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SaPI mutations affecting replication and transfer and enabling autonomous replication in the absence of helper phage

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Summary

The SaPIs are chromosomal islands in staphylococci and other Gram-positive bacteria that carry genes for superantigens, virulence factors, resistance and certain metabolic functions. They have intimate relationships with certain temperate phages involving phage-induced excision, replication and efficient packaging in special small-headed infective phage-like particles, resulting in very high transfer frequencies. They generally contain 18–22 ORFs. We have systematically inactivated each of these ORFs and determined their functional groupings. In other reports, we have shown that five are involved in excision/integration, replication and packaging. In this report, we summarize the mutational analysis and focus on two key ORFs involved in regulation of the SaPI excision–replication–packaging cycle *vis-à-vis* phage induction. These two genes are divergently transcribed and define the major transcriptional organization of the SaPI genome. One of them, *stl*, encodes a master repressor, possibly analogous to the standard cI phage repressor. Mutational inactivation of this gene results in SaPI excision and replication in the absence of any inducing phage. This replicated SaPI DNA is not packaged; however, since the capsid components are provided by the helper phage. We have not yet ascertained any specific function for the second putative regulatory gene, though it is highly conserved among the SaPIs.

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Introduction

Staphylococcus aureus pathogencity islands (SaPIs) are a family of related 15–17 kb mobile genetic elements that commonly carry genes for superantigen toxins and other virulence factors and are largely responsible for the spread of these. The key feature of their mobility and spread is the induction by certain phages of their excision, replication and efficient encapsidation into specific small-headed phage-like infectious particles (Lindsay *et al.*, 1998; Ruzin *et al.*, 2001; Ubeda *et al.*, 2005). This sequence of events is referred to as the SaPI excision–replication–packaging (ERP) cycle. As a consequence of their high-frequency transfer, the SaPIs are very widely distributed, with many *S. aureus* strains containing two or more (Novick and Subedi, 2007). The sequences of some 16 of these are currently available and reveal a well-conserved genome organization that is broadly similar to that of a typical temperate phage (Novick and Subedi, 2007). All SaPIs are integrated at specific chromosomal sites, are flanked by short direct repeats, which represent *att* site cores, and encode specific integrases that recognize these sites and are required for integration and excision (Maiques *et al.*, 2007; Ruzin *et al.*, 2001; Ubeda *et al.*, 2003). We refer to the chromosomal *att* site as att_C, the corresponding SaPI sequence as att_S, and the hybrid sites at the SaPI-chromosomal junctions as J_R and J_L, the latter being adjacent to the *int* gene in most cases. Five different SaPI *att* sites have been identified in the *S. aureus* genome, each of which is used by two or more of the known SaPIs (Novick and Subedi, 2007). These are not used by other mobile elements; they are usually at the 3′ ends of genes and reconstruct the coding sequences of these genes. Molecular genetic analyses of a few prototypical SaPIs, especially SaPI1, SaPI2, SaPIbov1, SaPI-

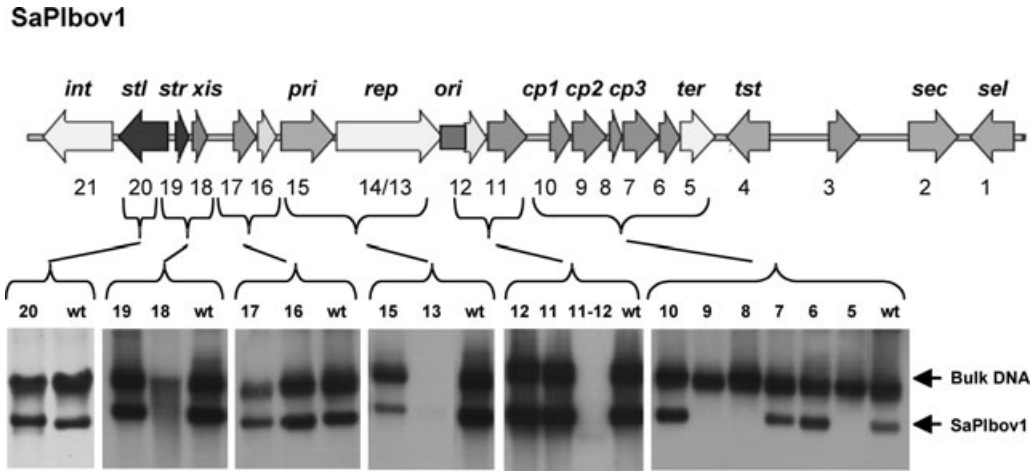

Fig. 1. SaPIbov1 maps and mutants. Below map is a Southern blot of SaPIbov1 mutant lysates obtained with samples taken 90 min after MC induction, separated on agarose and blotted with a SaPIbov1-specific probe. Upper band is 'bulk' DNA, including chromosomal, phage and replicating SaPI; lower band is SaPI linear monomers released from phage heads.

bov2 and SaPIn1 have been initiated and in Fig. S1 is a diagrammatic map of four of these: SaPI3, SaPI1, SaPIn1 and SaPIbov1, of which the latter is the primary subject of the present report. SaPIbov1 was identified in a bovine mastitis *S. aureus* isolate, RF122 (Fitzgerald *et al.*, 2001), and SaPIbov2 in a second such strain, V329 (Ubeda *et al.*, 2003). It is interesting that both of these occupy att_C site II in the staphylococcal chromosome and that no other known SaPIs have been found at this site; moreover, in a recent survey of human clinical isolates, SaPIs were identified at four of the five att_C sites, excluding site II (Subedi *et al.*, 2007). As can be seen, SaPIbov1 carries genes for TSST-1 (*tst*), plus enterotoxins C and L (*sec* and *sel*), and it contains some 17 ORFs that do not encode known toxins or other virulence genes. These are assumed to be involved in the ERP cycle, though not all of them are known to be translated.

In this study, we have constructed in-frame deletions in each of the SaPIbov1 ORFs shown in Fig. 1. We show that many of these genes have definable roles in the SaPI ERP cycle, and one of them is clearly the master regulator, in which mutations enable autonomous replication in the absence of helper phage. Several of the corresponding ORFs have been mutated in SaPI1 and SaPIbov2, and the behaviour of these mutants is similar to that of the corresponding SaPIbov1 mutants.

