Published in final edited form as:
Plasmid. 2012 March ; 67(2): 183–190. doi:10.1016/j.plasmid.2012.01.006.

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.

Keywords

pathogenicity island; replication origin; replication initiation; SaPI

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., 2008, 2005). In previous studies, we have analyzed several components of the SaPI lifecycle, including genome organization, phage

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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 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°C or 37°C overnight on glycerol-lactate agar medium (Novick, 1991), supplemented with antibiotics as appropriate. Broth cultures were grown at 32°C or 43°C in casamino acids–yeast extract broth (Novick, 1991) or TSB with shaking (240 rpm). Procedures for transduction and transformation in *S. 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 Figure 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 Figure 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.

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- ori s 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- ori s 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- l 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 g of bulk carrier DNA/50 g/ml BSA). After 20 min of incubation, the reaction mixtures were analyzed in a 4% native polyacrylamide gel. Gels were dried, and band shifts were analyzed with a Molecular Dynamics (Sunnyvale, CA) Phosphor Imager.

2.5. Melting assay

The procedure was a modification of the published procedure (Zzaman and Bastia, 2005). The reaction mixture containing 1 g each of plasmids containing different SaPIbov1- ori fragments, 40 mM HEPES/KOH (pH 7.6), 10 mM MgCl2, 20 mM KCl, 100 g/ml BSA and 1 to 25 ng of purified SaPIbov1-Rep protein, was incubated for 15 minutes at 37°C. After 15 min incubation, $KMnO_4$ in 3 M tetra ethyl ammonium chloride (TEAC) was added to a final concentration of 2 mM and incubated further for 5 min at 37°C. The reaction was quenched with 300 mM sodium acetate (pH 5.2) and 1 mM EDTA. The DNA was purified using Qiagen DNA purification column. The purified DNA was cleaved with 1U of nuclease S1 at

37°C for 4 hr. The DNA was purified again with a Qiagen DNA purification column. Nuclease S1-treated DNA was further digested with the different restriction enzymes combinations AvrII/SpHI, NotI, SacII/SphI or SacI/SphI, as indicated, at 37°C for 2 hr. The DNA was resolved in a 1.5% agarose gel, transferred to a nylon membrane using a vacuum unit and 20XSSC buffer as previously described (Sambrook and Maniatis, 1989), and hybridizated with a probe against plasmid sequences flanking the SaPIbov1- *ori* (Fig. 2).

2.6. Induction of prophages

Bacteria were grown in TSB broth to OD_{540} = 0.4 and induced by adding mitomycin C (2 g ml–1). Cultures were grown at 32°C with slow shaking (80 r.p.m.). Lysis usually occurred within 3 h. Samples were removed at various time points after phage induction, and standard SDS minilysates were prepared and separated on 0.7% agarose gels, as previously described (Lindsay et al., 1998).

3. Results

3.1. Deletion analysis of Rep-ori binding

The SaPIbov1- *ori* contains 11 hexanucleotide iterons, 10 with the sequence GTACCC and 1 with a single mismatch, (GTATCC), flanking the AT-rich region (Fig. 1A). To determine the role of these iterons in the initiation of replication, we constructed a series of deletions in the cloned replication origin and tested these for Rep binding, for melting and for their ability to support replication. In the first series of tests (Fig. 1B), we performed an electrophoretic mobility shift assay (EMSA) which confirmed Rep binding to the intact *ori* (probe 1), and showed that the same binding pattern, including a second retarded band, was obtained with the four iterons adjacent to and flanking the AT-rich region (probe 3). No gel shift was observed however, with just the intervening AT-rich region (probe 2), indicating that Rep does not detectably bind to this region. Binding was also observed with 4 other combinations of iterons, including the entire set on either side of the AT-rich region (probes 7 & 8), one pair only (probe 4), and even a single intact iteron (probe 5). In all cases in which iterons from only one side of the AT-rich region were present, no second retarded band was observed, suggesting that Rep binds separately to iterons on either side of the AT-rich region. Note that probes 5 and 6 contain the same fragment as probe 4 except that the sequence of one of the repeats for probe 5 and both repeats for probe 6, to which no binding was detected, had two induced nucleotide changes (GTACCC to GGATCC). This suggests that there is a rather stringent requirement for the native iteron sequence.

