNase-free DNase and the integrity of the purified RNA samples determined by bioanalyzer. Samples with a RIN >9.7 are suitable for RNA-seq. (**c**) The RNA enrichment procedure removes rRNA leaving mRNA transcripts. (**d**) The mRNA is fragmented, reverse-transcribed into cDNA, and sequenced using next-generation DNA-sequencing technology. (**e**) Sequencing reads corresponding to rRNA are filtered out and the remaining reads are aligned to a reference genome. Expression values for each gene are calculated based upon the number of reads aligning to that gene

3. MicrobExpress Bacterial mRNA Enrichment Kit (Life Technologies).
4. Magnetic rack for 1.5 ml tubes.
5. 0.2 ml tubes.
6. Thermocycler.
7. Ethanol (70 %).
8. Agilent RNA 6000 Pico kit.

2.4 RNA-Sequencing

1. Ion Total RNA-Seq Kit v2.
2. Ion PGM™ Template 200 Kit.
3. Ion 318™ Chip.
4. Ion PGM™ 200 Sequencing Kit.
5. Ion Xpress™ RNA-Seq Barcode Kit (optional).
6. IonTorrent PGM.
7. IonTorrent OneTouch.

2.5 Data Analysis

1. CLC Genomics Workbench software.

3 Methods

3.1 RNA Isolation

While a variety of RNA isolation procedures are available, the protocol below is based upon the Qiagen RNeasy kit. When isolating RNA, use dedicated RNase-free reagents throughout (*see* **Note 1**).

1. Grow bacterial cultures as required and pellet cells corresponding to 1×10^9 CFU (*see* **Notes 2** and **3**).
2. Immediately freeze pellets and store at −80 °C prior to RNA isolation (*see* **Note 4**).
3. Thaw pellets and resuspend in 100 μl TE buffer pH 8.0.
4. Transfer resuspended cells to a Lysing matrix B tube and bead beat for 60 s.
5. Add 650 μl of buffer RLT containing β-mercaptoethanol to each tube and repeat the bead beating procedure for an additional 60 s.
6. Centrifuge samples at full speed in a microcentrifuge for 1 min.
7. Withdraw 600 μl of supernatant from each tube, being careful not to disturb the pelleted beads/cellular debris, and mix with 900 μl of 100 % ethanol in a 1.5 ml tube (*see* **Note 5**).
8. Immediately transfer 600 μl of the lysate–ethanol mix to an RNeasy mini spin column.

9. Centrifuge for 30 s at maximum speed, discard the flow-through, reconnect the RNeasy mini spin column to the collection tube and transfer another 600 µl of the lysate–ethanol mix to the RNeasy mini spin column.
10. Repeat step 9, transferring the remainder of the lysate–ethanol mix to the RNeasy mini spin column, and centrifuge.
11. Wash the column with 700 µl of buffer RW1.
12. Transfer the spin column to a new 2 ml collection tube.
13. Wash the column twice with 500 µl of buffer RPE (*see* **Note 6**) discarding the flow-through each time.
14. Reconnect the spin column and the 2 ml collection tube and centrifuge at full speed for 2 min.
15. Place the spin column in a clean 1.5 ml tube and elute the RNA by adding 53 µl of nuclease-free water to the column, wait 1 min, then centrifuge at full speed for 1 min.

3.2 DNase Treatment of Purified RNA

To ensure complete removal of contaminating DNA, treat the isolated RNA samples with DNase I (*see* **Note 7**)

1. Set up DNase treatment reactions by adding 6 µl of 10× DNase buffer and 1 µl of DNase I to the 53 µl of RNA eluted in step 15 (above).
2. Incubate at 37 °C for 60 min.
3. Stop the DNase I reaction by adding 7 µl of DNase deactivation buffer, mix by pipetting, and centrifuge samples at 10,000 × *g* for 2.5 min.
4. Carefully remove 42 µl of the supernatant to a clean tube (taking care not to disturb the white pelleted material), and assess the quality and quantity of RNA using an Agilent bioanalyzer.
5. Store RNA samples at −80 °C.

3.3 RNA Enrichment

RNA samples for RNA-seq experiments must be of the highest quality. Any degradation of the ribosomal RNA will result in poor RNA enrichment and subsequently increase the number of rRNA reads in the RNA-seq data set. To ensure RNA samples are of the highest quality they must be assessed on an Agilent bioanalyzer instrument using an RNA 6000 nano chip.

1. Following the manufacturer's protocol assess the quality and quantity of RNA samples by running 1 µl of each on an Agilent RNA 6000 nano chip.

RNA samples with an RNA integrity number (RIN) greater than or equal to 9.8 are of a suitable quality for use in RNA-seq (*see* **Note 8**).

2. Assess the quality of the RNA samples and proceed to the enrichment step only with those where the RIN > 9.7.
3. Using quantification from the bioanalyzer, determine the volume of each RNA sample that equals 4 μg RNA and add nuclease-free water to bring the total volume to 24 μl (*see* **Note 9**).
4. Add 2 μl of a 1/100 dilution of ERCC RNA spike-in-mix to each sample (*see* **Note 10**).
5. Proceed to rRNA removal.

Efficient removal of rRNA is essential prior to performing RNA-seq reactions. The method below combines the use of two commercially available rRNA removal kits. The Ribo-Zero Magnetic Kit for Gram-Positive Bacteria is used first, followed by a second round of purification using a MicrobExpress Bacterial mRNA Enrichment Kit (*see* **Note 11**). *S. aureus* RNA samples processed according to this protocol routinely generate RNA-seq data sets where total rRNA reads <1 %.

6. Prepare the Ribo-Zero Magnetic beads according to the manufacturer's protocol (*see* **Note 12**).
7. For each reaction, pipette 225 μl Magnetic Beads into a 1.5 ml RNase-free tube.
8. Place the tube on a magnetic rack and allow the solution to clear.
9. Remove and discard the supernatant, remove the tube from the magnetic rack, and wash the beads by pipetting up and down with 225 μl of RNase-free water.
10. Repeat steps 8–9 for a total of two washes with RNase-free water.
11. After the second wash, remove tubes from the magnetic rack and resuspend beads in 65 μl of Magnetic Bead Resuspension Solution.
12. Add 1 μl of RiboGuard RNase Inhibitor to each tube and mix by pipetting, and/or gentle vortexing.
13. Store the beads at room temperature until used in step 16.
14. Set up the Ribo-Zero reaction in a 0.2 ml nuclease-free tube by combining the following in the order given:

26 μl	RNA in water (from step 3 above)
4 μl	Ribo-Zero Reaction Buffer
10 μl	Ribo-Zero rRNA Removal Solution
40 μl	Total volume

15. Mix by pipetting and incubate at 68 °C for 10 min, then at room temperature for 5 min (*see* **Note 13**).
16. After incubation, add RNA to the magnetic beads (from step 13) and immediately pipette up and down at least ten times to mix, followed by vortexing at medium speed for 10 s.
17. Incubate reactions at room temperature for 5 min, then vortex (medium speed for 10 s) and incubate at 50 °C for 5 min.
18. Place tubes on a magnetic rack for 1 min or until solution appears clear.
19. Remove supernatant (95 μl) to a new 1.5 ml tube, being careful not to disturb the magnetic beads (*see* **Note 14**).
20. Add 120 μl of MicrobExpress Binding Buffer and mix by pipetting (total = 215 μl).
21. Add 4 μl of MicrobExpress Capture Oligo Mix, mix by pipetting and incubate at 70 °C for 10 min, then 37 °C for 30 min.
22. During this incubation, prepare the MicrobExpress Oligo MagBeads.
23. Transfer 50 μl Oligo MagBeads (per sample) to a new 1.5 ml tube, capture the beads on a magnetic rack, remove and discard the supernatant, and resuspend beads in 50 μl of nuclease-free water.
24. Capture beads on a magnetic rack again, remove and discard the supernatant, and resuspend in 50 μl of Binding Buffer.
25. Repeat step 24 and then incubate the resuspended beads at 37 °C in a waterbath until ready to use in step 26.
26. Following the incubation step (step 21) add 50 μl of Oligo MagBeads (from step 25) to the RNA/Capture Oligo Mix reaction (from step 21).
27. Mix samples by pipetting, and incubate at 37 °C for 15 min.
28. Capture Oligo MagBeads on a magnetic rack and transfer the supernatant (approx. 250 μl) to a clean 1.5 ml tube (*see* **Note 15**).
29. Ethanol-precipitate RNA as follows:

250 μl	Enriched RNA sample (from step 28)
25 μl	3 M Sodium Acetate
5 μl	Glycogen
750 μl	100 % Ethanol
1,030 μl	Total volume

30. Precipitate RNA at −80 °C for at least 1 h (*see* **Note 16**).

31. Pellet the precipitated RNA by centrifugation at max speed in a refrigerated centrifuge, 4 °C for 30 min.
32. Carefully remove the supernatant, wash the pellet with 750 µl of ice-cold 70 % ethanol and centrifuge again (10 min, max speed, 4 °C).
33. Remove the supernatant, air dry pellets (no more than 5 min) and resuspend RNA in 10 µl of nuclease-free water.
34. Assess the efficiency of rRNA removal by running 1 µl of each sample on an Agilent RNA 6000 nano or pico chip (*see* **Note 17** and Fig. 1).

3.4 RNA-Sequencing

A variety of kits and protocols exist for performing RNA-seq on each of the various next-generation sequencing platforms. Any of these may be used to sequence enriched RNA, and detailed protocols are available from the manufacturers to achieve this. RNA-seq in our laboratory has been carried out using the IonTorrent PGM. Library construction is performed using Ion Total RNA-Seq v2 Kits from Life Technologies. Template positive ISPs are generated using an Ion PGM™ Template OT2 200 Kit, followed by sequencing on an Ion 318™ Chip v2 using Ion PGM™ 200 Sequencing Kits. Detailed protocols, as well as instructional videos for each of these kits, can be found on the Ion Community Web page (http://ioncommunity.lifetechnologies.com).

Using the above protocols approx. 700 Mb to 1 Gb of sequencing data can be generated per run. To ensure sufficient depth of coverage is achieved for the *S. aureus* transcriptome, we run one RNA-seq experiment per Ion 318™ Chip. For smaller genomes, or if less depth of coverage is required, samples may be multiplexed, sequencing two or more RNA-seq samples on one Ion 318™ Chip. To do this, each sample must be barcoded using an Ion Xpress™ RNA-Seq Barcode Kit.

Following sequencing the data can be analyzed using a variety of platform-specific tools and plug-ins, or exported (in a variety of formats) for further analysis. A number of software packages are available for downstream analysis of NGS data. The next section will outline how we analyze RNA-seq data using the CLC Genomics Workbench software package (*see* **Note 18**).

3.5 Data Analysis

Detailed protocols and instructional videos on performing RNA-seq analysis using CLC Genomics Workbench can be obtained from the CLC Web site (http://www.clcbio.com/products/clc-genomics-workbench). Below we outline the major steps performed for RNA-seq analysis of *S. aureus* transcriptomic data.

1. Export unaligned read data in .sff file format.
2. Import data into CLC Genomics Workbench using the "Import—IonTorrent data" function (*see* **Note 19**).

3. Download the reference genome of the most closely related strain/species as a Genbank file (*see* **Note 20**), and import into CLC Genomics Workbench using the "Standard Import" function.
4. Generate a FASTA file containing the sequences of all 5S, 16S, and 23S rRNA genes from the reference genome, and import this into CLC Genomics Workbench using the "Standard Import" function (*see* **Note 21**).
5. Use the "Map reads to a reference" function to map RNA-seq data against the FASTA file containing rRNA sequences.
6. Collect any unmapped reads into a separate file (*see* **Note 22**).
7. Use the unmapped reads file, and the reference genome Genbank file, to perform RNA-seq data analysis using the "RNA-seq Analysis" function in CLC Genomics Workbench.
8. Following RNA-seq analysis, the gene expression values generated for two analyzed samples can be compared using the "Set up experiment" function.
9. Normalize experimental data using the "Quantile Normalization" function (*see* **Note 23**).
10. Gene expression values may be calculated, and a variety of statistical analyses (including scatter plots, histograms, Principal Component Analysis and hierarchical clustering), can be performed using CLC Genomics Workbench. Alternatively the data may be exported in a variety of formats for analysis elsewhere.