Results

Most of the SaPIbov1 genes are implicated in its replication and transfer

To determine the roles of the different ORFs in the SaPIbov1 ERP cycle, we generated an in-frame deletion in each, using pMAD (Arnaud *et al.*, 2004). With one gene, *int*, we were unable to transfer the mutation to *S. aureus*. A possible reason for this is discussed below. These mutants were initially generated in strain JP45 (RN4220 SaPIbov1 *tst*::*tetM*; Table S1), and then transferred by transduction to strains RN27 (lysogenic for phage 80α) and RN451 (lysogenic for phage ϕ11; Table S1). We used ϕ11 as well as 80α because ϕ11 induces only SaPIbov1, whereas 80α induces both SaPIbov1 and SaPI1, as well as four other SaPIs thus far tested. This suggests that SaPIbov1 possesses one or more gene functions, lacking in the other SaPIs, that enable induction by ϕ11. Studies are in progress to identify these functions.

The SaPIbov1 pMAD mutants were each analysed for the three sequential and definable stages of the SaPI ERP cycle. Each strain was mitomycin-C induced, screening lysates were prepared after 90 min, separated on agarose, stained and photographed, and then Southern blotted with a SaPIbov1-specific probe. We have not, in this presentation, specifically analysed excision. We assume that mutants that produced a SaPI band or showed significant replication must have been excised. To quantify replication of SaPI DNA, we performed quantitative PCR (qPCR) with samples taken at the 90 min time point (Table 1). Using the phage lysates which resulted upon further incubation, we evaluated packaging efficiency by determining the SaPIbov1-specific transfer frequencies. These results are presented qualitatively as either high-frequency SPST (SaPI-specific transfer; H – roughly commensurate with the plaque-forming titre of the phage), low-frequency SPST (L – between 1% and 0.001% of the plaque-forming titre) or no SPST (0 – commensurate with transfer by generalized transduction).

It is noted that a key feature of the SaPI ERP cycle is the appearance following SaPI induction of a SaPI-specific band in screening gels of whole-cell lysates. This band

Table 1. Quantification of SaPIbov1 mutants DNA replication by 80α.

Strain	Δ SaPIbov1 DNA[a]
SaPbov1 wt	50.21
Δ ORFs 11–12	1
Δ ORFs 13	1.6
Δ ORF 14	1.75
Δ ORF 15	20
Δ ORF 17	38
Δ ORF 18	10.8
Δ ORF 19	50

a. Number of SaPIbov1 copies compared with the chromosomal *gyr* gene.

migrates ahead of the heavy band containing sheared phage and chromosomal DNAs (referred to as 'bulk' DNA), represents primarily linear SaPI monomers and is usually an indication of SaPI excision and replication. The primary results with the serial mutations in the SaPIbov1 ORFs, starting with ORF3, are shown in Fig. 1 and summarized in Tables 1 and 2. We have not obtained mutations in the superantigen genes (ORFs 1, 2 and 4), which are assumed not to be involved in the SaPI ERP cycle.

ORF3

An in-frame deletion in ORF3 had no detectable effect on SaPIbov1 replication or SPST (data not shown).

ORFS 5–10

A detailed study of ORFs 5–10, which form an operon, has been reported elsewhere (Ubeda *et al*., 2007a) and is summarized briefly here (Fig. 1; Table 2). ORF5 encodes a homologue of the terminase small subunit (STS) of typical phages from Gram-positive bacteria, and is henceforth designated *ter*. *ter* deletion eliminated the characteristic SaPI band (Fig. 1) and sharply reduced the frequency of SaPIbov1 transfer (Table 2) but did not affect SaPIbov1 replication (Fig. 1, Table 2). Thus, *ter* function is required for SaPI DNA packaging. The *ter* defect was fully restored by the cloned gene (data not shown). As no SaPI encodes a homologue of the phage terminase large subunit (LTS), it is concluded that *ter* couples SaPI packaging with that of the inducing phage by complexing with the LTS.

ORFs 7, 8 and 9 are required for the formation of small capsids and are designated *cp3*, *cp2* and *cp1* respectively (Ubeda *et al*., 2007a); however, mutation of any of these did not decrease the SaPIbov1 transfer frequency (Table 2). These results suggest that although *ter* is absolutely required, SaPI encapsidation can evidently function with full-sized phage heads as well as with the SaPI-induced small ones. SaPI Ter must interact with the LTS at least as effectively as the phage STS does. Interestingly, SaPIbov2, which lacks *cp2*, packages its DNA only in full-sized phage heads (Maiques *et al*., 2007). Deletions

Table 2. Properties of SaPI mutants.[a]

		SaPI band		Replication		Transduction[b]	
Mutated ORF	Function	80α	ϕ11	80α	ϕ11	80α	ϕ11
SaPIbov1		+++	+++	+++	+++	H	H
3		+++	+++	+++	+++	H	H
5	Terminase	–	–	+++	+++	L	L
6		+++	+++	+++	+++	H	H
7	Capsid	++	++	+++	+++	H	H
8	Capsid	–	–	+++	+++	H	H
9	Capsid	–	–	+++	+++	H	H
10		+++	+++	+++	+++	H	H
Δ11-12	*ori*	–	–	–	–	0	0
11		+++	+++	+++	+++	H	H
12		+++	+++	+++	++	H	L
13	*rep*	–	–	–	–	L	H
15	Primase	+	+	+	+	H	H
16		+++	+++	+++	+++	H	H
17		++	+++	++	+++	H	H
18	*xis*	–	–	+	+	L	L
19	Transcriptional regulator	+++	+++	+++	+++	H	H
20	Repressor	+++	+++	+++	+++	H	H
int	Integrase					0	0
SaPI1		+++	–	+++	–	H	0
15	*rep*	–	–	–	–	L	0
22	Repressor	+++	+++	+++	+++	H	H
int	Integrase	–	–	+	+	0	0

a. Table summarizes findings with the different mutants.
b. H – ≥1% of wt frequency; L – ≤ 1% of wt frequency; 0 – ≤ 10^{-5} of wt frequency.

in ORFs 6 and 10 had no detectable phenotype in these tests (Fig. 1, Table 2).

