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# Structure–function analysis of the SaPIbov1 replication origin in *Staphylococcus aureus*

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ABSTRACT

The SaPIs and their relatives are phage satellites and are unique among the known bacterial pathogenicity islands in their ability to replicate autonomously. They possess a phage-like replicon, which is organized as two sets of iterons arrayed symmetrically to flank an AT-rich region that is driven to melt by the binding of a SaPI-specific initiator (Rep) to the flanking iterons. Extensive deletion analysis has revealed that Rep can bind to a single iteron, generating a simple shift in a gel mobility assay; when bound on both sides, a second retarded band is seen, suggesting independent binding. Binding to both sites of the ori is necessary but not sufficient to melt the AT-rich region and initiate replication. For these processes, virtually the entire origin must be present. Since SaPI replication can be initiated on linear DNA, it is suggested that bilateral binding may be necessary to constrain the intervening DNA to enable Rep-driven melting.



## 1. Introduction

The staphylococcal pathogenicity islands are prototypes of a large family of phage-related chromosomal islands that are widely distributed among Gram-positive bacteria (Novick and Subedi, 2007). Following infection of their host organism by any of several helper phages, the SaPI genome excises, replicates autonomously, and is encapsidated in small infectious phage-like particles composed of phage virion proteins (Lindsay et al., 1998; Tormo-Más et al., 2010; Tormo et al., 2008; Ubeda et al., 2005, 2008). In previous studies, we have analyzed several components of the SaPI lifecycle, including genome organization, phage induction, excision and integration, and packaging (review: Novick et al., 2010). In this report, we describe the sequence requirements for the initiation of replication.

Classically, the initiation of replication in prokaryotes involves specific binding of a replication initiator protein to a unique replication origin followed by melting at an AT-rich region within or adjacent to the origin, which enables helicase-driven unwinding preparatory to the start of polymerization. This paradigm applies fully to the SaPIs, as the specific components of the SaPI replicon include a specific replication origin (*ori*) and an initiator protein (Rep) that recognizes and binds to it. All of the 16 known SaPI replication origins have a common, though rather unusual structure, consisting of two sets of short repeated sequences (iterons) flanking an AT-rich region of about 80 bp (Ubeda et al., 2007). The Rep protein, like analogous proteins of various phages and viruses (Briani et al., 2001), has helicase activity, which is required for initiation (Ubeda et al., 2007), and is predicted to be hexameric. It binds specifically to the isolated *ori* region, showing multiple bands in a gel mobility shift assay (Ubeda et al., 2007). The Rep–*ori* interaction is SaPI-specific and is determined by a matching interaction between the iterons and a specificity determinant in the C-terminal region of the Rep protein (Ubeda et al., 2007). Following initiation, replication is continued by host polymerization functions, probably aided by a SaPI-coded primase. The product of SaPI

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replication is a linear concatemer (Ubeda et al., 2007) which is packaged by the headful mechanism (Ruzin et al., 2001), initiated by a complex between the phage terminase large subunit and a SaPI-encoded version of the terminase small subunit.

In this study, we have sought to ascertain the roles of the several sequence elements in the unusual SaPI replication origin and to see how they interact with the Rep protein. We show that although Rep can bind to a single iteron segment, it can induce melting, which occurs within the AT-rich region as one might have expected, and can initiate replication only when essentially the entire *ori* is present.

## 2. Material and methods

### 2.1. Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table S1 (supplementary data). Bacteria were grown at 32 or 37 °C overnight on glycerol–lactate agar medium (Novick, 1991), supplemented with antibiotics as appropriate. Broth cultures were grown at 32 or 43 °C in casamino acids–yeast extract broth (Novick, 1991) or TSB with shaking (240 rpm). Procedures for transduction and transformation in *Staphylococcus aureus* were performed essentially as described (Novick, 1991).

### 2.2. DNA methods

General DNA manipulations were performed by standard procedures (Ausubel et al., 1987; Sambrook and Maniatis, 1989). Oligonucleotides used in this study are listed in Table S2 (supplementary data). Oligonucleotides pCN51-1m/pCN51-2c were used to generate the probe used in the melting assay. Oligonucleotides SaPIbov1-112mE and SaPIbov1-113cB were used to generate the probe used in Fig. 5. Labeling of the probes and DNA hybridization were performed according to the protocol supplied with the ECL Direct Nucleic Acid Labeling kit (Amersham, Piscataway, NJ).