3.2. Rep-induced melting within the SaPIbov1 replication origin

As noted, the initiation of replication in prokaryotes involves melting at an AT-rich site within or adjacent to the origin, involving a complex between *ori* DNA and the initiation protein, driven by the free energy of supercoiling (Zakrzewska-Czerwińska et al., 2007). Since the SaPIbov1 Rep protein, the only known initiator of SaPI replication, binds to repeat sequences flanking the AT-rich region, we hypothesized that it might induce melting in that region. We tested this by means of a standard DNA melting assay using $KMnO_4$, which oxidizes unpaired T residues in a melted region (Lu et al., 1998), preventing them from snapping back after removal of the protein that induced the melting (see Fig. 2A and methods). We incubated different amounts of purified SaPIbov1 Rep protein with plasmid pRN9256, which contains the SaPIbov1- *ori*, in the presence of 2 mM $KMnO_4$. After quenching the $KMnO_4$ reaction, we purified the plasmid DNA and digested it with single strand (SS)-specific S1 nuclease to linearize plasmid DNA containing any melted (i.e. single-stranded) regions. Following S1 nuclease treatment, we digested the plasmid DNA with AvrII and SphI and analyzed the digestion products by agarose gel electrophoresis followed by Southern blotting with a probe specific for the plasmid sequences flanking the

ori. As seen in Fig. 2B, following the Rep-$KMnO_4$-S1 treatment, the 1.2 kb AvrII-SphI ori-containing fragment has been replaced by fragments of 750 and 450 bp, consistent with S1 cleavage of melted DNA within the AT-rich region of the cloned SaPIbov1- ori. To confirm this result, we performed the same Rep-$KMnO_4$-S1 treatment but digested the plasmid with other restriction enzymes, which would give different fragment sizes on the Southern blot if the ori were melted. As shown in Fig. 2C, two fragments of the expected sizes (1750 and 750 bp) were observed when the Rep-$KMnO_4$-S1-treated plasmid was digested with NotI or SacII and SphI. Similarly, two fragments of 750bp were observed, as expected, when the treated plasmid was digested with SacI and SphI. It is noted that there are extra bands in this gel that are not accounted for by the restriction map. We suspect that there may be a mistake in the map.

These results are taken to indicate that Rep induces melting within the AT-rich region. It is suggested, therefore, that the initiation of SaPI replication conforms to the general pattern of replication initiation on prokaryotic replicons, starting with Rep binding, followed immediately by melting, and that the melting enables unwinding driven by the helicase activity of the protein The two activities of the protein are readily separable since the helicase activity of the Rep protein requires ATP (Ubeda et al., 2007) but binding and melting do not.

3.3. Sequence requirements for initiation

The above results indicate that SaPIbov1-Rep binds to the repeats but not to the AT-rich region, and that a second retarded band is observed in EMSA assays when iterons from both sides of the AT-rich region were present, but not when those from only one side were present. This suggests that Rep binds independently to the opposing iteron sets and we hypothesized that this dual binding would be required for both melting and initiation. Accordingly, we compared the sequence requirements for initiation with those for binding and melting. Here, we made used of pRN9263, a thermosensitive plasmid that contains the SaPIbov1 replicon, including the SaPI-coded primase gene, which facilitates SaPI-driven plasmid replication but is not absolutely required. This plasmid can replicate at 43°C only if the SaPIbov1- ori is present in cis. We constructed plasmid derivatives lacking different segments of the ori (see Fig. 3A and methods). The derivative plasmids were introduced into RN4220 by transformation, and the resulting strains were tested for thermosensitivity of plasmid replication. Bacteria were grown at 43°C to OD_{540}=0.6 and 1 ml samples were used to prepare mini-lysates that were separated on an agarose gel and Southern blotted with a plasmid-specific probe. Hybridization conditions were used that would show a plasmid-specific band only if there were an increase at 43°C in the amount of plasmid DNA over that present at the time of the temperature shift. As shown in Fig. 3B, some ori configurations supported replication as well as the intact ori; others did not generate a detectable band, and still others showed bands of intermediate intensity. The results with constructs 3, 4, & 5 show that iterons on both sides of the AT-rich region, as well as the AT-rich region itself must be present, and that removal of even the single rightmost iteron (construct 9) causes a considerable reduction in intensity of the plasmid signal. The only iteron deletion that seems compatible with full replication is that of the leftmost element (construct 8). Deletions in plasmids 6 & 7, which eliminate replication, are missing not only the iterons but also the 17 bp intervening region. However, this entire region does not seem to be required as construct 17, in which 12 of the 17 bp have been deleted, replicates as well as the wild-type (plasmid WT).