ORFs 11 and 12

Mutations affecting ORFs 11 and 12 did not affect SaPI replication (Fig. 1), or SPST frequency except that ΔORF12 appeared to reduce transduction by ϕ11 (Table 2). Importantly, a deletion of both ORFs that included parts of the flanking intergenic regions on both sides eliminated SaPIbov1 replication and SPST (Fig. 1, Tables 1 and 2), suggesting that either or both of the intergenic regions are essential (or, less likely, that ORFs 11 and 12 are redundant essential genes). Examination of the intergenic region between ORFs 12 and 13 has revealed that this region represents the replication origin (*ori*) (Ubeda *et al*., 2007b). However, deletion of the replication origin would not account for the profound reduction in SPST seen with the ORFs 11–12 deletion, as replication is not required for SPST (see mutant in ORF13). It is suggested therefore that the *pac* site may be in the deleted region between ORFs 10 and 11, and this possibility is currently under study.

SaPIbov1 ORFs 13 (SaPI1 ORF15) and 15

Deletion in SaPIbov1 ORF13 (initially thought to be two consecutive genes – ORFs 13 and 14 – now known to be only one) and insertional inactivation of the corresponding gene in SaPI1 (ORF15) abolished SaPI replication (Fig. 1, Table 1), but did not eliminate SPST (Table 2), leading to the identification of its product as the replicon-specific initiator, Rep. Full replication capability was restored by the cloned *rep* gene, confirming its identification (not shown). The predicted product of this gene has been annotated in the *S. aureus* genomes as helicase-like, which brings to mind the fact that many Rep proteins of small extrachromosomal elements, such as the α protein of coliphage P4, have both helicase and initiation functions (Lindqvist *et al*., 1993). The replication initiation function of this gene was confirmed in a separate study in which it was demonstrated that a cloned segment including SaPIbov1 ORFs 13 (*rep*) and 15 plus the *ori* could drive replication of a suicide plasmid in *S. aureus*, and that this replication function was SaPI specific (Ubeda *et al*., 2007b).

Deletion in SaPIbov1 ORF15, encoding a conserved primase homologue (*pri*), reduced replication but did not eliminate it (Fig. 1, Table 1), and seemed to have no effect on SPST with 80α but may have caused a relative increase in SPST with ϕ11 (Table 2). As this gene is not required for SaPIbov1 replication, its importance for replication of the cloned *pri–rep–ori* segment remains to be determined.

ORFs 16 and 17

Mutation of ORFs 16 and 17 had no obvious effect on SaPIbov1 replication; the ORF16 mutation appeared to decrease the SPST frequency moderately (Table 2).

ORF18

The predicted product of ORF18 is homologous to Xis proteins; mutation of this gene caused a severe decrease in replication (Fig. 1, Table 1) and in SPST frequency (Table 2). Moreover, the Southern blotting pattern of this mutant (Fig. 1) is essentially the same as that seen with an *int*⁻ mutant of SaPI1 (unpublished results), we suggest that ORF18 may be required for excision, and designate it henceforth as *xis.* Perhaps the possession of an active *xis* function may be what distinguishes SaPIbov1 and SaPIbov2 from SaPI1, which does not excise spontaneously. If this is correct, it would mean that the inducing phage cannot supply the required *xis* function for SaPIbov1. Experiments to test directly for the role of *xis* in SaPIbov1 excision are in progress.

ORFs 19 and 20

All known SaPIs have a pair of divergently oriented ORFs that define their overall transcriptional organization; one of these, SaPIbov1 ORF19, is oriented in the rightward direction as the map is conventionally drawn and the other, ORF20, leftward. Both of the predicted products contain a helix–turn–helix motif typical of transcriptional regulators, and for this reason are here designated *str* (SaPI transcription rightward; ORF19) and *stl* (SaPI transcription leftward; ORF20). Rather surprisingly, however, an in-frame deletion in SaPIbov1 ORF19 had no effect on phage induced SaPI replication or SPST (Fig. 1, Tables 1 and 2).

ORF20 (stl) *is the master regulator of the SaPI ERP cycle*

In RN4220, either an in-frame deletion in SaPIbov1 ORF20 or an insertional inactivation of SaPI1 ORF22 caused slow growth and the development of yellow pigment. It is noted that RN4220 is unpigmented owing to a deletion in *rsbU*, which greatly reduces the activity of s^B (Kullik and Giachino, 1997). Pigmentation is restored under conditions of stress, such as subinhibitory concentrations of antibiotics (Herbert *et al*., 2001), and we suggest that the stress of unrestricted SaPI replication is responsible for the pigmentation and slow growth seen with this mutant. With either mutant, in a non-lysogen, the production of SaPI DNA was sharply increased, as shown by Southern blotting (Fig. 2), clearly representing excision

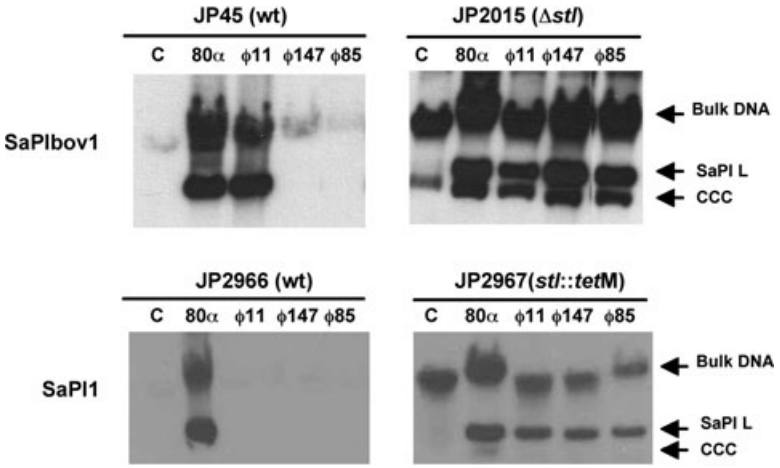

Fig. 2. Derepression of SaPI replication by *stl* (ORF20/22) mutation. Indicated SaPI derivatives of RN4220 were uninfected (C), 80α, ϕ11, ϕ147 or ϕ85 infected. At 45 min after infection, 3 ml samples were collected and used to prepare standard minilysates, which were run on a 0.7% agarose gel overnight, then Southern blotted, probed for SaPI *ter* and visualized by ECL. L, SaPI linear monomers; CCC, covalently closed circular SaPI.