The SaPIbov1-*ori* mutants used in the experiments shown in Fig. 5 and Table 1 were constructed using the plasmid pMAD as previously described (Ubeda et al., 2008). The combination of primers (SaPIbov1-ori-63cS, SaPIbov1-ori-14m/SaPIbov1-ori-13c, SaPIbov1-ori-64mE), (SaPIbov1-ori-63cS, SaPIbov1-ori-65m/SaPIbov1-ori-66c, SaPIbov1-ori-64mE), (SaPIbov1-ori-63cS, SaPIbov1-ori-48m/=SaPIbov1-ori-47c, SaPIbov1-ori-64mE), (SaPIbov1-ori-63cS, SaPIbov1-ori-53m/SaPIbov1-ori-54c, SaPIbov1-ori-64mE), (SaPIbov1-ori-63cS, SaPIbov1-ori-39m/SaPIbov1-ori-38c, SaPIbov1-ori-64mE) were used to generate SaPIbov1 deletion mutants 5, 6, 10, 12 and 14, respectively.

**Table 1**
Effect of SaPIbov1 *ori* mutations on SaPIbov1 transfer frequency.

| | Phage titer | SaPI titer |
|---|---|---|
| RN451 | $4.6 \times 10^8$ | |
| RN451 SaPIbov1 | $1.2 \times 10^7$ | $2.0 \times 10^7$ |
| RN451 SaPIbov1*Δrep*[*] | $1.0 \times 10^7$ | $1.0 \times 10^6$ |
| RN451 SaPIbov1Δ5 | $2.0 \times 10^7$ | $6.7 \times 10^5$ |
| RN451 SaPIbov1Δ6 | $2.0 \times 10^7$ | $5.4 \times 10^8$ |
| RN451 SaPIbov1Δ10 | $1.4 \times 10^7$ | $1.8 \times 10^8$ |
| RN451 SaPIbov1Δ12 | $3.4 \times 10^7$ | $4.8 \times 10^8$ |
| RN451 SaPIbov1Δ14 | $1.8 \times 10^7$ | $5.4 \times 10^8$ |

See Fig. 3 for *ori* deletion genotypes.
[*] Deletion of Rep protein gene.

### 2.3. Plasmid constructs

All plasmids used in this study are listed in Table S3 (supplementary data). The primers used for each construction are indicated in Table S2. Plasmids pRN9256, pRN9257, pRN9258, pRN9259 and pRN9261, which contain different SaPIbov1-*ori* fragments were constructed by cloning PCR products obtained with the appropriate primers into the plasmid pRN9210. In order to generate plasmids pRN9260 and pRN9262, which contain SaPIbov1-*oris* with internal deletions, two separate PCR reactions with overlapping sequences were performed using the pair of primers indicated in Table S3. The generated PCR products contained the fragments of SaPIbov1-*ori* flanking the sequence to be deleted. A second PCR was performed with external primers to obtain a single fragment, which contains a modified SaPIbov1-*ori* with the desired deletion. The obtained PCR products were cloned in the plasmid pRN9210 using the appropriate restriction enzymes as indicated in Table S2. Plasmid pRN9263, was constructed by cloning a PCR product containing SaPIbov1-*pri-rep* into the thermosensitive plasmid pRN9220. Plasmids pRN9264, pRN9265, pRN9266, pRN9267, pRN9268, pRN9270, pRN9271, pRN9272 and pRN9273, which contain different SaPIbov1-*ori* fragments, were constructed by cloning PCR products obtained with the appropriate primers into the plasmid pRN9263. In order to generate plasmids pRN9269, pRN9274, pRN9275, pRN9276, pRN9277, pRN9278, pRN9279, pRN9280 and pRN9281, which contain SaPIbov1-*oris* with internal deletions, two separate PCR reactions with overlapping sequences were performed using the pair of primers indicated in Table S3. The generated PCR products contained the fragments of SaPIbov1-*ori* flanking the sequence to be deleted. A second PCR was performed with external primers to obtain a single fragment, which contains a modified SaPIbov1-*ori* with the desired deletion.

### 2.4. Mobility-shift assays

SaPIbov1 Rep protein, containing N-terminal histidine tag, was purified using the plasmid pRN9208, encoding SaPIbov1-Rep, as previously described (Ubeda et al., 2007). SaPIbov1 Rep-*ori* complexes were detected by electrophoretic gel mobility shift assay by using purified SaPIbov1-Rep protein and PCR $^{32}$P end-labeled *ori* probes 1–8, described in Fig. 1, that were obtained with primers P984/Sbovori-4mK, Sbovori-10mK/Sbovori-9cE, Sbovori-5mK/Sbovori-6cE, Sbovori-59m/Sbovori-9cE, Sbovori-60m/Sbovori-9cE, Sbovori-63m/Sbovori-9cE, Sbovori-4mK/Sbovori-9cE and Sbovori-10mK/P984, respectively. Typical 20-μl reactions containing 0.6 ng of labeled probe and different amounts of Rep protein were incubated in binding buffer (10 mM Hepes, pH 8/10 mM Tris–HCl, pH 8/5% glycerol/50 mM KCl/1 mM EDTA/1 mM DTT/1 μg of bulk car-