4 Notes

1. If possible use a dedicated clean area, and pipettes that have been cleaned using an RNase decontamination reagent such as RNaseZAP.
2. Washing pelleted bacteria once with an equal volume of ice-cold PBS may improve the quality of RNA samples taken from bacterial cultures in post-exponential and stationary phase.
3. A variety of RNA protecting reagents are available that may be added to bacterial cultures prior to pelleting samples. While the purpose of these reagents is to preserve RNA integrity, improved quality RNA samples can be obtained by simply working swiftly to freeze the bacterial pellet as quickly as possible.
4. Long-term storage of pellets is not recommended and will result in decreased quality of RNA samples. Short-term storage (i.e., less than 5 days) is acceptable.
5. Before beginning the RNA isolation procedure, aliquot 900 μl of 100 % ethanol into a 1.5 ml tube for each sample being processed. Following transfer of the 600 μl lysate into the

ethanol, mix immediately and thoroughly, before immediately transferring to the column.

6. Ensure ethanol has been added to buffer RPE prior to use, as per the manufacturer's instructions.
7. We have obtained variable results using "on column" DNase treatment protocols, and therefore, prefer to DNase treat samples following elution from columns. A variety of kits are available for DNase treatment. The turbo DNA-free kit from Ambion is used in our protocol.
8. The RIN number is calculated by the bioanalyzer software, and reflects the degree of degradation of ribosomal RNA. Samples with a RIN < 9.8 have some degree of rRNA degradation. Further processing of these samples will result in incomplete rRNA removal, and consequently, a high percentage of RNA-seq reads will be of rRNA origin.
9. The maximum starting volume for the RNA enrichment procedure is 24 μl; therefore, the starting concentration of RNA samples in water should be approximately 167 ng/μl.
10. The ERCC RNA spike-in-mix acts as an internal control when comparing expression between RNA-seq data sets. Adding the ERCC RNA spike-in-mix prior to rRNA depletion controls for variation throughout the sample processing steps.
11. For steps 6–19 use reagents from the Ribo-Zero Magnetic Kit for Gram-Positive Bacteria. For steps 20–30 use reagents from the MicrobExpress Kit.
12. The manufacturer's protocol contains detailed instructions and suggestions for each step of the procedure. These instructions are summarized in steps 7–15; however, it is highly recommended that the manufacturer's protocol is read prior to commencing experimentation.
13. For consistency, do these incubation steps in a thermocycler.
14. The supernatant contains the enriched RNA, while rRNA is bound to the magnetic beads. Be careful not to transfer any of the magnetic beads along with enriched RNA. It is preferable to leave a small amount of supernatant behind in tubes containing the magnetic beads, than to transfer magnetic beads along with the enriched sample.
15. As with step 19, it is preferable to leave a small amount of supernatant behind in tubes containing the magnetic beads, than to transfer magnetic beads along with the enriched sample. Omit the washing steps included in the MicrobExpress protocol as they only slightly increase the yield of RNA, whilst at the same time greatly increasing the chances of carrying over rRNA into the enriched sample.
16. Ethanol precipitation can also be carried out overnight.

17. The amount of RNA remaining in each sample will be <90 % that of the starting amount; therefore, running the enriched samples on an Agilent RNA 6000 Pico kit may be necessary to obtain reliable data. The bioanalyzer trace for enriched samples should show no discernable peak for 5S, 16S, or 23S rRNA (*see* Fig. 1 panel b). If any peaks are observed corresponding in size to these rRNA species, then the enrichment was not successful and samples should not be used in RNA-seq analysis.
18. While other software programs are available for data analysis (and therefore the specifics of data handling will vary depending on the package used) the basic steps outlined will be similar.
19. Specific import functions exist for each type of next-generation sequencing platform.
20. When performing RNA-seq a "reference genome" must be used. Use the strain/species that most closely matches, genetically, that of the experimental organism. *S. aureus* genomes may be downloaded from NCBI (http://www.ncbi.nlm.nih.gov/genome/154).
21. The purpose of RNA enrichment procedures (Section 3.3) is to remove the majority of ribosomal RNA from samples; however, some rRNA will remain. As this amount of residual rRNA can vary among samples, all reads in the RNA-seq data set corresponding to rRNA should be removed prior to calculating RPKM expression values for each gene. RPKM values are calculated based on the total number of reads in a data set, and therefore, any variation in the number of rRNA reads across different samples could have inadvertent effects on the expression values in each sample.
22. The unmapped reads file now contains all RNA-seq reads corresponding to messenger RNA, with any remaining rRNA reads removed. Use this file for further analysis.
23. Quantile normalization is recommended during analysis of bacterial RNA-seq data sets (1).

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Quantitative Real-Time PCR (qPCR) Workflow for Analyzing *Staphylococcus aureus* Gene Expression

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Abstract

Quantitative real-time polymerase chain reaction (qPCR) is a sensitive tool that can be used to quantify and compare the amount of specific RNA transcripts between different biological samples. This chapter describes the use of a "two-step" qPCR method to calculate the relative fold change of expression of genes of interest in *S. aureus*. Using this work-flow, cDNA is synthesized from RNA templates (previously checked for the absence of significant genomic DNA contamination) using a cocktail of random primers and reverse-transcriptase enzyme. The cDNA pools generated can then be assessed for expression of specific genes of interest using SYBR Green-based qPCR and quantification of relative fold-change expression.

Keywords: Real-time PCR, Relative quantification, Livak calculation, RNA, cDNA, SYBR Green

1 Introduction

Quantitative real-time polymerase chain reaction (qPCR) represents an important advancement in molecular biology, whereby the sensitivity of PCR has been combined with the ability to monitor amplification of specific double-stranded DNA products "in real time" at the end of each cycle of the PCR reaction (1, 2). This is achieved by the use of specialized thermocyclers that can measure the fluorescence of specific primer or probe sequences (i.e., "TaqMan" hydrolysis probes, "LightCycler" hybridization probes, molecular beacons) or intercalating dyes (i.e., SYBR Green) to detect the amplified product of interest (3). Regardless of the detection method used, the cycle number at which enough amplified product accumulates to yield a detectable fluorescent signal (termed the "threshold cycle" or "C_T value") is the actual measure used to calculate and quantify the initial amount of template present in a qPCR reaction (4). The amount of starting template in a qPCR reaction and the measured C_T value have an inverse relationship, in that the greater the amount of initial target template, the fewer cycles that are needed to produce detectable fluorescence (2). Because C_T values are measured in the exponential phase of amplification (when reagents are not limiting), they can be used to

reliably and accurately calculate the initial amount of template present in a reaction (4).

In order to utilize qPCR for measuring changes in RNA transcript levels, there are several factors that must be taken into account. For one, it is important to ensure that RNA samples are of high quality and are not contaminated with significant amounts of genomic DNA that may yield false-positive amplification in the downstream qPCR reaction. A second parameter to consider is the choice of a "one-step" (cDNA synthesis and qPCR occur sequentially in the same master mix) verses "two-step" (cDNA is synthesized in a separate reaction before being used as template in qPCR). "Two-step" qPCR typically uses a mixture of oligo(dT) and random primers to amplify the entire cDNA pool, which can be advantageous if subsequent expression studies require the measurement of many different transcripts. In contrast, "one-step" PCR must use a gene-specific oligonucleotide to prime reverse-transcription of the RNA of interest, but this technique cuts out pipetting steps that could potentially introduce experimental error and lesson the accuracy of the overall qPCR (4). Third, a detection method must be chosen, using either fluorescently labeled primers/probes, or intercalating dyes that fluoresce when bound to the double-stranded PCR product. Labeled primers or probes are sequence-specific, but are less cost-effective since a new probe must be synthesized for each gene of interest. Intercalating dyes such as SYBR Green bind indiscriminately to all double stranded DNA products (including potential nonspecific PCR products and/or primer dimers), therefore they lack the target specificity conferred by the labeled primers/probes. However, these dyes are an economical alternative and are preferred for assays requiring testing of many different genes, eliminating the need to design specific probes for each gene of interest. A melt-curve analysis should always be included with qPCR reactions using intercalating dyes, as this will indicate the presence of nonspecific products and/or primer dimers (4, 5). Fourth, a choice must be made between using absolute or relative quantification methods to analyze gene expression data. In absolute quantification the C_T value of each unknown sample is compared to a standard curve of C_T values generated from known amounts of template. By comparison, relative quantification does not calculate the absolute starting quantity of unknown template, but instead reports fold change values compared to a control condition (4). Finally, publication of qPCR results should adhere to the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines, a series of recommendations created to promote publication of sufficient experimental detail that enables the reader to assess the quality of the presented results and/or to be able to reproduce the described experiments (6, 7).

qPCR has become popular in the field of *S. aureus* biology as a means of measuring changes in gene expression, as well as a validation tool for RNA microarray (8–11) and RNA-seq studies (12–14). The two-step qPCR workflow presented in this chapter has been optimized for analyzing *S. aureus* gene expression in our lab by relative quantification using the Livak method (15). The typical experimental workflow is (1) "quality-control" check of RNA samples for genomic DNA contamination, (2) cDNA synthesis from RNA templates using reverse-transcriptase, (3) detection of transcripts of interest using qPCR, and (4) quantification of relative fold-change expression.

2 Materials

1. Nuclease-free PCR water, non-DEPC treated (*see* **Note 1**).
2. Forward and Reverse Primer pairs for reference gene and target gene (*see* **Note 2**).
3. Bio-Rad iQ™ SYBR® Green Supermix (2× concentrate).
4. Ambion RNase away (*see* **Note 3**).
5. DNAase/RNase-free water (*see* **Note 3**).
6. 70 % ethanol (*see* **Note 3**).
7. PCR cabinet with filtered airflow and UV light (*see* **Note 3**).
8. Pipettes (*see* **Note 3**).
9. Vortex Genie.
10. DNase/RNase-free aerosol barrier pipette tips (*see* **Note 3**).
11. 1.7 ml microcentrifuge tubes, sterile, DNase/RNase free.
12. RNA Template (*see* **Note 4**).
13. Ambion Turbo-DNase kit (*see* **Note 4**).
14. Bio-Rad iScript™ cDNA Synthesis Kit.
15. Thermocycler.
16. Thin-walled PCR tubes, sterile, DNase/RNase free.
17. qPCR machine (*see* **Note 5**).
18. qPCR well plate (*see* **Note 5**).
19. Film for qPCR plate (*see* **Note 5**).

3 Methods

For all steps below, all samples and reaction components should be thawed and stored on ice while in use. Perform all work in a PCR cabinet using DNase/RNAse-free pipettes and filter tips (*see* **Note 3**).

3.1 Check of RNA Samples for Genomic DNA Contamination

1. Dilute each RNA sample in sterile nuclease-free water (non-DEPC treated) to a final concentration of 37.5 ng/μl in a volume of 20 μl. Place diluted samples on ice until step 5 below.
2. Set up a master mix containing all of the components (but do not add template) listed in Table 1, column 1, using the reference gene primer set. This master mix should contain enough volume to analyze each RNA template, a no template control (NTC), and one extra sample to account for pipetting error. In this example experimental setup, the master mix recipe in column 2 of Table 1 is designed for triplicate 15 μl qPCR reactions per template, and is enough to analyze two RNA templates, one positive control (genomic DNA), one no template control (NTC; nuclease free water), and one extra sample to account for pipetting error.
3. Vortex master mix from step 2 above, and aliquot into mini master mixes for each RNA sample, DNA template, and NTC. Each mini master mix should have enough volume for setting up triplicate qPCR reactions per template, plus one extra volume to account for pipetting error. In this experimental example, 52 μl of the master mix is aliquoted into 4 × 1.7 ml microcentrifuge tubes.
4. Add the appropriate template (RNA sample, DNA sample, or NTC) to each mini master mix. In this experimental example, 8 μl of template is added to 52 μl of mini master mix.
5. Vortex each mini master mix and aliquot in triplicate into wells of the qPCR plate. In this experimental example, 15 μl of each

Table 1
Sample qPCR reaction setup for analyzing four templates (two RNA samples, one DNA sample, one NTC)

Component (initial concentration)	Amount to add to master mix (260 μl total volume) (μl)	Volume per mini master mix (60 μl total volume) (μl)	Final volume per 15 μl reaction (μl)	Final concentration or amount per 15 μl reaction
Sterile, nuclease free water (non-DEPC treated)	50	10	2.5	N/A
Forward primer (2.5 μM)	30	6	1.5	0.25 μM
Reverse primer (2.5 μM)	30	6	1.5	0.25 μM
iQ™ SYBR® Green Supermix (2×)	150	30	7.5	1×
Template (37.5 ng/μl)	Not added at this step	8	2	75 ng

mini master mix is aliquoted in triplicate to the wells of the qPCR plate (*see* **Note 6**).