As seen in Fig. 3A, the repeats are organized in 6 groups, 3 on the left (A, B and C,) and 3 on the right (D, E and F). We tested the requirements of groups B & E by changing 2 of the 6 bp in each repeat, which is predicted to eliminate direct Rep binding as suggested by the abolition of Rep binding to a single repeat when 2 out of 6 bp were modified (see Fig. 1B,

fragment 6). In both cases (plasmids 10 & 11), replication was moderately diminished. In contrast, changing two of the six nucleotides in the group C and D iterons, eliminated replication (plasmids 12 and 13). These results showed that iteron groups A, C, D and F are crucial for plasmid replication.

As can be seen in Fig. 3A, the SaPIbov1 origin of replication has great symmetry not only in the number and direction of the repeats but also in the spacing between groups. This spacing, however, appears to have only a moderate role in replication: deletions affecting the B-C spacing (plasmid 14) or the D-E spacing (plasmid 15) had only a minimal effect; even deletions affecting the spacing in both groups did not totally block replication (plasmid 16), though the effect was rather severe in this case.

Although we have not performed melting assays on all of the deletion derivatives illustrated in Fig. 3, we have done the melting assay on a few critical examples, as shown in Fig. 4. In these few examples, melting is fully correlated with initiation. However, there appear to be very weak Rep-induced bands with all of the non-replicating constructs. Perhaps Rep binding may distort the DNA sufficiently to allow the $KMnO_4$ reaction on a small number of molecules. Such very weak melting is not compatible with the initiation of replication.

3.4. Ori requirements for melting

The above results showed that both the repeats and the AT-rich region are important for SaPI replication. We wished to confirm the predicted correlation between Rep-induced melting and replication. Accordingly, we cloned some of the *ori*s showed in Fig. 3A to plasmid pRN9210. We then tested the ability of Rep to melt the resulting plasmids using the same assay as in Figure 2. As shown in Fig. 4, tests of *ori* derivatives 1, 2, 4, 5, 8 and 14 showed that the AT-rich region is required, as are the flanking sets of repeats. In fact, any significant deletion eliminated melting - only *ori*-8 and *ori*-14 which lacked either a single repeat (*ori*-8) or one of the intervening segments (*ori*-14) – i.e., minor changes - could support melting. These results clearly confirm the predicted correlation between melting and replication. We suggest that Rep binding to repeats flanking the *ori* is required for melting; however, this is apparently not sufficient, since *ori*-2 shows the second retarded band (Fig. 1) but does not support melting.

3.5. Effects of ori deletions on phage-induced SaPIbov1 replication and transfer

As described above, we have used a somewhat artificial system – a plasmid containing the cloned *rep* and *ori* functions - to enable the study of *ori* requirements for melting and the initiation of replication. We next analyzed some of these *ori* constructs for their effects on function of the intact SaPIbov1 genome. Here, we substituted some of the *ori* constructs illustrated in Fig. 3 for the native SaPIbov1- *ori* in situ, in a ϕ11 lysogenic strain containing SaPIbov1, then tested SaPIbov1 containing the different incomplete *ori*s for SaPIbov1 replication and transfer following mitomycin C induction of the resident ϕ11 prophage. As reported previously (Ruzin et al., 2001; Ubeda et al., 2005), electrophoresis of whole-cell DNA prepared after mitomycin-C induction of a ϕ11 lysogenic strain containing SaPIbov1 showed a DNA species that migrates faster than the bulk DNA (Fig. 5B). This species represents monomeric SaPI DNA released from phage particles and is seen only if the phage-induced SaPI replication cycle has been completed. A southern blot with a SaPIbov1-specific probe confirmed that this band contained monomeric SaPIbov1 DNA (Fig. 5C). In contrast, no such band was observed when SaPIbov1 lacked the AT rich region (*ori*5) or, as previously shown (Ubeda et al., 2008), with a derivative of SaPIbov1 lacking Rep (Fig 5B, 5C). This result confirms that the AT rich region is absolutely necessary for SaPI replication. We next analyzed origin derivatives lacking some of the iterons. The *ori* was replaced by deletion derivatives 6, 10, 12, or 14 which lack iteron groups A, B, C or the B-C