and replication of the island. Note especially that in the absence of phage, there is no classical SaPI band (SaPI L) with either of the mutants, although there is a more rapidly migrating band (SaPI CCC) that has been shown previously to represent supercoiled monomers, presumably SaPI excision products (Lindsay *et al*., 1998). Note also that with the SaPIbov1 and SaPI1 *stl* mutants, infection with different phages, two of which, ϕ147 and ϕ85, cannot induce the wt SaPIs, induced the formation of the classical SaPI band, representing encapsidation (Fig. 2). This was accompanied by high SaPI transfer frequencies with these phages (Table 3). Infection or induction of an 80α lysogen containing the ORF20 mutant resulted in the typical pattern of SaPI replication with these ordinarily non-inducing phages (Fig. 2). A similar effect was seen with the corresponding SaPI1 mutant, which is also shown in Fig. 2. These findings suggest that for SaPIbov1 and SaPI1 the ERP cycle is repressed by the product of this gene and therefore that the key effect of an inducing phage is to relieve this repression, and that these non-inducing phages cannot induce the SaPI ERP cycle because they cannot induce derepression. Consistent with this is the effect of the cloned SaPIbov1 ORF20 and SaPI1 ORF22 (under P*cad* control), as shown in Fig. 3. As can be seen, SaPIbov1 ΔORF20 and SaPI1 ORF22::*tetM* replication are dramatically repressed in the complemented strains. Interestingly, although the products of SaPIbov1 ORF20 and SaPI1 ORF22 have unrelated sequences, they may cross-react to a moderate extent (data not shown).

It seems likely that the pigmentation and poor growth of bacteria containing SaPIbov1 or SaPI1 with this gene mutated is caused either by SaPI overreplication or by overexpression of a potentially toxic SaPI gene. In support of this possibility, the cloned ORF20 eliminated autonomous SaPIbov1 Δ20 replication in the absence of

Table 3. Transfer of the SaPI *stl* mutants.[a]

Donor strain	SaPI	Phage	SaPI transfer titre[b]	Phage titre[c]
JP45	SaPIbov1	ϕ80α	5.7×10^8	6.1×10^8
		ϕ11	3.4×10^7	3.2×10^7
		ϕ147	1.4×10^4	7.1×10^9
		ϕ85	5.8×10^4	5.3×10^9
JP2015	SaPIbov1 Δ*stl*	ϕ80α	3.7×10^8	6.1×10^8
		ϕ11	3.7×10^7	1.1×10^8
		ϕ147	3.1×10^8	2.8×10^9
		ϕ85	2.4×10^7	8.1×10^8
JP2966	SaPI1	ϕ80α	2.4×10^8	4.1×10^8
		ϕ11	710	5.1×10^9
		ϕ147	940	2.8×10^{10}
		ϕ85	5.5×10^4	5.6×10^9
JP2967	SaPI1 Δ*stl*	ϕ80α	3.8×10^8	3.8×10^9
		ϕ11	2.5×10^8	1.3×10^9
		ϕ147	4.5×10^8	3.2×10^9
		ϕ85	1.7×10^8	3.3×10^9

a. The means of results from three independent experiments are presented. Variation was within ±5% in all cases.
b. Number of transductants × ml of infected culture, using RN4220 as recipient strain.
c. Number of phage plaques × ml of infected culture, using RN4220 as recipient strain.

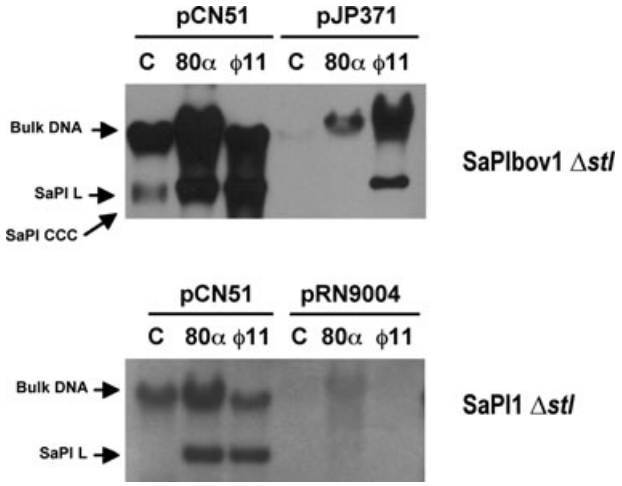

Fig. 3. Effect of *stl* complementation on SaPIbov1 ΔORF20 and SaPI1 ORF22::*tetM* replication. Complemented *stl* mutants of SaPIbov1 or SaPI1, derivatives of RN4220, were uninfected (C), 80α or ϕ11 infected. At 1 h after infection, 1 ml samples were collected and used to prepare standard minilysates, which were run on a 0.7% agarose gel overnight, then Southern blotted, probed for SaPI *ter* and visualized by ECL. pCN51, empty vector; pJP371, pCN51 expressing SaPIbov1 ORF20; pRN9004, pCN51 expressing SaPI1 ORF22.

phage (Fig. 3), greatly reduced the SPST frequency (Table 4), and restored normal growth and pigmentation. Interestingly, it also completely eliminated the SaPI-specific interference with phage growth (Table 4). These effects are almost certainly owing to overproduction of the cloned repressor.

Regulatory functions of stl

To define the regulatory function of Stl, we analysed the expression of *stl*, *str*, *xis* and *rep* (ORF13, essential for SaPI replication) in the presence of SaPIbov1 or SaPIbov1 Δ20. We used plasmids pJP446, pJP447, pJP448 and pJP449 (see Fig. 4A for a diagram), in which several different segments of the regulatory region have been cloned to pCN41, creating transcriptional fusions to β-lactamase. pJP446 contains the entire intergenic region, including *str–xis* fused to β-lactamase, plus the N-terminal end of *stl*. pJP447 contains the intergenic region, neither of the intact regulatory genes, and the N-terminal end of *str* fused to β-lactamase. pJP448 is similar to pJP447 except that β-lactamase is fused to the N-terminal end of *stl*. pJP449 contains the entire intergenic region, from *str* to *rep*, which is fused to β-lactamase, plus the N-terminal end of *stl*. These plasmids were introduced into RN4220 containing wt SaPIbov1 (JP45) or the *stl* mutant (Δ20; JP2015) (Table S1). As shown in Fig. 4B, expression of *str*, *xis* and *rep* were inhibited by the wt SaPIbov1, but not by the Δ20 mutant. In contrast, the expression of *stl* was inhibited by the SaPIbov1 Δ20 mutant. We conclude from these results that Stl is an autoinducer, and that the spontaneous excision and replication of SaPIbov1 seen with the *stl* mutant suggests that Stl is a general repressor of SaPIbov1 gene expression.