6. Apply plastic film to seal the top of the qPCR plate (*see* **Note 7**).
7. Transfer sealed qPCR plate to qPCR machine, and run qPCR reaction, using denaturation, annealing/extension times and temperatures that have been optimized for the primers and templates to be analyzed (*see* **Note 8**).
8. Examine the average C_T values generated from each qPCR reaction. The NTC reaction should not yield a C_T value (or a very high C_T value close to 40). In order to proceed with cDNA synthesis, each RNA template should yield a C_T value of 30 or larger. If RNA C_T values are less than 30, an Ambion Turbo-DNase treatment may be performed to eliminate residual DNA contamination.

3.2 cDNA Synthesis from RNA Templates

1. Based on the RNA concentration of each sample (*see* **Note 4**), calculate the volume for each that contains 0.75 μg RNA.
2. Setup a 20 μl cDNA synthesis reaction in a thin-walled PCR tube for each RNA sample using the Bio-Rad iScript™ cDNA Synthesis Kit and recipe listed in Table 2 (*see* **Note 9**).
3. Transfer cDNA reactions to a thermocycler programmed as follows: 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, 4 °C hold.
4. When cDNA synthesis reactions are complete, use cDNA immediately for qPCR or store at −20 °C until you are ready to proceed with qPCR (*see* **Note 9**).

3.3 qPCR Detection of Target and Reference Gene Expression

1. Set up a master mix for each of the reference primers and the target primers, as outlined in Fig. 1. Each master mix will contain all of the components listed in Table 1, column 1, with the exception of template, which will be added in step 4 below.

Table 2
Recipe for cDNA synthesis

Reaction component	Volume added per 20 μl reaction	Final concentration or amount per 20 μl reaction
5× iScript reaction mix (contains polyA and random hexamer primers)	4 μl	1×
iScript reverse transcriptase enzyme	1 μl	
RNA template	Volume necessary to add 0.75 μg RNA to reaction	37.5 ng/μl
Sterile, nuclease-free water (non-DEPC treated)	Volume necessary to bring reaction volume to 20 μl	

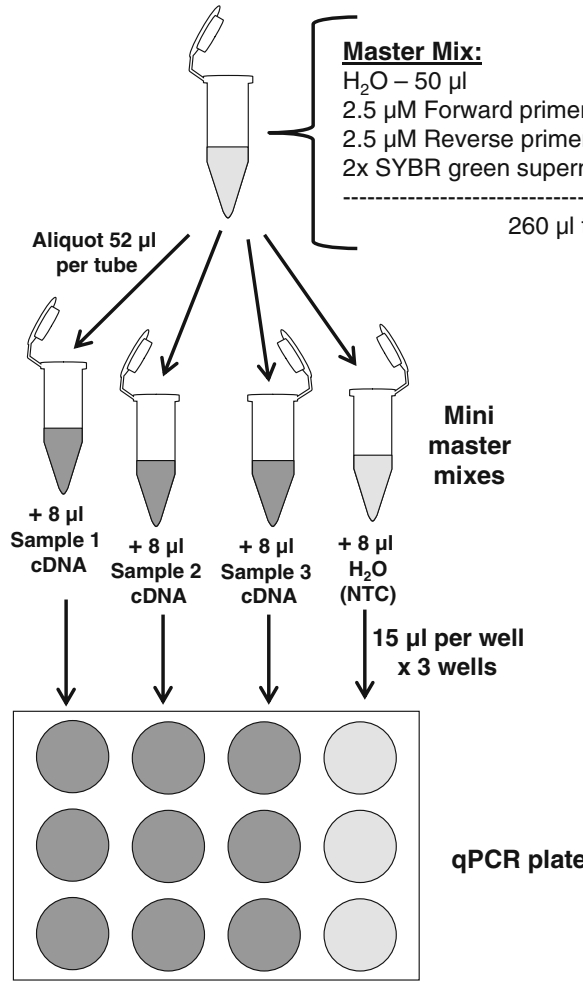

Fig. 1 Schematic of qPCR setup

The master mix should contain enough volume to analyze each cDNA template, a no template (negative) control, and one extra sample to account for pipetting error. In this example experimental setup, the master mix recipe in column 2 of Table 1 is designed for triplicate 15 µl qPCR reactions per template, and is enough to analyze three cDNA templates, one NTC control, and one extra sample to account for pipetting error.

2. Vortex each master mix from step 1 above, and aliquot each into mini master mixes for each cDNA sample and NTC. Each mini master mix should have enough volume for setting up triplicate qPCR reactions per template, plus one extra volume to account for pipetting error. In this experimental example, 52 µl of each master mix is aliquoted into 4 × 1.7 ml microcentrifuge tubes.

3. Add the appropriate template (cDNA, or sterile nuclease-free water for NTC) to each mini master mix. In this experimental example, 8 μl of template is added to 52 μl of each mini master mix.
4. Vortex each mini master mix, and aliquot in triplicate into wells of the qPCR plate. In this experimental example, 15 μl of each mini master mix is aliquoted in triplicate to the wells of the qPCR plate (*see* **Note 6**).
5. Apply plastic film to seal the top of the qPCR plate (*see* **Note 7**).
6. Transfer sealed qPCR plate to qPCR machine, and run qPCR reaction, using denaturation, annealing/extension times and temperatures that have been optimized for the primers and templates to be analyzed (*see* **Note 8**).
7. Examine the average C_T values generated from each qPCR reaction (cDNA templates and NTC). The NTC reaction should not yield a C_T value (or a very high C_T value close to 40). Also examine the melt curve to ensure the presence of one distinct peak per primer set (*see* **Note 10**).

3.4 Quantification of Relative Fold-Change Expression Using the Livak Method (15) (See Note 11)

1. For the experimental setup being analyzed, one of the cDNA samples must be assigned as the calibrator, and the rest of the cDNA samples as the "test" samples. For the example data graphed in Fig. 2, the aerobic 2 h growth cDNA sample was assigned as the calibrator, whereas the 6 and 12 h aerobic samples and all low-oxygen samples were considered the "test" samples.
2. Normalize the C_T value of each target gene to that of its corresponding reference (ref) gene C_T value for each test sample and the calibrator sample using the following formulas:

$$\Delta C_T(\text{test}) = C_T(\text{target, test}) - C_T(\text{ref, test})$$
$$\Delta C_T(\text{calibrator}) = C_T(\text{target, calibrator}) - C_T(\text{ref, calibrator})$$

3. Normalize the ΔC_T of each test sample to the ΔC_T of the calibrator using the following formula:

$$\Delta\Delta C_T = \Delta C_T(\text{test}) - \Delta C_T(\text{calibrator})$$

4. Calculate the expression ratio for each test sample $\Delta\Delta C_T$ using the Livak equation:

$$2^{-\Delta\Delta C_T} = \text{Normalized expression ratio}$$

5. The normalized expression ratio for each test sample can be graphed as the fold-change relative to the calibrator sample (*see* Fig. 2), or alternatively can be presented in a table. The example data presented in Fig. 2 were obtained by following the qPCR

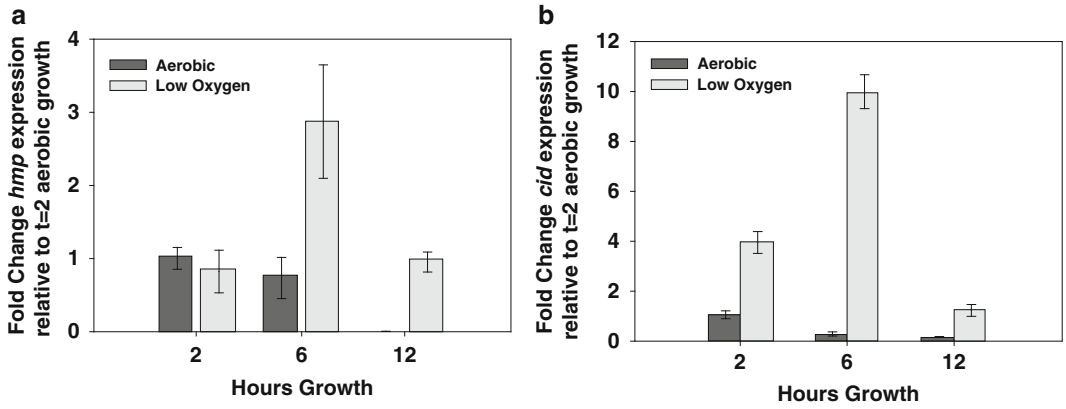

Fig. 2 Sample qPCR data analyzing *hmp* (**a**) and *cidA* (**b**) gene expression in *S. aureus*. Total RNA was isolated at the indicated time points from $n = 3$ biological replicates of UAMS-1 grown under highly aerated (*dark grey bars*; 250 rpm, 1:10 volume-to-flask ratio) or low-oxygen (*light grey bars*; 0 rpm, 7:10 volume-to-flask ratio) conditions. Quantitative real-time PCR was performed on reverse-transcribed cDNA from each sample using *hmp* (**a**) and *cidA* (**b**) specific primers. The Livak method was used to determine relative fold-change expression of each gene, using *sigA* expression as the reference gene and the 2 h aerobic sample as the calibrator. Error bars = Standard Error of the Mean

workflow presented in this chapter, and the resulting data indicated that both the *cidA* and *hmp* genes are more highly expressed during low-oxygen growth relative to aerobic growth. These results correlated very well to previous qPCR expression data for these two genes published by other groups (16, 17).

4 Notes

1. It is recommended that non-DEPC-treated nuclease-free sterile water be utilized for qPCR-based applications, as trace amounts of DEPC that have not been inactivated are thought to have the potential to inactivate the PCR polymerase and inhibit the qPCR reaction.
2. Primer design for qPCR: It is recommended that primer design for genes of interest be performed with software such as Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi), which has a preprogrammed qPCR option. In general, qPCR primers should amplify a target product of 75–200 bp, as smaller products tend to have higher efficiencies but less specificity. At the same time, qPCR products need to be large enough to make them distinguishable from primer dimers. Primers must also be designed for a reference gene, which by definition should be expressed at a similar level over all of the different experimental conditions you plan to test (18). Our lab has found that primers specific for the *S. aureus* sigma-A (*sigA*)

gene satisfy this criteria under most of the experimental conditions we have tested. Other researchers have also utilized *sigA* as a reference gene (17). Alternatively, it has recently been suggested that the geometric mean of several reference genes be used for normalization of *S. aureus* gene expression studies using qPCR (19). For both target and reference primers, it is recommended that these be tested in a traditional PCR reaction using genomic DNA as template, and analyzed by gel electrophoresis to verify amplification of the correctly sized product and the absence of nonspecific products or primer dimers. In addition to the primer considerations stated above, each target and reference primer pair must be used to generate a standard curve using serial dilutions of cDNA template, in order to calculate the efficiency of each primer set (4). This analysis will also give you an idea of how much you need to dilute your sample cDNA template (if at all), to ensure that the C_T values fall into the linear range of the curve. Hallmarks of a good standard curve include linearity ($R^2 > 0.98$), efficiency (E) between 90 and 105 %, and an ideal slope of −3.3 (4).

3. Nuclease-free work environment considerations: To minimize degradation of RNA and cDNA samples, it is imperative to maintain a clean workspace that is free from contamination by DNase and RNase enzymes. The first safe-guard to protecting these samples is wearing gloves and changing them often throughout the experiment. If RNA or cDNA degradation appears to be problematic in the qPCR workflow, the use of additional precautions such as wearing disposable sleeve protectors over your lab coat, and performing all steps of the experiment under a PCR cabinet equipped with UV light and filtered airflow is highly recommended. Prior to working with RNA or cDNA, all pipettes and work surfaces should be wiped down thoroughly with Ambion RNase Away. As this solution is irritating to skin and can rust metal surfaces, it is important to wash the pipettes and work surfaces a second time with 70 % (vol/vol) ethanol (made with nuclease-free water) or 100 % nuclease-free water to remove the residual RNase Away. All disposable plastics to be used in the qPCR workflow (tubes, aerosol barrier pipette tips) should also be certified DNase/RNase free and autoclaved before use (if not already pre-sterilized).