spacer, respectively (Fig. 3A). In contrast to the above results with plasmids carrying these same deletions (Fig 3), *ori*s6 and 12 supported clearly detectable replication, though the levels seemed to be somewhat diminished in comparison to the WT ori, but more robust than with the *ori*-containing plasmid, whereas, *ori*s10 and 14 were not appreciably different from the plasmid model. It thus appears that the plasmid model used for the results shown in Fig. 3 represents a considerably more stringent test of SaPIbov1 *ori*-driven replication than the intact island for deletions 6 and 12, but not for 10 or 14. The reasons for this discrepancy are not immediately apparent, and will require further study.

In a previous study, we demonstrated that deletion of the SaPIbov1 rep protein caused only a modest diminution of SaPIbov1 transfer whereas deletion of the *ori* eliminated transfer (Ubeda et al., 2008). Since the *ori* deletion in that study included ORFs 11 & 12, the apparent discrepancy could have been related to the extent of the *ori* deletion. As shown in Table 1, *ori* deletion 5, which eliminated the AT-rich region of the *ori* and completely blocked SaPIbov1 replication, with either the plasmid or the intact island, supported SaPIbov1 transfer at about the same modestly reduced level as deletion of the *rep* gene. These results confirm that SaPI-specific replication is not required for high-frequency transfer. As also shown in Table 1, all of the other *ori* deletions tested, 6, 10, 12, and 14 supported SaPIbov1 transfer at an even higher frequency than that seen with the WT island, and there was no correlation with the severity of the replication defect caused by these deletions. The basis of this 10-50-fold increase in SaPIbov1 transfer frequency seen with these *ori* deletions remains to be determined.

4. Discussion

The overall SaPI replication process is similar to that of a bacteriophage: there is a SaPI-specific replication initiation protein with helicase activity that binds to the SaPI origin in a sequence-specific manner and initiates melting within the origin, followed by helicase-driven unwinding (Ubeda et al., 2007). As the SaPIs do not encode homologs of any other replication proteins, it is assumed that all of the polymerization functions are provided by the host cell. The focus of the present investigation was the sequence organization of the SaPI replication origin, which seems unique among the known replication origin sequences of the most closely related elements, the bacteriophages (Denniston-Thompson et al., 1977; Horiuchi, 1997; Ravin et al., 2003; Saito et al., 1980). The key feature of this organization is its symmetry, with the iterons on either side of the central AT-rich region being mostly in opposite orientations. The iterons are required for binding of the Rep protein and this binding results in primary and secondary retarded bands on a gel mobility shift assay (Fig. 1 and (Ubeda et al., 2007)). Interestingly, only the primary band is observed when only iterons from one side are present. The appearance of this band is independent of the number of iterons. In fact, the same kind of shift was observed with a single iteron or six iterons. It is also interesting that a second retarded band is seen only when oppositely oriented iterons flanking the AT-rich region are present. This result suggests that Rep binds independently to oppositely oriented iterons flanking the AT-rich region. Experiments performed to test the contribution of the different iterons to *ori* function showed that nearly the entire *ori* is needed for replication (Fig. 3) and perhaps also for melting (Fig. 4). Since binding at about the same level was seen in the EMSA with the 4 iterons of groups C+D (Figs. 1 & 3) as with the WT *ori*, we conclude that binding per se is not sufficient for replication initiation or *ori* melting. Future work should clarify the nature of the involvement of the additional iterons. As is true of all prokaryotic double-stranded (DS) DNA replication origins, initiator protein binding induces melting, usually – perhaps always - (in an AT-rich region adjacent to or within the Rep protein binding site. In the case of SaPIbov1, we have demonstrated Rep-induced melting of the AT-rich region, and shown using a plasmid containing the SaPI *ori*

that the flanking, oppositely oriented iterons are required. Also predictably, this melting is required for replication of a plasmid containing the SaPIbov1 replication origin (Figs. 3 & 4)

