

## Role of Staphylococcal Phage and SaPI Integrase in Intra- and Interspecies SaPI Transfer<sup>∇</sup>

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**SaPIbov2 is a member of the SaPI family of staphylococcal pathogenicity islands and is very closely related to SaPIbov1. Typically, certain temperate phages can induce excision and replication of one or more of these islands and can package them into special small phage-like particles commensurate with their genome sizes (referred to as the excision-replication-packaging [ERP] cycle). We have studied the phage-SaPI interaction in some depth using SaPIbov2, with special reference to the role of its integrase. We demonstrate here that SaPIbov2 can be induced to replicate by different staphylococcal phages. After replication, SaPIbov2 is efficiently encapsidated and transferred to recipient organisms, including different non-*Staphylococcus aureus* staphylococci, where it integrates at a SaPI-specific attachment site, *att<sub>C</sub>*, by means of a self-coded integrase (Int). Phages that cannot induce the SaPIbov2 ERP cycle can transfer the island by *recA*-dependent classical generalized transduction and can also transfer it by a novel mechanism that requires the expression of SaPIbov2 *int* in the recipient but not in the donor. It is suggested that this mechanism involves the encapsidation of standard transducing fragments containing the intact island followed by *int*-mediated excision, circularization, and integration in the recipient.**

Pathogenicity islands (PTIs), a subset of horizontally transferred genetic elements now known generically as genomic islands, are largely responsible for the difference in overall pathogenic potential between otherwise closely related enteric organisms. In gram-positive bacteria, the role of PTIs seems to be somewhat different; pathogenesis is largely due to constant chromosomal genes, and PTIs are more frequently involved in diseases caused by single protein toxins. With this perspective, we considered a remarkable family of highly mobile PTIs in *Staphylococcus aureus* that are induced to excise and replicate by certain resident prophages, are encapsidated into small-headed phage-like particles, and are transferred at frequencies commensurate with the plaque-forming titer of the phage (17). Late in infection, a SaPI-specific DNA band appears in an agarose gel containing whole-cell lysates and represents monomeric SaPI DNA that is released from the small-headed particles (11). This process is referred to as the SaPI excision-replication-packaging (ERP) cycle, and the high-frequency SaPI transfer is referred to as SaPI-specific transfer (SPST) to distinguish it from classical generalized transduction (CGT) (see below).

The SaPIs have a highly conserved genetic organization which parallels that of bacteriophages and clearly distinguishes them from all other horizontally acquired genomic islands

(16). The SaPI1-encoded (17) and SaPIbov2-encoded (25) integrases are required for both excision and integration of the corresponding elements, and it is assumed that the same is true for the other SaPIs. The known SaPIs are mostly 14 to 17 kb long, and many carry superantigen or other virulence genes. An exception is SaPIbov2, carried by bovine mastitis isolates, which is 27 kb long and carries a transposon (6). This transposon, which is 12 kb long, contains *bap*, the gene for a ~240-kDa adhesin that is involved in biofilm formation (5) and has a significant role in bovine mastitis (6). *bap* is common not only in bovine mastitis *S. aureus* strains but also in various non-*S. aureus* staphylococci of veterinary origin (22); although it does not seem to be part of any specific mobile element, it has almost certainly been horizontally transferred. Although the functionality of the SaPIbov2 transposon has not been demonstrated, it is likely to have been responsible for the insertion of *bap* into SaPIbov2.

Although the overall genetic organization of the SaPIs is largely conserved, there are significant differences in the details, which has led to the development of several individual SaPIs as prototypes for molecular genetic analyses. In addition to SaPII (11, 17), we have developed SaPIbov1 (7, 13, 23), the prototype for bovine strains, and SaPI2 (11, 21), the prototype for human toxic shock syndrome, and in this report we describe analysis of a fourth SaPI, SaPIbov2, that has previously been sequenced (25). SaPIbov2 is closely related to SaPIbov1 (7) and uses the same *att* site, and both are unusual among the SaPIs described thus far in that they show spontaneous integration and excision, similar to the SCCmec elements (9), which leads to a low level of hereditary instability. SaPIbov2 is

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included in our set of prototypes, although the closely related SaPIbov1 is better characterized (23), both because we have succeeded in obtaining a stable mutation of SaPIbov2 *int* but not of SaPIbov1 *int* and because SaPIbov2 lacks one of the genes that we have recently found to be required for SaPI capsid morphogenesis (24). Our present focus is on the role of SaPIbov2 *int* in the ERP cycle and on phage specificity in SaPIbov2 induction and transfer. Our results, obtained using SaPIbov2 as a model, suggest three possible modes of SaPI transfer: (i) typical high-frequency SPST accompanying induction of the ERP cycle; (ii) specific *recA*-independent transfer by phages that do not induce replication, which requires a functional SaPIbov2 *int* gene in the recipient strain but not in the donor; and (iii) standard, *recA*-dependent generalized transduction. Additionally, we found that SaPIbov2 can be transferred by several different phages to a variety of non-*S. aureus* staphylococci.

#### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Bacterial strains used in these studies are listed in Table 1. Bacteria were grown at 37°C overnight on tryptic soy agar supplemented with antibiotics for plasmid maintenance. Broth cultures were grown aerobically (with shaking at 240 rpm) at 37°C in tryptic soy broth (TSB) without antibiotics.