4. RNA samples can be isolated from *S. aureus* by any optimized method of preference, as long as the concentration of RNA is ≥70 ng/μl and the A_{260}/A_{280} ratio is ≥2.0. Our lab utilizes the FASTPREP lysing matrix B and Qiagen RNeasy kit according to a previously published protocol, and elute the RNA using non-DEPC-treated nuclease-free water (20). For RNA to be analyzed by qPCR we routinely incorporate a second

DNase treatment following RNA isolation, using the Ambion Turbo DNase kit. RNA samples should be stored at −80 °C prior to cDNA synthesis and qPCR analysis. Although we have not encountered any issues with degradation of RNA upon repeated freeze-thawing of samples, RNA can be stored as working aliquots to minimize this concern.

5. Use the plates and sealing film that are compatible with the qPCR platform to be used in the experiment. Our lab routinely uses the Illumina Eco real-time PCR instrument, which has a small 48-well plate format.
6. It is important to minimize the introduction of air bubbles into the wells of the qPCR plate, as these can sometimes interfere with fluorescent detection by the qPCR instrument. Air bubbles can be removed by gently tapping the plate, or, if there is access to a centrifuge and rotor compatible with the qPCR plate, a short, low-speed spin should greatly reduce or eliminate air bubbles from the wells.
7. When applying the sealing film to the plate, it is helpful to use the edge of a pipette box lid or some other solid edged surface to smooth out the film evenly and securely over the plate. Wrinkles in the applied sealing film can sometimes interfere with fluorescent detection by the qPCR instrument, and if the sealing film is not secured properly, evaporation can occur in the wells during the qPCR reaction.
8. In our lab we use the Illumina Eco qPCR machine, and with most *S. aureus* primers and templates, the program (including melt curve analysis) outlined in Table 3 works well.
9. cDNA synthesis reactions may be scaled up to 40 μl if a large amount of cDNA template is required. If this option is pursued, 1.5 μg RNA template should be added to each 40 μl cDNA synthesis reaction. For best results, repeated freeze-thawing of

Table 3
Sample qPCR cycling protocol

Step	# Cycles	Temperature	Time
Polymerase activation	1	95 °C	3 min
Data collection	40	95 °C (denaturation)	15 s
		58 °C (annealing/extension)	30 s
Melt curve	1	95 °C	15 s
		58 °C	15 s
		58 °C + 0.5 °C/s	
		95 °C	15 s

cDNA samples should be avoided. Therefore it is recommended that cDNA be stored at −20 °C in working aliquots.

10. When performing qPCR using non-sequence specific detection methods such as SYBR Green, it is essential that a melt curve is performed at the end of the qPCR reaction. This step detects the melting temperatures (based on the size and nucleotide composition) of each PCR product formed in each well of the qPCR plate. One distinct peak on the melting curve generally indicates that only one PCR product is being synthesized and detected. However, multiple peaks indicate the presence of nonspecific products and/or formation of primer dimers, which can artificially lower the C_T of the reaction. In this latter scenario, the qPCR primers should be redesigned.
11. To use the Livak calculation for relative quantification in qPCR, the efficiencies of the reference and target primers must be within 5 % of each other, and both efficiencies should ideally be close to 100 %. The efficiencies of each primer set can be calculated by performing a standard curve with serial dilutions of cDNA template as described in **Note 2**. If the efficiency of each primer set is high (greater than 85 %) but not within 5 % of each other, an alternative relative fold-change calculation can be performed, such as the Pffafl method (21).

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Electrophoretic Mobility Shift Assays

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Abstract

Experimental demonstration of regulatory protein interactions with the sequences upstream of potential target genes is an important element in gene expression studies. These experiments termed electrophoretic mobility shift assays (EMSAs) provide valuable insight into the mechanism of action of transcription factors. EMSAs combined with downstream applications such as transcriptional analysis help uncover precisely how regulatory proteins control target gene expression. This chapter comprises a guideline for expression and purification of recombinant transcription factor proteins followed by a detailed protocol for EMSAs.

Keywords: Electrophoretic mobility shift assay, Transcription regulator, Promoter, Gene regulation

1 Introduction

Transcription factors are regulatory proteins that bind to specific DNA sequence motifs upstream of genes in order to modulate their expression. In molecular biology EMSAs, also called gel retardation assays, are employed to ascertain if a protein of interest or several proteins bind to a specific DNA fragment. The method involves investigating whether a labeled DNA probe can interact specifically with a purified recombinant protein under in vitro conditions. A specific interaction between the DNA fragment and recombinant protein will generate a complex with a higher molecular weight than the labeled DNA fragment alone. Electrophoretic migration of a protein–DNA complex through a polyacrylamide gel will be slower or "retarded" compared to migration of DNA fragment alone. In order to determine if the protein–DNA complexes are specific, an excess of unlabeled specific DNA is added to the reaction, which competes with the labeled DNA fragment resulting in reduced levels of interaction between the labeled DNA fragment and the protein. In contrast, the addition of excess nonspecific DNA will have no effect on the specific interaction between a protein and a labeled DNA fragment. If the protein–DNA complex is nonspecific, addition of excess unlabeled specific or nonspecific DNA will reduce the interaction of the complex (Fig. 1).

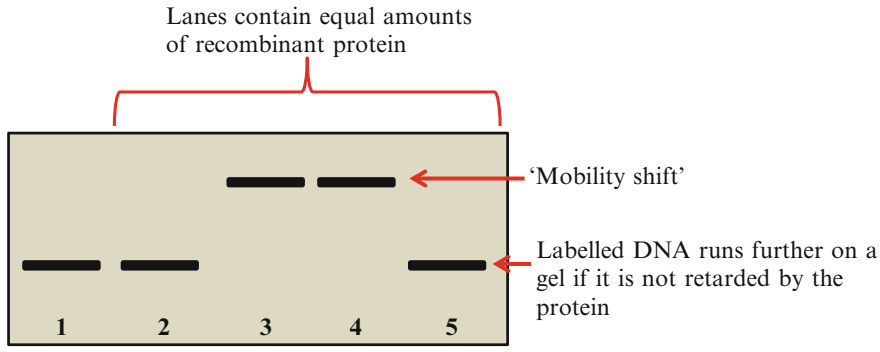

Fig. 1 Schematic representing an EMSA. Only labeled DNA probes are visible in the EMSA. They run slower if they are bound by the recombinant protein. *Lane 1* is a negative control containing a labeled DNA probe of interest (specific DNA) and no protein. *Lane 2* contains a labeled nonspecific DNA probe predicted not to interact to the protein of interest. *Lane 3* contains a labeled specific DNA probe. *Lane 4* contains the labeled specific DNA probe and 10× nonspecific unlabeled competitor DNA. *Lane 5* contains a labeled specific DNA probe and 10× specific unlabeled competitor DNA. *Lanes 2–5* contain the same amount of recombinant protein. An excess of unlabeled competitor DNA disrupts the gel shift only if it is specific

2 Materials

2.1 Protein Purification and Western Blot

1. Overnight expression autoinduction media: EMDmillipore
2. Lysis buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0
3. Lysozyme (50 mg/ml) stock
4. 2× SDS reducing sample buffer: 100 mM Tris–HCl (pH 6.8), 4 % (w/v) SDS (sodium dodecyl sulfate; electrophoresis grade), 0.2 % (w/v) bromophenol blue, 20 % (v/v) glycerol, 200 mM DTT (dithiothreitol). Store the SDS gel-loading buffer without DTT at room temperature. Add DTT from a 1 M stock just before the buffer is used. 200 mM β-mercaptoethanol can be used instead of DTT.
5. 4–12 % Bis-Tris pre-cast mini gel, 10 well, 1.5 mm thick
6. Immobilon P PVDF membrane 0.45 μm
7. 100 % methanol
8. Denionized water (dH_2O)
9. NuPAGE MES SDS Running Buffer (20×): Invitrogen or similar buffer
10. NuPAGE 20× Transfer Buffer: Invitrogen or similar buffer
11. Mini cell electrophoresis chamber
12. Transfer Module
13. His tag mouse monoclonal antibody-

14. Chemiluminescent Kit, anti-mouse
15. Ni-NTA Agarose: Invitrogen
16. Wash buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0
17. Elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 8.0
18. Bradford Assay reagents
19. *E. coli* strain carrying expression plasmid

2.2 Purification of a Biotinylated DNA Probe

1. 10 % TBE precast gel
2. TE buffer: 10 mM Tris pH 8.0, 1 mM EDTA
3. Ethidium bromide
4. 70 % Ethanol
5. 3 M sodium acetate
6. Spectrophotometer capable of quantifying DNA

2.3 EMSA

1. Binding buffer: 100 mM Tris pH 7.5, 500 mM KCl, 10 mM DTT, 5 % glycerol, 5 mM $MgCl_2$, 0.2 μg poly-(dI-dC)
2. Precast 6 % DNA Retardation Gels
3. Biodyne-B nylon membrane: Pall Corporation, AGB
4. Chemiluminescent Electrophoretic Mobility Shift Assay Kit: (we prefer the LightShift kit from Thermo Scientific).

3 Methods

3.1 Tag Selection for Recombinant Protein

Tags can interfere with protein function so it is important to consider their size, application, and other properties before deciding which tag to fuse to your protein of interest. Table 1 summarizes the applications and size of the following tags GST, His, HA, myc, FLAG, MBP. Small tags such as HA, His, FLAG, and myc are ~1 kDa in size and are therefore less likely to interfere with protein function. These tags can be added to either the N- or C-terminal of the protein (Fig. 2) (*see* **Note 1**). Another advantage of choosing a small tag is that the tag sequence can be added to your cloning primer to facilitate amplification of a tagged version of your gene of interest. The PCR product containing the tagged-gene can then be subcloned into an expression vector of your choice rather than purchasing a recombinant expression system required when using the larger MBP or GST tags. However, some proteins, such as AraC-type proteins are insoluble and notoriously difficult to purify. Addition of an MBP tag can facilitate the purification of such insoluble proteins [1].

Table 1
Tags commonly added to N- or C- terminal proteins in order to facilitate downstream applications such as protein purification, pull-down assays, western blots

Name	Sequence	MW (kDa)	Notes on application	Notes on construction
FLAG	DYKDDDDK	1	Pull down assays Very good for western blot	Can add sequence to primer
His (polyhistidine)	HHHHHH	1	Western blot, pull down ssays Very good for purification	Can add sequence to primer
MBP (maltose binding protein)		42	Good tag for purification of typically insoluble proteins	Need a plasmid system
GST (Glutathione *S*-transferase)		26	Pull down assays, protein purification, western blot	Need a plasmid system
HA (human influenza hemagglutinin)	YPYDVPDYA	1	Western blot, purification, pull down assays	Can add sequence to primer
Myc	EQKLISEEDL	1.2	Western blot, protein purification	Can add sequence to primer

Sequence information is shown for small tags

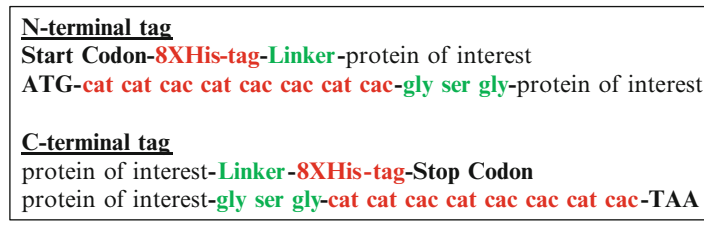

Fig. 2 Schematic representing the orientation 8×Histidine tags (DNA sequence in *red*) and linker (amino acid sequence in *green*) on the N- and C- terminal of a gene of interest. If the tag is added to the N-terminal of the gene, the start codon must be included in front of the tag, followed by a linker and the sequence of the gene (ensuring the codons are in frame). Similarly, when a tag is added to the C-terminal of a gene, the gene codons must run in frame with the linker, followed by the tag and then the stop codon

The His-tag will be described in this chapter because the His purification system is relatively straightforward and available from multiple commercial suppliers. When designing oligonucleotide primers to amplify a gene of interest, a linker can be added between the gene itself and the His-tag. For example, linkers encoding gly-gly-gly or gly-ser-gly can be used (Fig. 2). This helps to reduce the

possibility of the tag affecting the function of the recombinant protein (*see* **Note 2**). For best results, the tagged gene should be cloned into an inducible expression vector, for example under the control of an IPTG-inducible promoter and transformed into an cloning *E. coli* strain such as DH5α [2] (*see* **Note 3**).