When testing the *ori* deletions in the intact, integrated SaPI, as predicted, the AT-rich region was essential for replication. However, as we have observed previously with a SaPI mutant with a deletion of the Rep protein (Ubeda et al., 2008), the SaPI transfer frequency was reduced only 10 fold. This result indicates that high frequency SaPI transfer can occur without replication (although excision is required (Novick et al., 2010)). It is noted also that the phage titer was not increased, indicating that non-replicating SaPI interferes with helper phage reproduction (Ruzin et al., 2001) to the same extent as does the WT. As we have previously shown, the SaPIs encode a small terminase subunit which is expressed by the non-replicating island (Novick et al., 2010) and directs packaging of SaPI DNA. Given that the number of infected bacteria in a typical culture prior to lysis is 10^9/ml, and that each cell contains at least one SaPI molecule, if at least one cell in 100 released a single SaPI particle upon lysis, and if at least 10% of these resulted in a SaPI transfer event, the observed transfer frequency of 10^6/ml (Table 1) would be easily reached. Regarding the absence of the so-called "SaPI band" (faster migrating band) shown in Fig. 5, with the *ori* 5 mutant, we note first, that when the blot was overexposed, a SaPI band representing encapsidation could be seen, and second, that the relation between SaPI and phage packaging in large and small capsids is complex and is far beyond the scope of this report. Suffice it to say that both phage and SaPI DNAs can be packaged in both large and small capsids and that both phage and SaPI genes are involved (Novick et al., 2010; Ram et al, in preparation).

Interestingly, two of the *ori* mutations, 6 and 12 had a less severe impact on replication of the intact SaPI than on replication of the plasmid containing the cloned *ori*. A similar discrepancy was observed in a study of the *Escherichia coli* chromosome origin, *oriC* (Bates et al., 1995). In this study, the insertion of a 2 kb fragment at the HindIII site between the R3 and R4 DnaA boxes within *oriC* completely inactivated *oriC* when cloned on a plasmid, while the same insertion was tolerated in the chromosome. One possibility that could explain the differences between the activity of the 6 and 12 *ori* derivatives in the plasmid vs. the SaPI is that the plasmid encodes only the SaPI Rep and primase – which are sufficient but may not be optimal for the replication of a supercoiled plasmid -whereas the intact island may encode other proteins important for replication, and its functional organization – programmed for optimal expression of replication genes – as well as its structure, may also contribute. Future studies are planned to address this possibility.

Together, these results are entirely consistent with the general picture of replication initiation for a prokaryotic DSDNA replication origin. It is noted that SaPI replication can apparently be initiated on a linear substrate (Ruzin et al., 2001), raising the question of how the molecule is constrained to allow helicase-driven unwinding. We suggest that Rep binding may constrain the iteron regions flanking the AT-rich region, allowing the latter to be melted and to bind the Rep helicase. Alternatively, *ori* unwinding may be initiatied by transcription, as is the case for T7 (Romano et al., 1981). Further studies should elucidate the initiation mechanism for the uniquely organized SaPI replication origins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research Highlights

- Demonstration of iterons in a replication origin from a gram-positive organism
- Symmetrical arrangement of iterons flanking an AT-rich region
- Requirement of flanking iterons for melting of the AT-rich region
- High frequency SaPI transfer in the absence of replication

