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An *rpsL*-based allelic exchange vector for *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus is one of the most successful bacterial pathogens, harboring a vast repertoire of virulence factors in its arsenal. As such, the genetic manipulation of *S. aureus* chromosomal DNA is an important tool for the study of genes involved in virulence and survival in the host. Previously reported allelic exchange vectors for *S. aureus* are shuttle vectors that can be propagated in *Escherichia coli*, so that standard genetic manipulations can be carried out. Most of the vectors currently in use carry the temperature-sensitive replicon (pE194ts) that was originally developed for use in *Bacillus subtilis*. Here we show that in *S. aureus*, the thermosensitivity of a pE194ts vector is incomplete at standard non-permissive temperatures (42 °C), and replication of the plasmid is impaired but not abolished. We report *rpsL*-based counterselection vectors, with an improved temperature-sensitive replicon (pT181 *repC3*) that is completely blocked for replication in *S. aureus* at non-permissive and standard growth temperature (37 °C). We also describe a set of temperature-sensitive vectors that can be cured at standard growth temperature. These vectors provide highly effective tools for rapidly generating allelic replacement mutations and curing expression plasmids, and expand the genetic tool set available for the study of *S. aureus*.

1. Introduction

Staphylococcus aureus is a major human pathogen that carries a plethora of virulence factors, including toxins, immunomodulatory factors, and coenzymes that allow it to survive in adverse conditions within a human host. *S. aureus* pathogenesis is further compounded by the emergence of methicillin-resistant *S. aureus* (MRSA) strains. Therefore, knowledge of staphylococcal factors involved in infection, growth, pathology and transmission is important for the understanding of staphylococcal pathobiology.

In recent years, *S. aureus* genome sequencing projects have led to the generation of a large amount of sequence information. High-throughput methods to identify open reading frames (ORFs) important for *S. aureus* virulence and survival have implicated a large number of ORFs of unknown function that are of particular interest. The development of complementation vectors (Charpentier et al., 2004) and allelic exchange systems (Arnaud et al., 2004; Bae and Schneewind, 2006) have made *S. aureus* more amenable to molecular genetic analysis, and this has greatly facilitated studies of its biochemistry, physiology and pathogenicity (Chatterjee et al., 2013; Geiger et al., 2012; Pereira et al.,

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2013; Price-Whelan et al., 2013; Tormo-Mas et al., 2010; Valle et al., 2012). To date, most of the temperature-sensitive allelic exchange vectors developed for *S. aureus* rely on the pE194ts replicon that was originally developed for use in *Bacillus subtilis* (Villafane et al., 1987). In *S. aureus*, the replication of a vector with pE194ts is not blocked, but only severely impaired at 42 °C (see Results), and higher incubation temperature (43 °C) or liquid passaging is often required to reliably lose the vector (Bae and Schneewind, 2006). High incubation temperature can be problematic in *S. aureus*, as it is one of the primary environmental stresses that have been linked to spontaneous secondary mutations, such as those reported in the *sae* locus (Sun et al., 2010).

Here, we describe an additional system for allelic replacement in *S. aureus*, adapted from an *rpsL*-based counterselection strategy (Ortiz-Martin et al., 2006; Russell and Dahlquist, 1989; Sander et al., 1995; Skorupski and Taylor, 1996). Streptomycin belongs to the aminoglycoside class of antibiotics that inhibit prokaryotic protein synthesis by binding to the small subunit of the ribosome. Bacteria have evolved several resistance mechanisms to streptomycin, and one of them involves common single-step mutations within the *rpsL* gene coding for ribosomal protein S12 (Funatsu and Wittmann, 1972). However, in merodiploid strains with both wild type and mutant alleles, streptomycin sensitivity is dominant over resistance (Lederberg, 1951). Vectors pJC1202, pJC1600, and pJC1619 carry a wild type copy of the *S. aureus rpsL* gene (*rpsL*$^+$), and can be used for counterselection in strains that are streptomycin resistant due to mutations in *rpsL* (*rpsL**). A key feature of these vectors is improved temperature-sensitivity, which is based on a pT181 *repC3* replicon that is not only completely blocked for replication at standard non-permissive temperature (42 °C) (Novick et al., 1982), but also at standard growth temperature (37 °C), and permits the entire allelic exchange to be carried out on a series of agar plates by streaking for single colonies at standard growth temperatures.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains (TOP 10 or XL1-Blue) were grown on LB agar and LB broth supplemented with ampicillin: 100 μg/ml as needed. *S. aureus* strains were usually grown on glycerol lactate (Novick, 1991) or tryptic soy (TS) broth or agar supplemented with antibiotics (cadmium chloride: 0.1 mM; chloramphenicol: 10 μg/ml; erythromycin: 5 μg/ml; streptomycin: 300 μg/ml; tetracycline: 5 μg/ml) as needed.