3.2 Confirming Expression of Recombinant Protein in E. coli

It is important to confirm the nucleotide sequence of the recombinant expression construct to ensure that the gene and its tag will be expressed in frame before transformation into a strain of *E. coli* suitable for protein expression, such as BL21(DE3) (*see* **Note 4**) [3].

Once the expression strain has been selected, a western blot can be performed to verify the expression level, size, and quality of the recombinant protein. A western blot can also provide insight into whether the recombinant protein is present in the soluble fraction under various growth and induction conditions (*see* **Note 5**). Many types of expression systems are commercially available; an IPTG-inducible promoter will be used as an example here.

3.3 Pilot Expression Protocol: Preparation of Cell Extracts for Western Blot

1. Inoculate 10 ml overnight expression autoinduction media containing selection antibiotic with a single colony of the *E. coli* strain harboring either the IPTG-inducible recombinant expression plasmid or the empty plasmid.
2. Incubate overnight (approximately 16 h) at 37 °C with good aeration.
3. Pellet cells by centrifugation at 8800 × *g* for 10 min.
4. Decant supernatant and resuspend cell pellet in 1 ml lysis buffer containing 1 mg/ml lysozyme and incubate on ice for 20 min with gentle agitation.
5. Sonicate cells in an ice bath for 2 min (15 s bursts with 30 s intervals). Samples must be kept cold at all times. It is also important to ensure that no frothing occurs during sonication, which may result in protein denaturation.
6. Separate the soluble and insoluble fractions of the cell extracts by centrifugation at 16,000 × *g* for 30 min at 4 °C.
7. Aliquot the soluble fraction into a clean labeled tube on ice and resuspend the insoluble fraction in 1 ml lysis buffer. Remaining particulate insoluble material is likely not protein.
8. Add 30 µl aliquots of the soluble or insoluble fractions to 30 µl 2× SDS reducing sample buffer in a PCR tube and heat to 95 °C for 5 min.
9. Centrifuge the samples at 16,000 × *g* for 2 min to pellet cell debris.
10. Load the supernatant (10–30 µl aliquots) on a 4–12 % Bis-Tris acrylamide gel and separate by electrophoresis at 180 V for approximately 45 min.

11. During electrophoresis, prepare the PVDF membrane and filter paper (*see* **Note 6**).
12. Soak the sponges and filter paper in 1× transfer buffer until required.
13. Submerge the membrane in 100 % methanol for 10 s (*see* **Note 7**) and then transfer into dH_2O using a plastic forceps (*see* **Note 8**).
14. Transfer the separated proteins in the polyacrylamide gel to the PVDF membrane at 30 V for 1 h using an electroblotting apparatus.
15. Immuno-detection is performed using commercial antibodies specific for the tag fused to the expressed protein. For example, an anti-polyhistidine antibody (or antibody against an alternative tag fused to the protein of interest) is used as a primary antibody. His-tag antibodies can nonspecifically react with *E. coli* proteins containing histidine residues and it is therefore important to include a control *E. coli* strain carrying only the empty expression vector to distinguish between specific and nonspecific bands on the Western blot (*see* **Note 9**).
16. Confirm that the recombinant protein is in the soluble fraction. If it is in the soluble fraction, then further optimization is not needed before purification. If the recombinant protein is only detectable in the insoluble fraction, the conditions need to be optimized to increase solubility (*see* **Note 10**).

3.4 Purification of Recombinant Protein

When the protein expression conditions have been optimized, culture volumes can be scaled up before proceeding to the protein purification procedure.

1. Dilute the soluble recombinant His-tagged protein 1/50 in cold lysis buffer. For example, 1 ml of soluble protein is added to 50 ml cold lysis buffer and stored on ice.
2. Add 1 ml of Ni-NTA agarose (slurry) into the soluble protein and allow to rotate slowly on a shaker at 4 °C for 1 h or overnight. This allows the His-tagged recombinant protein to bind to the nickel in the agarose.
3. Prepare the column by passing 1 column volume of wash buffer through the column.
4. Cap the column and add the protein-slurry to the column.
5. Incubate for at least 15 min until the agarose settles to the bottom of the column.
6. Uncap the column and allow to flow through, some of which can be retained for analysis by Western blot.
7. Wash the column resin with 5 column volumes of wash buffer at a speed of 1 ml/min using a peristaltic pump.

8. Recap the column and add 500 µl of elution buffer to the resin and incubate for 5 min.
9. Uncap the column and collect the eluate from the column in a micro-centrifuge tube.
10. Repeat steps 8 and 9 *four* more times.
11. Collect purified elution fractions 1–5 in 500 µl aliquots and analyze on a 4–12 % Bis-Tris acrylamide gel. Determine the protein concentrations by Bradford Assay.
12. Protein samples can be stored at 4 °C for use within 1 week or in the longer term at −80 °C in 50 µl aliquots.

3.5 Preparation of a Biotinylated DNA Probe

A DNA probe can be labeled radioactively, fluorescently, or with biotin. Radioactively labeled probes are the most sensitive but are less frequently used due to the cumbersome safety requirements. Biotin-labeled oligonucleotide primers are routinely available from commercial suppliers and when used for amplification, the DNA fragments generate biotinylated DNA probes. The probe chosen can comprise only the upstream regulatory sequences for a given gene/operon or extended intergenic regions (*see* **Note 11**). Although the probe can be extracted and purified from an agarose gel, for best results purification should be performed from a non-denaturing 10 % polyacrylamide gel using the following technique.

1. Mix the probe PCR with 6× loading dye and load into a 10 % polyacrylamide gene TBE gel.
2. Perform electrophoresis at 100 V for 65 min in 1× TBE buffer.
3. Following electrophoresis, remove the gel and stain with ethidium bromide.
4. Visualize the probe very briefly under UV light and extract from the gel using a scalpel (*see* **Note 12**).
5. Cut a small hole in the bottom of a 1.5 ml micro-centrifuge tube and place in a 2 ml microcentrifuge tube. Then place the acrylamide "slice" in the 1.5 ml tube, which sits into the 2 ml microcentrifuge tube.
6. Centrifuge the tubes for 1 min at 16,000 × *g*. This macerates the gel "slice" through the hole as it passes into the 2 ml microcentrifuge tube for collection.
7. Mix 200 µl of TE buffer with the macerated acrylamide and incubate at 37 °C overnight and then centrifuge at 16,000 × *g* for 5 min.
8. Decant the supernatant into a clean micro-centrifuge tube.
9. Add another 200 µl of TE buffer to the acrylamide, vortex, centrifuge again at 16,000 × *g* for 2 min and collect the supernatant.

10. Pool both supernatant fractions and centrifuge at 16,000 × *g* for a further 2 min.
11. Harvest the supernatants, leaving the last 10 μl to avoid recovery of any residual acrylamide.
12. Precipitate the DNA with 1 ml 70 % EtOH containing 40 μl of 3 M sodium acetate and centrifuge at 16,000 × *g* for 10 min.
13. Discard the supernatant and wash the DNA pellet with 200 μl 70 % EtOH then centrifuge at 16,000 × *g* for 10 min.
14. Discard the supernatant again and allow the DNA pellet to air dry at room temperature for 10–15 min.
15. Resuspend the DNA pellet in 30 μl TE buffer and determine the DNA concentration using a spectrophotometer.

3.6 EMSA

To investigate if the recombinant protein binds to the DNA sequence of interest, a pilot reaction should be performed with the purified recombinant protein, purified biotinylated DNA probe, and binding buffer. If a shift is observed in this pilot experiment, the next step is to repeat the reaction with doubling dilutions of the purified protein in order to determine the minimum protein required to shift a known concentration of DNA (*see* **Note 13**).

The protocol for carrying out the EMSA is as follows:

1. Prepare a 20 μl binding reaction containing a minimum of 10 μl binding buffer, 2–10 ng biotinylated probe and the purified protein.
2. Incubate the reaction at room temperature for 20 min before loading directly onto a 6 % DNA Retardation Gel and electrophoresing at 100 V for 65 min. These gels contain 0.5× TBE which provides an environment of low-ionic strength to promote protein–DNA interaction (*see* **Notes 14** and **15**).
3. Transfer the DNA or DNA–protein complex onto a Biodyne-B nylon membrane (Pall Corporation, AGB) at 4 °C in prechilled 0.5 % TBE at 80 V for 60 min.
4. Cross-link the membrane under UV light for 10 min (*see* **Note 16**).
5. Detection of the bands representing labeled probes or interaction complexes can be performed using various commercial chemiluminescent imaging kits (such as the LightShift chemiluminescent electrophoretic mobility shift assay kit) and visualized using a chemiluminescent imager (*see* **Notes 17** and **18**).

3.7 Determination of Protein–DNA Interaction Specificity Using Competitor DNA

To determine if the observed protein–DNA interaction is specific, competitor DNA is added to the reaction. Generally competitor DNA is unlabeled DNA that is either specific (i.e. identical to the labeled probe) or nonspecific (a DNA fragment that is not predicted to interact with the protein). Competitor DNA is added to

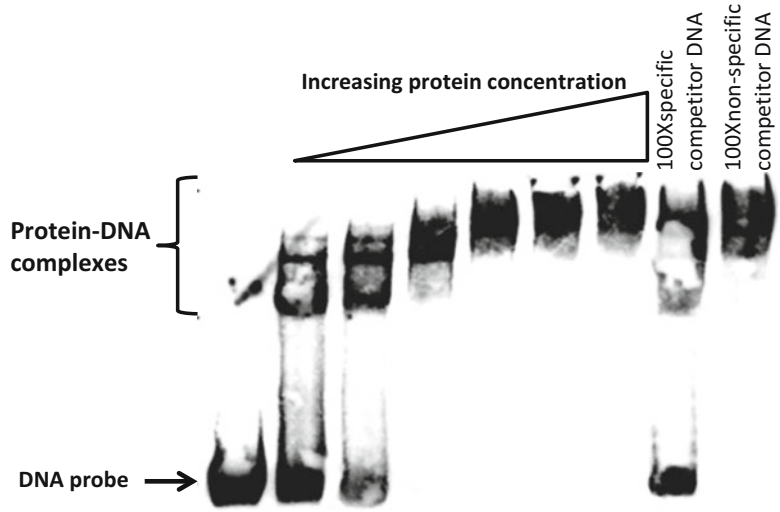

Fig. 3 A standard amount of biotinylated DNA probe is added to each well. *Lane 1* contains probe and no protein. *Lanes 2–7* include increasing amounts of recombinant protein. *Lane 8* and *9* also contain 100× specific and nonspecific competitor DNA respectively

the binding reaction at a final concentration of 1×, 10×, and 100× the concentration of the labeled probe. An excess of specific competitor DNA but not nonspecific competitor DNA should disrupt the interaction of a protein bound to its target probe (Fig. 3) [4].

4 Notes

1. N- or C-terminal tags can interfere with protein function. Therefore, it is recommended that both recombinant proteins (an N- terminal and a C-terminal version) are tested in EMSAs.
2. A minimum of five histidine residues are required for purification of tagged proteins, although this can be increased to eight residues to facilitate an improved Western blot signal using anti-polyhistidine antibodies.
3. This strain contains several mutations to aid the cloning procedure: *recA1* increases insert stability, *endA1* increases plasmid yield, *lacZΔM15* allows blue/white screening. Blue/white screening is a useful tool to identify candidate recombinant clones. β-galactosidase is an enzyme encoded by the *lacZ* gene. When X-gal, an analog of lactose, is added to the growth media, β-galactosidase breaks it down producing a blue 5-bromo-4-chloro-indoxyl pigment which is visible in the *E. coli* colony. However, strains carrying the *lacZΔM15* mutation are deficient in many N-terminal residues of *lacZ* gene [5]. Strains

carrying this mutation are unable to produce the blue break down product when X-gal is added to the media unless transformed with a plasmid expressing a complementing fragment of *lacZ* in a process called α-complementation [6]. A multiple cloning site within the *lacZ* fragment of vectors pUC19 or pBluescript facilitates blue/white screening [7]. During cloning, the *lacZ* fragment will be disrupted and will no longer complement the chromosomally encoded *lacZ* gene causing a white colony to form. Therefore, blue colonies express intact LacZα which breaks down X-gal and do not contain an insert. White colonies however, have an interrupted LacZα due to the presence of an insert. White colonies can be streaked on media containing selective antibiotic for further testing.