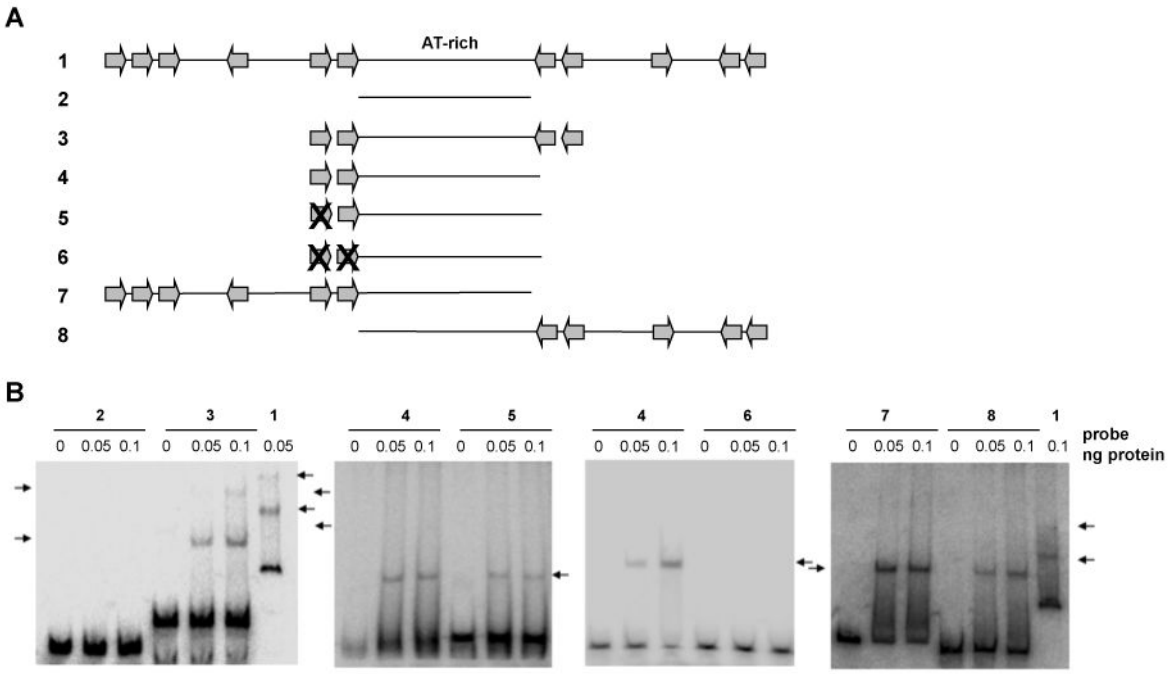

Figure 1. Rep binds to the SaPI-*ori* repeats

A. Schematic representation of the different SaPIbov1- oriprobes used in the electrophoretic mobility shift assay (EMSA). Arrows represent the hexanucleotides repeats containing the sequence (gtaccc). Crossout arrows represent repeats where the sequence gtaccc have been modified to ggatcc. The AT-rich region is indicated. Probe-1 contains the whole SaPIbov1-ori(301 bp).

B. EMSA using different amounts of SaPIbov1-Rep protein. The different probes used are indicated. The different DNA-protein complexes are indicated by arrows.