Autonomous replication

It is clear from the above results that SaPIbov1 and SaPI1 are capable of autonomous replication in the absence of any inducing phage and that this is inhibited by Stl. As noted above, the *pri–rep–ori* segment of SaPIbov1 can drive replication of a suicide plasmid in *S. aureus*. In this experiment, however, the *pri–rep–ori* unit was cloned behind an exogenous promoter. To analyse the effect of the native regulatory circuitry on this autonomous replication, we cloned to a suicide plasmid two PCR products, one containing SaPIbov1 ORFs 11–19, and the other ORFs 11–20 generating pJP382 and pJP381 respectively. Both of these subclones produced colonies in *S. aureus* on selective media, consistent with autonomous replication. In both cases, however, the colonies were very small and appeared only after several days of incubation. As we have found with the cloned *pri–rep–ori* unit (Ubeda *et al*., 2007b), these plasmids were extremely unstable, presumably owing to defects in segregation. As with the highly unstable *pri–rep–ori* plasmid (Ubeda *et al*., 2007b), they generated multimeric products that yielded monomeric plasmid DNA upon digestion with a singly cutting enzyme (data not shown). Although autonomous maintenance of the clone containing ORFs 11–19 (pJP382) was consistent with the *pri–rep–ori* plasmid results, maintenance of the clone containing ORFs 11–20 (pJP381) was unexpected as ORF20 encodes the SaPIbov1 repressor, Stl, which would have been expected to repress the SaPIbov1 replication module. This result is unexplained at present and is a subject of ongoing study.

Table 4. Effect of overexpression of the SaPI *ci*-like repressor in SaPIbov1 transfer.[a]

Donor strain	SaPI	Complemented with	Infected with	SaPI (titre)[b]	Phage titre[c]
JP2977	SaPIbov1 Δ*stl*	Empty vector	80α	1.4×10^8	3.1×10^8
JP3074	SaPIbov1 Δ*stl*	SaPIbov1 ORF20	80α	9.4×10^4	1.4×10^{12}

a. The means of results from three independent experiments are presented. Variation was within ±5% in all cases.
b. Number of transductants × ml of infected culture, using RN4220 as recipient strain.
c. Number of phage plaques × ml of infected culture, using RN4220 as recipient strain.

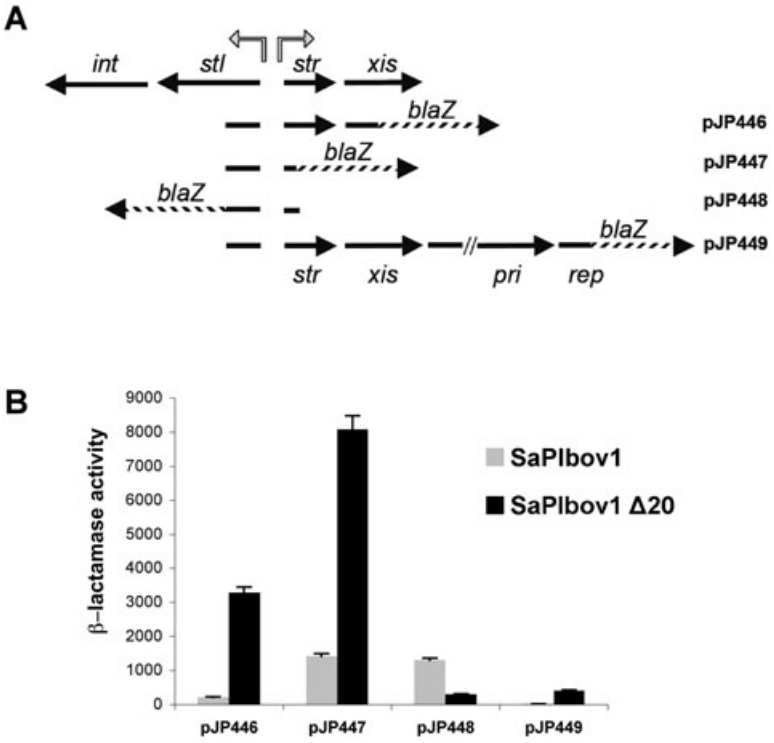

Fig. 4. Effect of SaPIbov1 regulatory genes on transcription.
A. Schematic representation of the different *blaZ* transcriptional fusions.
B. JP45 (RN4220 SaPIbov1) and JP2015 (RN4220 SaPIbov1 Δ*stI*) strains containing plasmids pJP446, pJP447, pJP448 or pJP449 (*blaZ* transcriptional fusions) were assayed for β-lactamase activity under standard conditions. Samples were normalized for total cell mass.

Finally, to investigate the generality of autonomous replication with SaPI subclones, we analysed one additional SaPI, SaPIn1 (Kuroda *et al*., 2001), by cloning the region of SaPIn1 corresponding to SaPIbov1 ORFs11–19 (SA1826 to SA1833) to the same suicide plasmid, generating plasmid pJP397. Slow-growing colonies were obtained, similar to those seen with the other self-replicating units, suggesting unstable autonomous replication.

ORF21 (int)

As an in-frame deletion mutation inactivating SaPIbov1 *int* (formerly referred to as '*sip*') caused a major reduction in cell growth and was extremely unstable, it could not be characterized. Perhaps a truncated Int protein is produced that is toxic. However, an inactivating insertion in SaPI1 *int* (P. Barry and R.P. Novick, unpubl. results) and an in-frame deletion in SaPIbov2 *int* (Maiques *et al*., 2007) were isolated and characterized. We find that SaPI1 and SaPIbov2 integrases are necessary for both phage-induced excision of the island and for replication and that the *int* mutants are comparable to the SaPIbov1 *xis* mutant as described above. Thus, a SaPI1 *int* mutant was unable to carry out any step in the ERP cycle: no SaPI-specific band was produced following phage induction; SaPI1-specific replication could not be demonstrated; and the transduction frequency was reduced by ~10^8-fold, with the residual transfer presumably representing CGT, as it was at about the same frequency as that by ϕ11, which does not induce SaPI1. This defect was complemented in the donor strain by the cloned *int*: 80α-specific induction of the SaPI1 ERP cycle was observed, with generation of the SaPI-specific band. However, transfer frequency was not increased, indicating that the integrase is required in the recipient as well as in the donor (P. Barry and R.P. Novick, unpublished results).