4. This strain has mutations in the *lon* and *ompT* genes which encode proteases that can affect protein yield [8]. DE3 indicates that the host strain is a lysogen of λDE3 phage and encodes a chromosomal copy of T7 RNA polymerase. This polymerase gene is under control of the *lacUV5* promoter, facilitating IPTG-induced expression of recombinant genes cloned in pET vectors [9]. There are many commercial strains of *E. coli* harboring various genetic mutations and/or plasmids which can be tested for optimal expression of various recombinant proteins. For example, expression of recombinant proteins that contain rare codons not commonly expressed in *E. coli*, such as proline, can be achieved using strain Rosetta(DE3) pLysS (MerckMillipore). This is a BL21 derivative harboring a plasmid which supplies the tRNAs for rare *E. coli* codons AGG, AGA, AUA, CUA, CCC, and GGA. In addition, there are several *E. coli* strains specifically engineered for expressing toxic proteins [10].
5. Induction of the protein of interest, for example from an IPTG-inducible system generally necessitates optimization. The recombinant *E. coli* strain can be grown in standard media such as Luria-Bertani and inducer added as the cells enter exponential phase. However, this process can require significant optimization to determine the optimal time of induction, length of induction, and concentration of IPTG. It is best to avoid high concentrations of IPTG as this can be associated with production of recombinant protein mainly in the insoluble fraction. As an alternative to IPTG, a mixture of glucose and lactose can be added to buffered media and expression of the recombinant protein allowed to proceed overnight without the need to optimize induction times. In such cultures *E. coli* first utilizes the glucose which helps to repress the expression of recombinant protein. As the glucose is exhausted, the *E. coli* will begin to simultaneously utilize the lactose and express the recombinant protein. Media of this type can be prepared in the laboratory or purchased commercially, for example overnight

expression (OnEx) autoinduction media from EMDmillipore can prove to be very helpful for protein expression using pET plasmids. Cultures grown in overnight expression media should be grown with good aeration to ensure maximal yield of recombinant protein.

6. The PVDF membrane required will differ depending on the size of the recombinant protein. For example, Immobilon P membrane^{SQ} is best suited for proteins less than 10 kDa while the Immobilon P membrane is more suited to higher molecular weight proteins. Six sponges are required for the western blot. One sponge is used to measure the size of filter paper required. Two pieces of filter paper are cut slightly smaller than the sponge, and the PVDF membrane cut slightly smaller than the filter paper. The top left corner of the membrane is cut to indicate "lane 1."
7. The membrane is very sensitive to handling especially if it will be subsequently exposed to X-ray film. To avoid observing black marks and creases prior to X-ray film exposure, always use a plastic forceps in the top left corner of the membrane (above lane 1).
8. Following submersion of the membrane in methanol, the membrane is transferred to deionized H_2O. The membrane will rise to the top of the water dish and is at risk of drying out. To avoid this, vigorously shake the water dish from side to side until the membrane sinks to the bottom. The dish can then be placed on a shaker table for all remaining wash steps.
9. Low concentrations of imidazole present in the lysis buffer should reduce nonspecific reaction of antibodies with histidine residues in native *E. coli* proteins.
10. Reducing the growth temperature from 37 to 30 °C or even room temperature can increase the solubility of the recombinant protein
11. It is also possible to incorporate base pair changes into the DNA probe to investigate if certain sequences are crucial for protein binding.
12. UV light introduces mutations into DNA. If used to visualize ethidium bromide stained DNA, exposure to UV light must be very brief. There are alternatives to ethidium bromide that do not require UV visualization if required [11, 12].
13. Many factors affect the specificity and strength of the protein–DNA interaction, including the ionic strength and pH of the binding buffer; the presence or absence of divalent cations such as Zn^{2+} and Mg^{2+}, nonionic detergents and carrier proteins such as bovine serum albumin. These factors need to be considered and can be determined empirically by manipulating

the binding buffer. There may also be information available in the literature on the DNA-binding requirements of specific proteins or families of proteins with shared structural characteristics.

14. Bromophenol blue can interfere with the reaction and it is recommended that loading dye be avoided. Glycerol is generally a constituent of the binding reaction and can be used to load the binding reaction into the wells of polyacrylamide gels.
15. EMSAs must be electrophoresed under nondenaturing conditions. SDS will disrupt protein–DNA interactions so it is important to use a protein electrophoresis tank that is never exposed to SDS-containing gels.
16. Following cross-linking, the membrane can be stored safely in a cool, dry place until detection is performed.
17. For increased sensitivity, the membrane can be exposed to X-Ray film and developed in a dark room.
18. If a recombinant protein does not bind to a labeled DNA fragment under the above conditions, it does not necessarily mean that the protein does not interact with the DNA sequence. The temperature and time of the binding reaction can be optimized or it is also possible that the protein requires modification in vivo before it binds a segment of DNA. For example, EMSAs revealed that the phosphorylated MgrA transcriptional regulator from *S. aureus* binds to the *norB* promoter but not the *norA* promoter, whereas dephosphorylated MgrA bound to the *norA* promoter and not *norB* [13].

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Rapid Amplification of cDNA Ends for RNA Transcript Sequencing in Staphylococcus

Eric Miller

Abstract

Rapid amplification of cDNA ends (RACE) is a technique that was developed to swiftly and efficiently amplify full-length RNA molecules in which the terminal ends have not been characterized. Current usage of this procedure has been more focused on sequencing and characterizing RNA 5′ and 3′ untranslated regions. Herein is described an adapted RACE protocol to amplify bacterial RNA transcripts.

Keywords: Amplification of cDNA ends, mRNA sequencing, Transcriptional start site, cDNA amplification, RNA mapping

1 Introduction

The *r*apid *a*mplification of *c*DNA *e*nds (RACE) is a procedure used to replicate and amplify full-length RNA transcripts that are minimally expressed [1]. More recently, this procedure has been utilized for identifying and characterizing the 5′ and 3′ untranslated regions of RNA transcripts to determine secondary structures, signal sequences, or cleavage sites [2–7]. With the recent surge of investigations into small regulatory RNA molecules, the RACE protocol has been instrumental in defining and sequencing the transcriptional units that are expressed. In addition, RACE and primer extension are two protocols used to identify transcriptional start sites. While both procedures utilize an internal primer for cDNA synthesis, RACE does have some distinct advantages. First, RACE exponentially amplifies the cDNA products, while primer extension amplifies cDNA linearly [1, 8–10]. This means transcripts that are minimally expressed will be more readily detected using RACE over primer extension. Second, RACE can be used to amplify the 3′ end of RNA as well [6] allowing for the entire RNA molecule to be reconstructed and cloned from the 5′ to the 3′ end. Mapping 3′ ends is not possible with primer extension [1, 8–10] since reverse transcriptase can only synthesize cDNA in 3′–5′ direction of an RNA molecule. Therefore, the 3′ end must already be known for primer extension to amplify cDNA. Finally, while not pertinent to

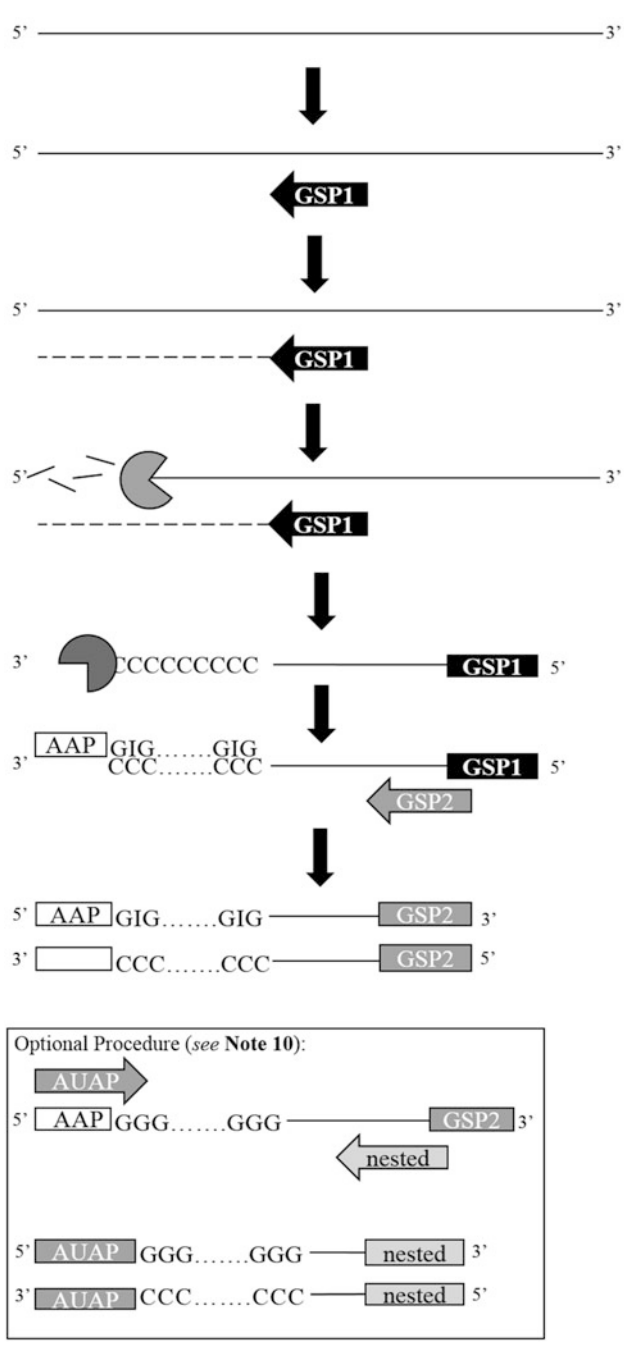
Table 1
Primer sequences for 5′ and 3′ RACE amplification of cDNA

Primer name	Sequence	Application
Abridged anchor primer	5′-GGCCACGCGTCGACTAGTAC GGGIIGGGIIGGGIIG-3′[a]	5′ RACE
Abridged universal amplification primer	5′GGCCACGCGTCGACTAGTAC-3′	5/3′ RACE
Universal amplification primer	5′-CUACUACUACUAGGCCACGC GTCGACTAGTAC-3′	5′/3′ RACE
Anchor primer	5′-CUACUACUACUAGGCCACGC GTCGACTAGTACGGGIIGG GIIGGGIIG-3′[a]	5′ RACE
Adapter primer	5′-GGCCACGCGTCGACTAGTAC TTTTTTTTTTTTTTTT-3′	3′ RACE

The GI regions of the Abridged Anchor and Anchor primers use deoxyinosine as a base. This provides high efficiency and specificity of binding for initial PCR cycles. A guanosine base can be substituted if needed

prokaryotes, primer extension is unable to detect splicing events within transcripts [11]. Due to the sequencing requirement of RACE, these regions can be easily identified. Thus, while primer extension and RACE are both viable options for multiple applications, RACE does provide several key advantages depending on the intended result.

The RACE protocol was first developed by Martin and colleagues in 1988 [1]. Since this publication, several modifications to the protocol have been documented [4, 12–17]. Regardless, the basic protocols have remained the same. For 5′ RACE, a gene-specific internal primer is used to amplify the first strand of cDNA and ligation of either homopolymeric tail or known primer to the 3′ end of the cDNA [5, 7, 16, 18]. Then, utilizing the known 3′ end sequence (homopolymeric tail or primer sequence (*see* Table 1)), the cDNA strand is amplified using PCR to make a double stranded DNA molecule, which can be cloned into a plasmid and sequenced. For 3′ RACE, eukaryotic mRNA contains a homopolymeric adenosine (poly-A) tail. However, bacterial mRNA does not contain a 3′ poly-A tail and requires additional steps to incorporate a string of adenosine nucleotides to the RNA molecules prior to synthesizing the first strand of cDNA [6, 16, 18]. By utilizing a poly-T primer to anneal to the synthetic poly-A tail, first strand cDNA synthesis can be accomplished. Finally, with the addition of a gene specific internal primer, the cDNA product is replicated with PCR and sequenced. This chapter contains workflows for both 5′ and 3′ RACE, each of which are summarized in Figs. 1 and 2, respectively.