2.2. Plasmid construction

The pT181 *repC3* replicon was sequenced with primers JCO 830, JCO 831, JCO 832, and JCO 833. The plasmids (see Table 1) used in this study were constructed by cloning PCR products amplified with oligonucleotide primers purchased from Integrated DNA Technologies (Coralville, IA).

Table 1
Strains, plasmids, and primers used in this study.

S. aureus strains		
Strain	Genotype or description	Reference
RN4220	Restriction-defective derivative of RN450	(Kreiswirth et al., 1983)
JCSA17	RN4220 *rpsL**	(This work)
JCSA133	JCSA417 *Δagr*	(This work)
JCSA416	RN0001 *rpsL**	(This work)
JCSA417	RN6734 *rpsL**	(This work)
JCSA418	RN6390 *rpsL**	(This work)
JCSA434	MW2 *rpsL**	(This work)
JCSA436	USA300 LAC *rpsL**	(This work)
Plasmids		
pUC18	*E. coli* cloning vector	(Yanisch-Perron et al., 1985)
pCN33	*E. coli*/*S. aureus* shuttle vector	(Charpentier et al., 2004)
pCN47	*E. coli*/*S. aureus* shuttle vector	(Charpentier et al., 2004)
pCN49	*E. coli*/*S. aureus* shuttle vector	(Charpentier et al., 2004)
pRN7145	*E. coli*/*S. aureus* shuttle vector	(Charpentier et al., 2004)
pSA0321	*S. aureus* temperature sensitive plasmid	(Novick et al., 1982)
pI524	*S. aureus* natural plasmid	(Novick, 1963)
pJC1125	pJC1079 with pT181 *repC3*	(Chen and Novick, 2007)
pJC1200	pUC18 with *blaZ* promoter cloned	(Chen and Novick, 2007)
pJC1201	pUC18 with *rpsL* gene cloned	(Chen and Novick, 2007)
pJC1202	pJC1125 *cat194*, Pbla-*rpsL*$^+$	(Chen and Novick, 2007)
pJC1204	pJC1202 with *agr::ermC* knockout construct	(This work)
pJC1306	*E. coli*/*S. aureus* shuttle vector *tetM*	(Chen et al., 2014b)
pJC1600	pJC1202 *ermC*, Pbla-*rpsL*$^+$	(This work)
pJC1601	pJC1125 *ermC*	(This work)
pJC1619	pJC1202 *tetM*, Pbla-*rpsL*$^+$	(This work)
pJC1945	pJC1125 *tetM*	(This work)
pJC1983	pJC1600 with *agr::cadCA* knockout construct	(This work)
Primers	5′ to 3′	
JCO 134	CTCATCCCTTCTTCATTAC	(This work)
JCO 136	CCTAGGTGCTGTTCCACGTTTACCATCTAAC	(This work)
JCO 176	GGGCCCAGCTTACTATGCC	(This work)
JCO 177	CTCGAGAATAAACCCTCCG	(This work)
JCO 178	CTCGAGCGTCAATGCGACAATAGTAGCATTG	(This work)
JCO 337	GGTACCTGAAGCGGGCGAGCGAG	(This work)
JCO 830	CTCATATATCAAGCAAAGTGACAGGC	(This work)
JCO 831	GCACATACTGTGTGCATATCTGATC	(This work)
JCO 832	CCGCAGTGCGTTGGATAG	(This work)
JCO 833	GGAGGTGTAGCATGTCTCATTC	(This work)

Clones were sequenced by Macrogen (Rockville, MD). Blunt-ended PCR products were cloned into the *Hinc*II site of pUC18. The *Hpa*I fragment from pSA0321, containing the *repC3* temperature sensitive replicon, replaced the pT181 replicon in pJC1079 to generate pJC1125. The *blaZ* promoter was amplified from *S. aureus* plasmid, pI524, using primers JCO 176 and JCO 177. The wild type *rpsL* gene was amplified from strain RN0001 with primers JCO 178 and JCO 136. A 3-way ligation with the pJC1200 *Apa*I-*Xho*I fragment and pJC1201 *Xho*I-*Avr*II fragments were cloned into pJC1125 to generate pJC1202. The *ermC* from pCN33 was used to replace the *cat194* of pJC1202 and pJC1125 to generate pJC1600 and pJC1601, respectively. The *tetM* from pJC1306 was used to