Discussion

In this paper, we have attempted to define the roles of the 17 open reading frames, largely conserved between the distantly related SaPIs, SaPI1 and SaPIbov1, that do not contribute to the manifest phenotype of the SaPI-carrying strains. We have constructed an in-frame deletion in each of these ORFs in SaPIbov1, and have insertionally inactivated several in SaPI1. These mutations have demonstrated clearly definable roles for nine of these genes in the SaPI ERP cycle, but not for eight others. These 'functionless' ORFs, 3, 6, 10, 11, 12, 16, 17 and 19 had no important phenotypes as revealed by in-frame deletions. As it is not known whether any of these are expressed or translated, though all have credible translation initiation signals, experiments are currently in progress to analyse their expression and translation. Further attempts to define possible functions will then be undertaken. Nevertheless, the studies reported here have enabled the for-

mulation of a detailed outline of the SaPI ERP cycle and it is unlikely that analysis of these 'functionless' ORFs will significantly modify this outline. The results presented here have amply confirmed and fleshed out our initial concept of the ERP cycle: under the control of certain phages, the SaPI genome is excised from its chromosomal site, replicates extensively as an autonomous unit, generating a multimeric form that is ultimately encapsidated into special small-headed, infectious, phage-like particles that are released upon phage-induced lysis and go on to infect other bacteria, where they integrate into the corresponding att_C site, thus perpetuating their existence and conferral of toxigenicity.

Using a mutational approach, we have defined three sets of genes that are responsible for the sequential unfolding of the SaPI ERP cycle – excision and re-integration, autonomous replication and packaging, and one master regulator that appears to control the entire cycle. The activities of some of these genes were, of course, readily predictable from sequence analysis and these predictions have been confirmed.

Integration/excision

Although it had been proven earlier that SaPI integrase homologues were required for integration (Ruzin *et al*., 2001; Ubeda *et al*., 2003; Maiques *et al*., 2007), no *xis* function had been defined, nor had the role of integrase in excision been clearly demonstrated: although the integrase of SaPIbov1 could catalyse (rare) spontaneous excision of the cloned SaPIbov1 *att–int* segment (Ubeda *et al*., 2003), the possibility that *int* alone could catalyse excision from the chromosome has not been tested and we report here that a separate gene appears to be required for excision. This gene (initially listed as ORF18) is homologous to typical phage *xis* genes. We have not found any gene similar to *xis* in SaPI1. However, the 80α *xis* gene does not seem sufficient for SaPI1, as an integrated plasmid containing only SaPI1 *int* and att_S cannot be induced to excise by the phage; however, this could simply mean that *int* is not expressed under these conditions. Experiments are in progress to investigate this possibility and to identify the putative *xis*-like gene in SaPI1. Excision is predictably required for the full ERP cycle; replication is apparently initiated *in situ* by mutants unable to excise, as is the case with lambda prophage; this replication extends into neighbouring chromosomal regions (Hirai and Fukasawa, 1976), and, in the case of int^- SaPI1 or xis^- SaPIbov1, eliminates SPST because replication is rather limited and because any encapsidation initiated at the *pac* site is predicted to extend into chromosomal DNA and would include only one of the SaPI-chromosome junctions, which would not enable integration. Also predictable is a requirement for integrase in the recipient; as wt SaPIs cannot replicate autonomously, failure to integrate would result in an abortive infection analogous to the abortive lysogeny seen with int^- phages.

Replication

A conserved SaPI gene (SaPIbov1 ORF13 or SaPI1 ORF15) usually annotated as 'helicase-like', is the replicon-specific initiator protein, Rep; we have shown elsewhere that the SaPI replication origin lies immediately 3′ to the *rep* gene (Ubeda *et al*., 2007b) and that the *rep–ori* segment plus an adjacent primase gene can drive replication of a suicide plasmid in *S. aureus*. The *rep–ori* complex is SaPI specific, as would be expected for any prokaryotic replicon. The *pri–rep–ori* complex is thus far the smallest SaPI unit that has been shown to be capable of autonomous replication. As described here, larger cloned segments are also functional, but seem to be even more unstable than the *pri–rep–ori* plasmid; however, we were surprised that the inclusion of ORF20 did not seem to inhibit autonomous replication. This unexpected finding is currently under study.

Packaging

The third set of ERP genes, SaPIbov1 ORFs 5–10 (corresponding to SaPI1 ORFs 3–8), is widely but not universally conserved among the SaPIs and is involved in packaging (encapsidation) (Ubeda *et al*., 2007a). The key gene in this set is *ter*, a homologue of the phage STS. Ter is absolutely required for SaPI encapsidation and we suggest that it acts as a subunit of the phage terminase, which it directs to recognize and cleave SaPI DNA at a SaPI-specific *pac* site, for insertion into the capsid. Three other genes in this set (SaPIbov1 ORFs 7, 8 and 9) are responsible for remodelling the phage capsomeres to fit the smaller SaPI genome. The similarity of this to the remodelling by coliphage P4 of the P2 capsid to fit its smaller genome (review: Lindqvist *et al*., 1993) is remarkable; whether the remodelling mechanisms are similar remains to be determined. Not all SaPIs encode such functions and, in one case, that of SaPIbov2, we have shown that SaPI DNA is encapsidated in normal sized phage heads (Maiques *et al*., 2007). This is especially relevant as SaPIbov2 is 27 kb in length and could not be accommodated by the small heads used for SaPI1 or SaPIbov1 and SaPIbov1 mutants defective in small capsid formation package their DNA very efficiently in full-sized phage heads. In retrospect, it is clear that encapsidation is rather non-specific – a fraction of the small SaPI heads contain phage DNA and a fraction of the normally sized phage heads contain SaPI DNA; the interaction of STS and *pac* site is clearly sufficient for encapsi-

dation into any available phage head; what gets packaged where is presumably a matter of competition; evidently the SaPI Ter is much better at packaging than the phage STS, at least in the few examples that have been studied. The presumptive evolutionary advantage for small-headed SaPI particles is that they cannot accommodate the entire phage genome and are thus a dead-end for the phage.

Regulation

It seems clear that Stl is a master-repressor of SaPIbov1 gene expression, and is also an autoinducer – autoinduction would aid in maintaining the SaPIbov1 genome in the off state. It is predicted that the primary target of SaPI induction is relief of repression by Stl. This could involve blockage of *stl* transcription, inactivation or titration of the protein, or both. The role of Str is less clear. Very probably, its promoter controls the major rightward transcription of the SaPI genome, which is activated by relief of Stl repression. It seems paradoxical, therefore that an in-frame deletion of the *str* gene had no significant phenotype. One possibility is that the inducing phage supplies this function. Further studies are clearly required to clarify this situation.