Isolate RNA from bacteria under appropriate growth conditions

Incubate GSP1 primer with RNA sample to anneal

Synthesize first strand of cDNA using SuperScript™II Reverse transcriptase

Degrade RNA transcripts with addition of RNase Mix and purify cDNA using S.N.A.P columns

Incorporate poly-C tail on 3' end of purified cDNA TdT enzyme

PCR amplify first strand cDNA using Abridged Anchor Primer and GSP2 primer

Purify PCR product by agarose gel electrophoresis and prepare sample for plasmid ligation or continue to Optional Procedure

Utilizing Abridged Universal Amplifying Primer and 2nd nested primer PCR amplify purified cDNA sample

Purify PCR product by agarose gel electrophoresis and prepare sample for plasmid ligation

Fig. 1 An illustrated summary of 5′ RACE protocol

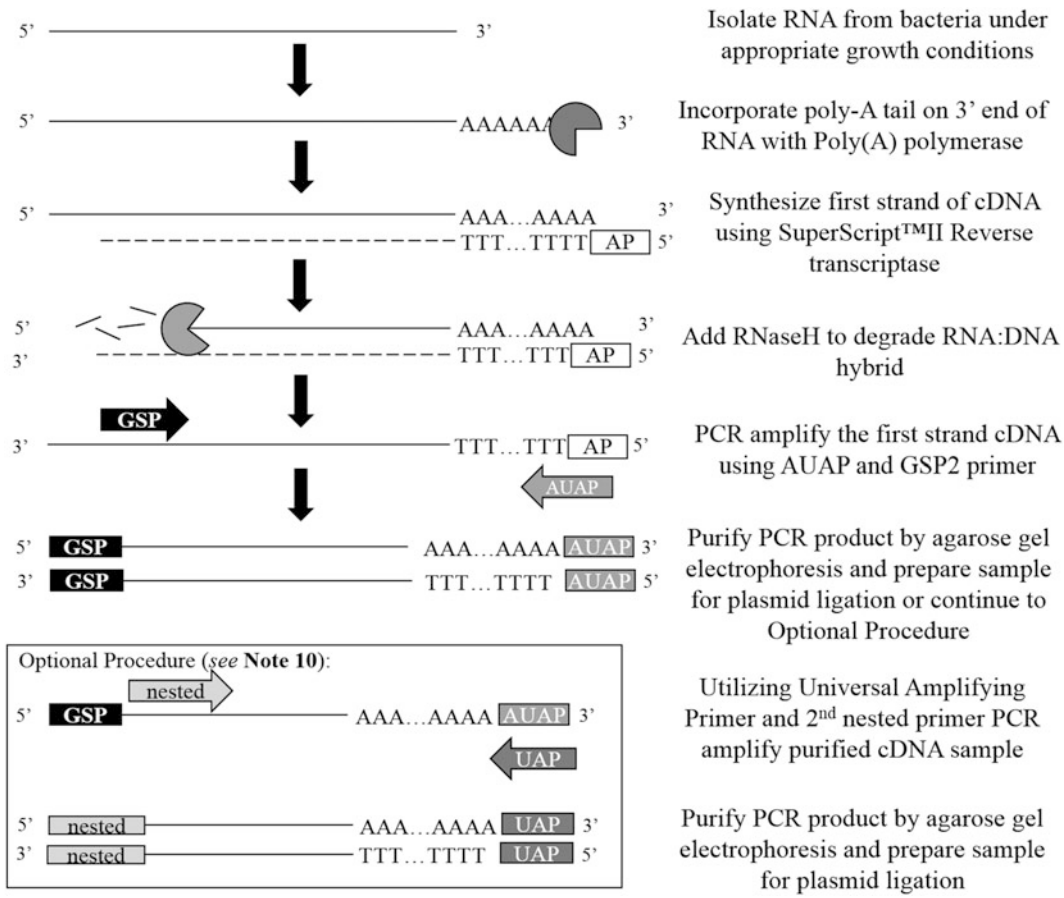


Fig. 2 An illustrated summary of 3' RACE protocol

2 Materials

If any solutions are made, use autoclaved diethylpyrocarbonate (DEPC) treated (*see* **Note 1**) or RNase-free water to ensure that RNA will not be degraded by contaminating RNases.

2.1 5′ RACE

While many kits are available, this protocol is written using the 5′ RACE System for Rapid Amplification of cDNA Ends v2.0 (Invitrogen/Life Technologies, Grand Island, NY, USA). All reagents should be stored at −20 °C, unless otherwise indicated. Contents include:

1. 10× PCR buffer: 200 mM Tris–HCl (pH 8.4), 500 mM KCl.
2. 25 mM $MgCl_2$.
3. 10 mM dNTP mix: 10 mM each nucleotide base (dATP, dCTP, dGTP, dTTP).
4. 1 M Dithiothreitol (DTT).

5. SuperScript™ II Reverse Transcriptase (200 units/μL).
6. RNase mix.
7. 5× tailing buffer: 50 mM Tris–HCl (pH 8.4), 125 mM KCl, 7.5 mM $MgCl_2$.
8. 2 mM dCTP.
9. Terminal deoxynucleotidyl transferase (TdT).
10. 5′ RACE abridged anchor primer (10 μM; AAP).
11. Universal amplification primer (10 μM; UAP).
12. DEPC-treated water.
13. S.N.A.P. columns; stored at room temperature.
14. Collection tubes; stored at room temperature.
15. DNA binding solution: 6 M sodium iodide; stored at 4 °C.
16. Wash buffer concentrate; stored at 4 °C.

2.2 3′ RACE

While many kits are available, this protocol is written using the 3′ RACE System for Rapid Amplification of cDNA Ends v2.0 (Invitrogen/Life Technologies, Grand Island, NY, USA). All reagents should be stored at −20 °C, unless otherwise indicated. Contents include:

1. 10× PCR buffer: 200 mM Tris–HCl (pH 8.4), 500 mM KCl.
2. 25 mM $MgCl_2$.
3. 10 mM dNTP mix: 10 mM of each nucleotide base (dATP, dCTP, dGTP, dTTP).
4. 0.1 M DTT.
5. SuperScript™ II Reverse Transcriptase (200 units/μL).
6. Adapter Primer (10 μM; AP).
7. Universal amplification prime.
8. Abridged universal amplification primer (10 μM).
9. DEPC-treated water.
10. *E. coli* RNase H (2 units/μL).

2.3 mRNA Poly(A) Tailing

Poly(A) Polymerase Tailing Kit.

1. Poly(A) Polymerase (4 units/μL).
2. Poly(A) Polymerase 10× reaction buffer.
3. 10 mM ATP.
4. RNase-free water.

1. Sterilized RNase-free 0.5 mL PCR tubes.
2. Sterilized RNase-free 1.5 mL tubes.
3. Sterilized RNase-free tips for pipettes.

2.4 Additional Reagents (Not Supplied with the Kits) and Equipment

4. Nuclease-free water.
5. Absolute 100 % ethanol.
6. 70 % ethanol stored at 4 °C.
7. User-designed gene-specific primers (GSP) (10 μM stock) (*see*-**Note 2**).
8. Microcentrifuge.
9. Mini centrifuge.
10. 37 and 42 °C water baths or heat block.
11. Thermal cycler.
12. Taq DNA polymerase.
13. RNase inhibitor.

2.5 Agarose Gels

1. Agarose.
2. 1× TBE running buffer: 0.1 M Tris base, 0.09 M boric acid, 0.001 M ethylenediaminetetraacetic acid (EDTA).
3. 10× DNA loading buffer: 5 mL water, 5 mL glycerol, and 25 mg bromophenol blue.

2.6 Sequencing

1. Agarose gel purification kit.
2. Cloning vector system.
3. *E. coli* DH5 alpha competent cells.

3 Methods

3.1 5′ RACE: GSP Design

1. GSP1: When designing the GSP1 primer, it should anneal approximately 300 bp into the coding region, to increase the yield of cDNA purified by the S.N.A.P. columns. Furthermore, the melting temperatures should be approximately to 42 °C (*see* **Note 3**).
2. GSP2: The nested GSP2 primer should anneal between the GSP1 and the 5′ end of the mRNA sequence (*see* Fig. 2). The melting temperature should range between 60 and 75 °C (*see* **Note 4**).

3.2 5′ RACE: First-Strand cDNA Synthesis

1. Remove required reagents and thaw on ice.
2. In a thin-walled 0.5 mL PCR tube, add:

   2 μL of 15 μg/μL primer GSP1 solution.

   1–5 μg RNA sample (*see* **Note 5**).

   DPEC-treated water to final volume of 15.5 μL.
3. Mix gently and place on ice.

4. Incubate for 10 min at 70 °C and then chill for 1 min on ice (*see* **Note 6**).
5. Collect solution at the bottom of the tube by brief centrifugation and add (in order):

   2.5 μL 10× PCR buffer.

   2.5 μL of 25 mM $MgCl_2$.

   1.0 μL of 10 mM dNTP mix.

   2.5 μL of 0.2 M DTT.

   Even with multiple samples, add reagent individually to each tube.
6. Gently mix the solution and collect at bottom by brief centrifugation.
7. Place tube in 42 °C water bath (or heat block/thermal cycler) and incubate for 1 min.
8. Add 1 μL SuperScript™ II RT and then mix gently yet thoroughly.
9. Place reaction in 42 °C incubator or water bath and incubate for 30–50 min (*see* **Note 7**).
10. Remove sample from heat source and incubate at 70 °C in thermal cycler for 15 min to terminate the reaction.
11. After a brief centrifugation, add 1 μL RNase mix and incubate for 30 min in a 37 °C water bath.
12. Collect reaction by brief centrifugation and place sample on ice before proceeding to next step.

3.3 5′ RACE: S.N.A.P. Column Purification of cDNA (see Note 8)

1. Remove binding solution from 4 °C to equilibrate solution to room temperature.
2. Equilibrate 100 μL water at 65 °C for each sample. This will be used in **step 12**.
3. Add 120 μL binding solution to each reaction and mix by pipetting.
4. Transfer the cDNA solution to S.N.A.P. column. Centrifuge at 13,000 × *g* for 20 s to bind cDNA to the columns.
5. Transfer flow-through to a clean 1.5 mL tube and store on ice until the final elution is collected and run on a gel. This is to confirm the cDNA has been isolated and is not still in the flow-through solution.
6. Remove wash buffer from 4 °C and immediately add 400 μL 1× wash buffer to spin cartridge. Centrifuge at 13,000 × *g* for 20 s.
7. Repeat this wash step three more times, for a total of four washes.

8. Add 400 µL cold 70 % ethanol to the column. Centrifuge at 13,000 × *g* for 20 s.
9. Repeat wash **step 7**.
10. After removing the final 70 % wash from the collection tube, centrifuge for 1 min to collect the residual ethanol from the column.
11. Transfer the column to a new 1.5 mL tube.
12. Add 50 µL of pre-heated (65 °C) water to the center of the column.
13. Let column stand for 1 min and then centrifuge at 13,000 × *g* for 20 s to elute cDNA sample.

3.4 5′ RACE: Terminal Deoxynucleotidyl Transferase Tailing of cDNA

1. Following the elution of cDNA (last step of Subheading 3.3), mix the following components together:

   6.5 µL of DEPC-treated water.

   5.0 µL of 5× tailing buffer.

   2.5 µL of 2 mM dCTP.

   10.0 µL SNAP-purified sample.
2. Incubate the reaction for 2–3 min at 94 °C.
3. Chill for 1 min by placing sample directly on ice.
4. Collect contents at the bottom of the tube by brief centrifugation and then place the sample back on ice.
5. Add 1 µL TdT to the solution and gently mix by pipetting.
6. Incubate sample for 10 min at 37 °C.
7. Incubate sample for 10 min at 65 °C to heat inactivate the TdT enzyme.
8. Collect sample at bottom of the tube by brief centrifugation and place on ice.

3.5 5′ RACE: PCR of dC-Tailed cDNA

1. Be sure to equilibrate the thermal cycler to 94 °C before incubating the reaction.
2. On ice, mix the following reagents:

   31.5 µL of water.

   5.0 µL of 10× PCR buffer.

   3.0 µL of 25 mM $MgCl_2$.

   1.0 µL of 10 mM dNTP mix.