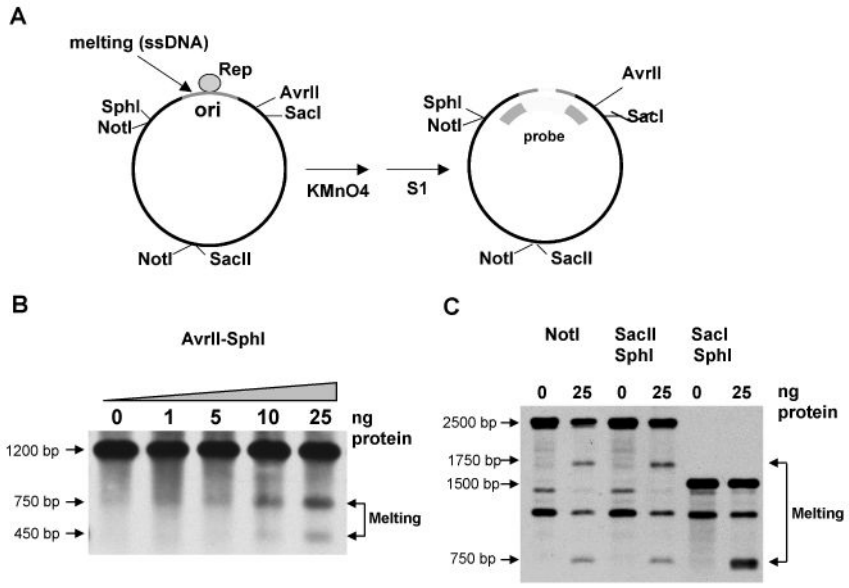

Figure 2. SaPIbov1-Rep melts SaPIbov1-*ori*

A. Schematic representation of the strategy used for Rep induction and detection of melting in the plasmid pRN9256 (4.3 Kb), which contains the SaPIbov1- ori The melted region was trapped by KMnO4, digested with S1 nuclease and cleaved with the indicated restriction enzymes. The digested plasmid was run on an agarose gel and blot with a plasmid-specific probe.

B. Southern blot of the reaction products obtained after incubation of the plasmid pRN9256 with increasing amounts of SaPIbov1-Rep. The plasmid was digested with AvrII and SphI for melting detection.

C. Southern blot of the reaction products obtained after incubation of the plasmid pRN9256 with 25 ng of SaPIbov1-Rep. The plasmid was either digested with NotI; SacII and SphI or SacI and SphI for melting detection.

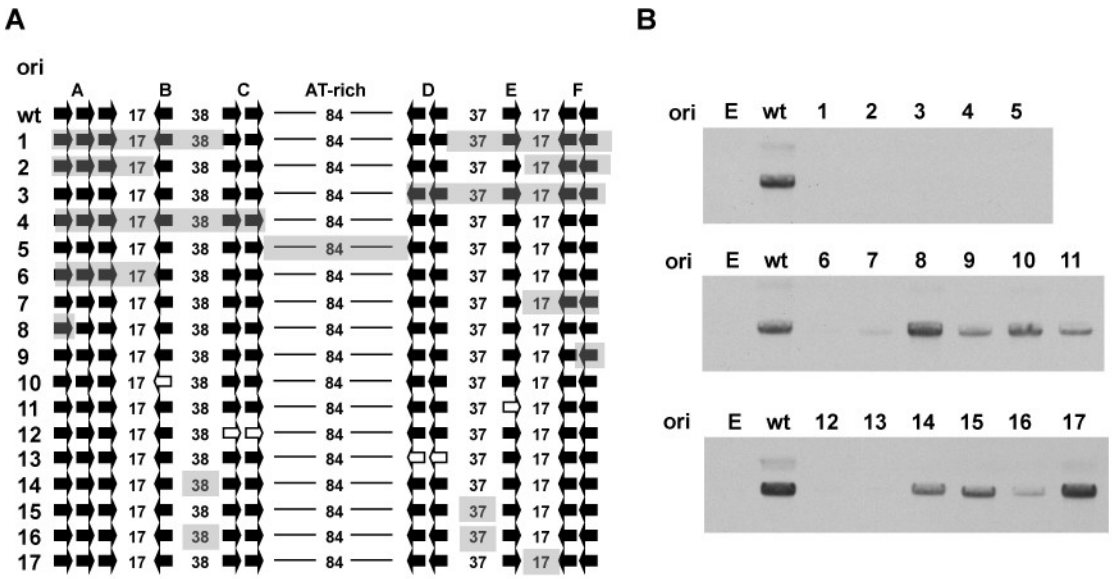

Figure 3. SaPI-*ori* requirements for replication

A. Schematic representation of the different SaPIbov1- *ori* constructs. Black arrows represent the hexanucleotides repeats. These repeats are grouped in 6 groups as indicated (A, B, C, D, E and F). Sequence deleted from the *ori* is gray-shadowed. Open arrows represent the modified hexanucleotides repeats where two of the six nucleotides have been changed. Number of bp between repeats is indicated.

B. Cultures of RN4220 strains containing the SaPIbov1 pri-rep-ori plasmids were grown on CY broth at 43°C until OD540=0.6. 1ml samples were removed and used to prepare minilysates. Lysates were separated by agarose gel electrophoresis and blot with a probe specific for the plasmid. E indicates empty vector.