An overall view of the SaPI genome is that it has been carefully crafted by evolution to encode just those functions that ensure its ability to replicate autonomously and to parasitize the encapsidation system of an inducing phage. SaPIs do not waste their time and energy replicating autonomously in the absence of a phage that can provide a useful encapsidation system; the similarity of this strategy to that of bacterial plasmids is interesting – these contain, at a minimum, only a replication origin, a means of activating it – usually a Rep protein – and a negative regulatory mechanism for controlling their replication rates. Some have low copy numbers and these also encode partitioning mechanisms. All other functions are provided by the host cell, so that the plasmid is the ultimate parasite. In addition to this minimum, many plasmids also encode a mechanism of conjugation that enables their intercell transfer. However, this transfer involves only the plasmid; chromosomal genes are expressly excluded, though other plasmids may get a free ride; SaPIs are also very good at promoting their own transfer at the expense of the inducing phage, but they are not quite as good as the plasmid in promoting only its own transfer, or as a similar element, coliphage P4, which completely prevents the formation of P2 capsids (Lindqvist *et al*., 1993).

An interesting question is the role of SaPI-coded *xis* functions. For SaPIbov1, it is very likely that *xis* is responsible for spontaneous excision, though *int* is, of course, also required (Ubeda *et al*., 2003). It seems, however, that spontaneous excision is disadvantageous, as, if re-integration fails, the island is lost. SCCmec elements can also undergo spontaneous excision; these are also unable to replicate or to be transferred, so here also spontaneous excision would seem disadvantageous. In the end, therefore, it is clear that there is still much to be learned about these fascinating subcellular creatures.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains used in these studies are listed in Table S1. Bacteria were grown at 37°C overnight on TSA agar medium, supplemented with antibiotics as appropriate. Broth cultures were grown at 37°C in TSB broth with shaking (240 r.p.m.). Procedures for preparation and analysis of phage lysates, transduction and transformation in *S. aureus* were performed essentially as described (Novick, 1991; Lindsay *et al*., 1998).

Induction of prophages

Bacteria were grown in TSB broth to OD_{540} = 0.4 and induced by adding mitomycin C (2 μg 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). Procedures for preparation and analysis of phages lysates, lysogens and transduction in *S. aureus* were essentially performed as described (Novick, 1991).

DNA methods

General DNA manipulations were performed by standard procedures (Sambrook *et al*., 1989; Ausubel *et al*., 1990). Oligonucleotides used in this study are listed in Table S2. Labelling of the probes and DNA hybridization was performed according to the protocol supplied with the PCR-DIG DNA-labelling and chemiluminescent detection kit (Roche).

To produce the mutant strains we used plasmid pMAD (Arnaud *et al*., 2004), as previously described (Ubeda *et al*., 2005). Briefly, two separate PCR products with overlapping sequence including the targeted sequence were combined. The oligonucleotides pairs are listed in Table S2. A second PCR was performed with external primers to obtain a single fragment. Specifically, 1 μl of each of the first PCR was mixed with 10 pM of the outside primers and PCR amplified. The fusion products were purified and cloned in the pGEM-T easy vector (Promega). The fragment was then cloned into the appropriate sites of the shuttle plasmid pMAD, and the resulting plasmids were transformed into *S. aureus* JP45 (RN4220 SaPIbov1 *tst*::*tet*M) by electroporation and subsequently in RN27 or RN451 by transduction (Novick, 1991). pMAD contains a temperature-sensitive origin of replication and an erythromycin resistance gene. The plasmid was integrated into the chromosome through homologous recombination at non-

permissive temperature (43.5°C). From the 43.5°C plate, one to five colonies were picked into 10 ml of TSB and incubated for 24 h at 30°C. Tenfold serial dilutions of this culture in sterile TSB were plated on TSA containing X-gal (5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside; 150 μg ml^{-1}). White colonies, which no longer contained the pMAD plasmid, were tested to confirm the replacement by DNA sequencing. Primers were obtained from Invitrogen Life Technologies.

To generate the *sip::pAZ106* mutant (JP1767) a fragment from within the *sip* gene was amplified using oligonucleotides SaPIbov-114mB and SaPIbov-115cE (Table S2). Cleavage sites for BamHI or EcoRI were incorporated in the 5′ ends to facilitate directional cloning. The single PCR product was purified using standard procedures and cloned into pAZ106 (Kemp *et al*., 1991). This yielded the plasmid pJP364. This plasmid (10–50 μg DNA) was transformed into *S. aureus* JP45 by electroporation, and erythromycin-resistant recombinants were selected. As the plasmid cannot replicate in *S. aureus*, Em^r transformants can only occur if the plasmid integrates into the chromosome at the site of shared homology in *int*.

For construction of the SaPI1 ORF15::*tetM* and SaPI1 ORF22::*tetM* mutants, segments containing ORFs 15 or 22 and flanking regions were PCR amplified using the oligonucleotides p702, p703 and p706, p707 respectively. 5′ cleavage sites for KpnI or PstI were included for directional cloning. The resulting plasmids were used as templates for inverse PCR with primers p716, p717 for ORF15 and p720, p721 for ORF22. These primers, containing sites for XhoI or SpeI, hybridize with the inner edges of the DNA flanking the knockout targets. The resulting PCR products were digested with XhoI and SpeI and ligated to a PCR product containing the *tetM* gene, obtained with primers p710, p711 or p712, p713, which have 5′ XhoI or SpeI cleavage sites. The plasmids containing *tetM* flanked by fragments flanking ORF15 or ORF22 were transformed into strain RN10430 (RN4220 SaPI1). Because pUC19 cannot replicate in *S. aureus*, the TcR transformants contained the plasmid integrated into SaPI1 by homologous recombination. SaPI1-plasmid recombinants were transduced to strain RN4282 (wt SaPI1). Transductants were analysed by PCR to identify colonies in which the target gene had been replaced by *tet*M.

Plasmid construction

Plasmid constructs were prepared by cloning PCR products obtained from oligonucleotide primers as listed in Table S2. All clones were sequenced by the Institute core sequencing lab. Plasmids pJP446, pJP447, pJP448 and pJP449, carrying *xis*, *str*, *stl* and *rep*–β-lactamase (*blaZ*) transcriptional fusions, were constructed by cloning different PCR-amplified fragment containing the promoter regions of these genes into pCN41, using KpnI and BamHI sites (see oligonucleotides used in supplementary material, Table S2).