   2.0 µL of 10 µM nested GSP2 stock.

   2.0 µL of 10 µM Abridged Anchor Primer.

   5.0 µL of dC-tailed cDNA.
3. After all other reagents have been added, add 0.5 µL Taq DNA polymerase and mix immediately.

4. Transfer tubes from ice to thermal cycler that was pre-equilibrated to the initial denaturation temperature of 94 °C.
5. The typical cycling protocol for a sample that is >1 kb is 94 °C for 2 min, repeat cycles 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and final extension at 72 °C for 7 min (*see* **Note 9**). Repeat the cycle for 30–35 rounds of PCR.
6. Hold reaction at 4 °C until able to proceed with agarose gel analysis and purification protocol (*see* **Note 10**) and continue with Subheading 3.10—RACE Sequence Analysis.

3.6 3′ RACE: GSP Primer Design

1. GSP: Similar to the 5′ RACE GSP1, the 3′ RACE GSP should anneal approximately 300 bp upstream of the 3′ end of the coding sequence to ensure appropriate size of amplification. The melting temperature of the GSP should range between 60 and 75 °C as it is utilized during PCR amplification with Adapter Primer (AP).

3.7 3′ RACE: Poly-A Tailing of RNA (see Note 11)

1. Determine the concentration of RNA by using a spectrophotometer (e.g., Nanodrop) or fluorometer (e.g., Qubit) (*see* **Note 12**).
2. Heat the mRNA at 65 °C for 5 min, and then immediately transfer to ice for 5 min (*see* **Note 13**).
3. In a 0.5 mL PCR tube, combine the following reaction components on ice:

   2.0 μL of Poly(A) 10× reaction buffer.

   2.0 μL of 10 mM ATP.

   0.5 μL RiboGuard RNase inhibitor.

   1–10 μg of total RNA (*see* **Note 5**).

   1.0 μL of Poly(A) Polymerase (4 Units).

   RNase-free water to bring up volume to 20.0 μL.
4. Incubate the reaction at 37 °C for 15–20 min.
5. Stop the reaction by either immediately storing solution at −20 °C for >1 h or by phenol/chloroform and salt-ethanol precipitation (*see* **Note 14**). Before proceeding to first-strand synthesis, determine the concentration of RNA by using a spectrophotometer or fluorometer.

3.8 3′ RACE: First-Strand cDNA Synthesis

1. In a 0.5 mL PCR tube, combine 50 ng of poly-A tailed RNA and DEPC-treated water to a final volume of 11 μL.
2. To the diluted RNA, add 1 μL of the 10 μM AP solution and mix gently by pipetting.
3. Collect the reaction in the bottom of the tube by brief centrifugation.

4. Heat mixture to 70 °C in the heat block for 10 min. Then, immediately chill on ice for at least 1 min.
5. Collect the contents in the tube by brief centrifugation.
6. To the RNA solution, add the following components (final total volume will be 18 μL) in order while on ice:

   2 μL of 10× PCR buffer.

   2 μL of 25 mM $MgCl_2$.

   1 μL of 10 mM dNTP mix.

   2 μL of 0.1 M DTT.
7. Mix gently with pipette tip and centrifuge briefly to collect the solution in the bottom of the tube.
8. Incubate the reaction for 2–5 min in a 42 °C water bath.
9. After the brief incubation, add 1 μL of Superscript II RT.
10. Mix gently by pipetting and incubate for 30–50 min in a 42 °C water bath or thermal cycler (*see* **Note 7**).
11. Once complete, terminate the reaction by incubating the solution for 15 min at 70 °C in the heat block or thermal cycler.
12. Immediately chill on ice for at least 1 min and collect the reaction by brief centrifugation.
13. Add 1 μL of RNase H to the tube and mix gently but thoroughly by pipetting up and down.
14. Incubate the mixture for 20 min in a 37 °C water bath.
15. Collect the reaction by brief centrifugation and place on ice.
16. If needed, the sample can safely be stored at −20 °C overnight; otherwise continue to PCR step.

3.9 3′ RACE: PCR of dT-Tailed cDNA

1. Add the following components to a 0.5 mL PCR tube sitting on ice:

   31.5 μL of nuclease-free water.

   5.0 μL of 10 PCR buffer.

   3.0 μL of 25 mM $MgCl_2$.

   1.0 μL of 10 mM dNTP mix.

   2.0 μL of 10 μM GSP.

   2.0 μL of 10 μM AUAP or UAP (*see* **Note 15**).

   5.0 μL of dT-tailed cDNA.
2. Add 0.5 μL of Taq DNA polymerase and mix gently with pipette.
3. Collect the reaction by brief centrifugation.
4. Transfer tubes directly from ice to the thermal cycler.

5. The typical cycling protocol for a sample that is >1 kb is 94 °C for 2 min, repeat cycles 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and final extension at 72 °C for 7 min (*see* **Note 10**). Repeat the cycle for 30–35 rounds of PCR.
6. Hold reaction at 4 °C until able to proceed with agarose gel analysis and purification protocol.

3.10 RACE Sequence Analysis

This section encompasses the protocol to analyze and sequence either 5′ or 3′ RACE products.

1. Directly to the PCR reaction, add 5–6 μL of 10× DNA loading dye and mix thoroughly.
2. Make a 1 % agarose gel by adding 1 g of agarose per 100 mL of TBE. Bring solution to a rapid boil on hot plate or in a microwave, until all agarose has completely melted. Allow agarose to cool, then add 1 μL of ethidium bromide or SYBR Gold per 50 mL of agarose gel, and mix. Pour gel solution into a gel caster.
3. Load the entire PCR reaction into one or two wells (*see* **Note 16**) and run gel at 5 V per cm^2 of gel for 30–50 min.
4. Remove gel from electrophoresis tank, capture an image in gel dock, and then excise the potential band of interest.
5. Use a gel extraction kit to purify the DNA.
6. It is then recommended to ligate the PCR product into cloning vector (*see* **Note 17**) and transform into an *E. coli* propagation strain, such as DH5α cells, using the appropriate antibiotic for selection.
7. Isolate the plasmids through a miniprep protocol. Either use plasmids directly for sequencing or run a PCR reaction from the plasmid for sequencing (*see* **Note 18**).

4 Notes

1. To make DEPC-treated water, add 1 mL of DEPC per 1 L of water (0.1 %) and incubate overnight (>12 h) at 37 °C. Following incubation, autoclave the water for sterility and to degrade residual DEPC left in solution. DEPC is a hazardous chemical and needs to be handled with care. Therefore, purchasing certified RNase-free water that has not been DEPC-treated is a better alternative.
2. These primers need to be target-specific primers designed by the researcher and have a Tm of approximately 60 °C. For 5′ RACE, two primers are required for efficient amplification of

the cDNA product, with the second primer internal to the first (*see* Fig. 1). For 3′ RACE, only one primer is required for cDNA synthesis. Details on how to make the primers can be found in **steps 1** and **2** of Subheading 3.1, and **step 1** of Subheading 3.6.

3. The melting temperature is the point at which 50 % of the primer has annealed to the target DNA sequence and therefore should be close to 42 °C for proper association to RNA during the reverse transcription reaction. The low melting temperature can be achieved by creating a small primer, 16–20 bp in length, or having a very low GC content.
4. When determining the location of GSP2, there can be overlapping sequence with GSP1 primer; however, this may reduce efficiency of amplification. The melting temperature of this primer is much higher than GSP1 due to being paired with the abridged anchor primer, which has a 68 °C melting temperature. The closer the melting temperatures of the primers, the more efficient the PCR reaction. These temperatures also need to be considered when setting up the temperatures for the PCR cycles.
5. The volume of the reaction is critical and cannot be overloaded. Therefore, the maximum volume of RNA is equivalent to the maximum volume of water that can be added. If the initial concentration of RNA is such that the minimum amount of RNA cannot be ascertained with the limited sample volume, the RNA sample should be concentrated using an RNA concentrating kit or the RNA will need to be re-isolated. Do not attempt to use an RNA sample below the suggested minimum level as results may be hampered.
6. It is important to place the sample directly on ice immediately following the incubation, so the RNA can remain linearized and will not reform secondary structures.
7. For most samples that are <1 kb, the 30 min incubation time is sufficient for first strand synthesis. However, if the product is >1 kb, the 50 min incubation time is required for full-length amplification. If you are unsure what size will result, use the longer incubation period.
8. If you do not purchase the RACE kit, the first strand of cDNA can be purified by lithium chloride and ethanol precipitation or utilizing another column based purification system, e.g., Qiagen PCR Purification Kit or Zymo Research DNA Clean and Concentrator kit.
9. For the PCR reaction, the annealing temperature may need to be increased to 60 or 65 °C depending on the melting temperature of GSP2, as previously discussed in **Note 4**. Furthermore, the final extension period needs to be ≥7 min if the product is

going to be ligated into a vector with T overhangs. This is due to Taq polymerase adding on A overhangs to the PCR product. If using a blunt-end cloning system or performing the optional nested primer (*see* **Note 10**), the final extension time should be reduced to <5 min.

10. There is an optional procedure to increase the yield of PCR product by performing a second round of PCR using a nested primer (*see* Figs. 1 and 2, Optional Procedure). The nested primer should anneal further upstream of the GSP2 primer. Furthermore, the Universal Amplification Primer (UAP) or Abridged Universal Amplification Primer (AUAP) should be used to complete the primer set.
11. Prokaryotic RNA does not have natural homopolymeric tail, and therefore requires an additional procedure to incorporate 3′ poly-A tail. If the target RNA is extremely rare, use mRNA-ONLY™ Prokaryotic mRNA isolation kit (Epicentre) to concentrate the mRNA. This protocol works to degrade 16S and 23S rRNA because rRNA molecules are processed, exposing 5′ monophosphates and allowing for targeted degradation. By eliminating rRNA from the sample, the target RNA concentration is enhanced and should be more readily amplified. However, if the target RNA is also posttranscriptionally processed, this kit may degrade the transcript. Also, due to eliminating rRNA from the sample, 50–200 ng of mRNA may be used for the reaction.
12. As RNA samples are precious, using systems such as Nanodrop or Qubit, which only require 1 μL for accurate readings, is highly valued. Furthermore, we recommend using Qubit if possible, as it can distinguish between free nucleotides and RNA transcripts, whereas Nanodrop cannot.
13. This step is optional. Heat denaturing the secondary RNA structures will allow for rapid access to the 3′ end for efficient poly-A tailing.
14. If starting with minimal (<2 μg) of RNA, do not attempt ethanol precipitation as it is possible to lose sample. Perform phenol extraction and ethanol precipitation as follows. Add 180 μL RNase-free water to give a total volume of 200 μL. Add 200 μL of phenol/chloroform. Mix well with gentle vortexing and centrifuge at 13,000 × *g* for 5 min. Transfer aqueous phase to a new RNase-free tube. Add 0.1 volume of 3 M sodium acetate, mix well by pipetting and let stand >30 s. Then, add 2.5 volumes of ethanol to the reaction and mix thoroughly. Incubate on ice or at −20 °C for 30 min. Pellet the RNA by centrifugation in a cooled microcentrifuge for 30 min at full speed at 4 °C. Carefully remove and discard the supernatant without disturbing the pellet. Wash the RNA pellet

with 70 % ethanol to remove residual salt. Use a short spin to collect residual ethanol at the bottom of the tube. Then pipette off ethanol and leave tube open upside-down for a not more than 3 min to air-dry the pellet. Resuspend the RNA pellet in 11 μL of RNase-free water.

15. The UAP should only be utilized when cloning via uracil DNA glycosylase (UDG). If T4 ligase cloning is used, then AUAP is recommended.
16. To obtain adequate quantities of cDNA product, the entire PCR reaction is required.
17. It is recommended to clone the sample into a plasmid for sequencing and stability of long-term storage purposes. For use with A overhanging products, the pCR4™ TOPO® vector (Invitrogen) is highly recommended for quick and efficient cloning. For blunt-end products, pCR™-Blunt II TOPO (Invitrogen) is recommended for quick and efficient cloning.
18. For sequencing reactions, either the plasmid or PCR product from the plasmid can be used as template for sequencing. It is important for 5′ RACE sequencing, to discuss using high GC content protocol with the sequencing facility. The 5′ RACE product contains a poly-C tail, which could create problems during sequencing as it is difficult for the enzymes to effectively amplify this region.

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