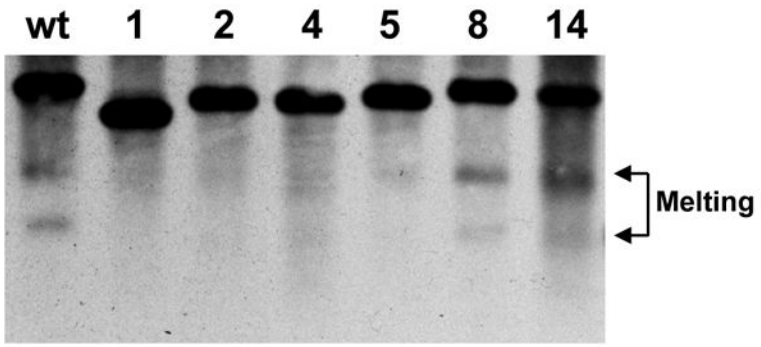

Figure 4. Ori requirements for melting

Plasmids containing different SaPIbov1- *ori* constructs were incubated with 25 ng of SaPIbov1-Rep. The melted region was trapped by KMnO4, digested with S1 nuclease and cleaved with AvrII and SphI. The digested plasmids were run on an agarose gel and blot with a plasmid-specific probe. Arrows indicate melting products. Numbers indicate the different SaPIbov1- *ori* constructs used, which are shown in Fig. 3A.

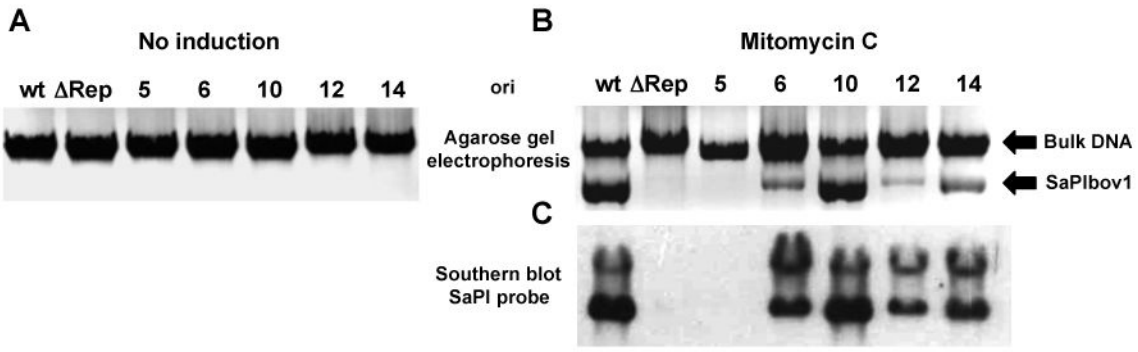

Figure 5. Ori requirements for SaPI replication

A and B. Bacterial cultures of staphylococcal strains containing phi11 and WT SaPIbov1 or derivatives of SaPIbov1 lacking *rep* or containing a partial *ori*, were incubated in broth at 32C with or without mitomycin C. Each number indicates the partial *ori* present in SaPIbov1, whose structure is shown in Fig. 3. Samples were removed 90 minutes after mitomycin C induction and used to prepare minilysates. Lysates were separated by agarose gel electrophoresis, stained with ethidium bromide and photographed.

C. Southern blot hybridization pattern of samples shown in (B) hybridized overnight with a SaPIbov1-specific probe.

Table 1

Effect of SaPIbov1 *ori* mutations on SaPIbov1 transfer frequency

	Phage titre	SaPI titre
RN451	4.6×10^8	
RN451 SaPIbov1	1.2×10^7	2.0×10^7
RN451 SaPIbov1 rep*	1.0×10^7	1.0×10^6
RN451 SaPIbov1 5	2.0×10^7	6.7×10^5
RN451 SaPIbov1 6	2.0×10^7	5.4×10^8
RN451 SaPIbov1 10	1.4×10^7	1.8×10^8
RN451 SaPIbov1 12	3.4×10^7	4.8×10^8
RN451 SaPIbov1 14	1.8×10^7	5.4×10^8

*Deletion of Rep protein gene.

See Fig. 3 for *ori* deletion genotypes.