Plasmids pJP381, pJP382 and pJP397 were constructed by cloning the different PCR-amplified fragments in a suicide plasmid generated by PCR amplification of pCN33 (Charpentier *et al*., 2004) with oligonucleotides NY-15mB and NY-16cX (Table S2). Strain JP45 (for SaPIbov1) or N315 (for SaPIn1) were used as template.

Enzyme assays

β-Lactamase assays, using nitrocefin as substrate, were performed as described (Ji *et al*., 1997), using a Thermomax (Molecular Devices) microtitre plate reader. Cells were obtained in exponential phase. β-Lactamase units are defined as $(Vmax)/OD_{650}$.

Real-time quantitative PCR

Total *S. aureus* DNA was prepared as previously described and was used for a real-time quantitative PCR using the iCycler machine (Bio-Rad) and the LC-DNA Master SYBR Green I mix (Bio-Rad). The SaPIs DNA was amplified using primers Sapibov-77mE and Sapibov-68cS (Table S2). The *gyr*B DNA was amplified as an endogenous control using the primers gyr-L and gyr-U (Table S2). The level of SaPIbov1 DNA was normalized with respect to *gyr* DNA. To monitor the specificity, the final PCR products were analysed by melting curves and electrophoresis. In each experiment, all the reactions were performed in triplicate. The relative levels in the different experiments were determined by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

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References

Arnaud, M., Chastanet, A., and Debarbouille, M. (2004) New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, Gram-positive bacteria. *Appl Environ Microbiol* **70:** 6887–6891.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1990) *Current Protocols in Molecular Biology*. New York: John Wiley & Sons.

Charpentier, E., Anton, A.I., Barry, P., Alfonso, B., Fang, Y., and Novick, R.P. (2004) Novel cassette-based shuttle vector system for Gram-positive bacteria. *Appl Environ Microbiol* **70:** 6076–6085.

Fitzgerald, J.R., Monday, S.R., Foster, T.J., Bohach, G.A., Hartigan, P.J., Meaney, W.J., and Smyth, C.J. (2001) Characterization of a putative pathogenicity island from bovine *Staphylococcus aureus* encoding multiple superantigens. *J Bacteriol* **183:** 63–70.

Herbert, S., Barry, P., and Novick, R.P. (2001) Subinhibitory clindamycin differentially inhibits transcription of exoprotein genes in *Staphylococcus aureus*. *Infect Immun* **69:** 2996–3003.

Hirai, K., and Fukasawa, T. (1976) Regional replication of the

bacterial chromosome induced by derepression of prophage lambda. II. Direction and origin. *Mol Gen Genet* **147:** 71–78.
Ji, G., Beavis, R., and Novick, R.P. (1997) Bacterial interference caused by autoinducing peptide variants. *Science* **276:** 2027–2030.
Kemp, E.H., Sammons, R.L., Moir, A., Sun, D., and Setlow, P. (1991) Analysis of transcriptional control of the *gerD* spore germination gene of *Bacillus subtilis* 168. *J Bacteriol* **173:** 4646–4652.
Kullik, I., and Giachino, P. (1997) The alternative sigma factor σ^B in *Staphylococcus aureus*: regulation of the *sigB* operon in response to growth phase and heat shock. *Arch Microbiol* **167:** 151–159.
Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa, H., Kobayashi, I., *et al.* (2001) Whole genome sequencing of meticillin-resistant *Staphylococcus aureus*. *Lancet* **357:** 1225–1240.
Lindqvist, B.H., Deho, G., and Calendar, R. (1993) Mechanisms of genome propagation and helper exploitation by satellite phage P4. *Microbiol Rev* **57:** 683–702.
Lindsay, J.A., Ruzin, A., Ross, H.F., Kurepina, N., and Novick, R.P. (1998) The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*. *Mol Microbiol* **29:** 527–543.
Livak, K.J., and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25:** 402–408.
Maiques, E., Ubeda, C., Tormo, M.A., Ferrer, M.D., Lasa, I., Novick, R.P., and Penades, J.R. (2007) Role of staphylococcal phage and SaPI integrase in intra- and interspecies SaPI transfer. *J Bacteriol* **189:** 5608–5616.
Novick, R.P. (1991) Genetic systems in staphylococci. *Methods Enzymol* **204:** 587–636.
Novick, R.P., and Subedi, A. (2007) The SaPIs: mobile pathogenicity islands of *Staphylococcus*. *Chem Immunol Allergy* **93:** 42–57.
Ruzin, A., Lindsay, J., and Novick, R.P. (2001) Molecular genetics of SaPI1 – a mobile pathogenicity island in *Staphylococcus aureus*. *Mol Microbiol* **41:** 365–377.
Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. New York: Cold Spring Harbor.
Subedi, A., Ubeda, C., Adhikari, R.P., Penadés, J.R., and Novick, R.P. (2007) Sequence analysis reveals genetic exchanges and intraspecific spread of SaPI2, a pathogenicity island involved in menstrual toxic shock. *Microbiology* **153:** 3235–3245.
Ubeda, C., Tormo, M.A., Cucarella, C., Trotonda, P., Foster, T.J., Lasa, I., and Penades, J.R. (2003) Sip, an integrase protein with excision, circularization and integration activities, defines a new family of mobile *Staphylococcus aureus* pathogenicity islands. *Mol Microbiol* **49:** 193–210.
Ubeda, C., Maiques, E., Knecht, E., Lasa, I., Novick, R.P., and Penades, J.R. (2005) Antibiotic-induced SOS response promotes horizontal dissemination of pathogenicity island-encoded virulence factors in staphylococci. *Mol Microbiol* **56:** 836–844.
Ubeda, C., Maiques, E., Tormo, M., Campoy, S., Lasa, I., Barbe, J., *et al.* (2007a) SaPI operon I is required for SaPI packaging and is controlled by LexA. *Mol Microbiol* **65:** 41–50.
Ubeda, C., Barry, P., Penades, J.R., and Novick, R.P. (2007b) A pathogenicity island replicon in Staphylococcus aureus replicates as an unstable plasmid. *Proc Natl Acad Sci USA* **104:** 14182–14188.

Supplementary material

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