**DNA methods.** General DNA manipulations were performed by following standard procedures (2, 18). Plasmid DNAs from *Escherichia coli* and staphylococci were purified with a Genelute plasmid miniprep kit (Sigma) used according to the manufacturer's protocol, except that the staphylococci were lysed by lysostaphin (12.5 µg/ml; Sigma) at 37°C for 1 h before plasmid purification. Plasmids were introduced into the staphylococcal strains by electroporation using a previously described method (5). Restriction enzymes were purchased from Roche and were used according to the manufacturer's instructions. Oligonucleotides were obtained from Invitrogen.

Staphylococcal chromosomal DNA was extracted using a Genelute bacterial genomic DNA kit (Sigma) according to the manufacturer's protocol, except that the bacterial cells were lysed by lysostaphin as described above.

For Southern blot hybridization, agarose gels containing electrophoretically separated DNA fragments were blotted onto nylon membranes (Hybond-N 0.45-µm-pore-size filters; Amersham Life Science) using standard methods (2, 18). Oligonucleotides SaPIbov-6c (5'-TAACGGCAAACAAGCGCG-3') and SaPIbov-65mBg (5'-GGAAGATCTCTATCTGTAAATAACTTATGG-3') were used to generate the specific SaPIbov2 probe. Labeling of the probes and DNA hybridization were performed according to the protocol supplied with a PCR-DIG DNA-labeling and chemiluminescence detection kit (Roche).

A SaPIbov2 derivative with *tetM* inserted into *bap* was constructed by PCR amplification of the 5' and 3' parts of the *bap* gene, including the promoter region. Oligonucleotides Bap-25mE (5'-CCGGAATTCGTCATATTAATTAACCATTCGCTAATC-3') and Bap-26cP (5'-AACTGTCAGTATAGCTAA CCACTAATATATCATGTC-3') were used for the 5' region of the gene, and oligonucleotides Bap-27mB (5'-CGCGGATCCTTGACGAGGTTGGTAATG GCAC-3') and Bap-28cX (5'-CTAGTCTAGAGATACATCACTTGTGTTGGC GTC-3') were used for the 3' region. These primers contained 5' EcoRI, PstI, BamHI, and XbaI restriction sites (underlined). The PCR products were inserted into either side of the *tetM* gene of plasmid pRN6680 (20). The resulting plasmid was cut at native EcoRI and HindIII sites internal to the 5' and 3' ends of the cloned *bap* fragments and ligated into the multiple cloning site of the temperature-sensitive plasmid vector pBT2 (3), generating pJP188.

Plasmid pJP188 was introduced by electroporation into *S. aureus* strain RN4220 (5). Once in strain RN4220, the plasmid was transduced into strain c104 using phage 80α. Allele replacement was carried out as described previously (15). The temperature-sensitive phenotype of the plasmid-facilitated integration by homologous recombination, and a double-crossover event was detected by plating on suitable antibiotics, through which a stable mutant was obtained. Bap loss was confirmed by loss of biofilm formation by Western blot (5) and Southern blot analyses.

A SaPIbov2 *int* mutant was constructed by combining two separate PCR products with overlapping sequences including the targeted sequence. The oligonucleotide pairs used were Sip-13mB (5'-CGCGGATCCCTAAGCATAAAAAGAGACTAA

TABLE 1. Strains used

Strain	Description	Reference
AK1	8325-4 <i>aur::ermB</i>	8
c104	Clinical isolate, SaPIbov2 <sup>+</sup>	25
JP1522	<i>S. xyloso</i> C482 (SaPIbov2 <i>bap::tetM</i> )	This study
JP1642	RN782 (SaPIbov2 <i>bap::tetM</i> )	This study
JP2025	c104 (SaPIbov2 <i>bap::tetM</i> )	This study
JP2130	RN27 (SaPIbov2 <i>bap::tetM</i> )	This study
JP2131	RN451 (SaPIbov2 <i>bap::tetM</i> )	This study
JP2310	RN4220 lysogenic for φ69	This study
JP2315	RN4220 lysogenic for φ80α	This study
JP2343	RN450 lysogenic for φ55	This study
JP2398	RN4220 lysogenic for φ85	This study
JP2487	RN27 (SaPIbov2 <i>bap::tetM Δint</i> )	This study
JP2488	RN451 (SaPIbov2 <i>bap::tetM Δint</i> )	This study
JP2489	RN782 (SaPIbov2 <i>bap::tetM Δint</i> )	This study
JP2506	PS 53 (SaPIbov2 <i>bap::tetM</i> )	This study
JP2509	PS 77 (SaPIbov2 <i>bap::tetM</i> )	This study
JP2517	PS X2 (SaPIbov2 <i>bap::tetM</i> )	This study
JP2521	JP2310 (SaPIbov2 <i>bap::tetM</i> )	This study
JP2522	JP2310 (SaPIbov2 <i>bap::tetM Δint</i> )	This study
JP2523	JP2315 (SaPIbov2 <i>bap::tetM</i> )	This study
JP2524	JP2315 (SaPIbov2 <i>bap::tetM Δint</i> )	This study
JP2525	JP2343 (SaPIbov2 <i>bap::tetM</i> )	This study
JP2526	JP2343 (SaPIbov2 <i>bap::tetM Δint</i> )	This study
JP2527	JP2398 (SaPIbov2 <i>bap::tetM</i> )	This study
JP2528	JP2398 (SaPIbov2 <i>bap::tetM Δint</i> )	This study
JP2557	<i>S. epidermidis</i> CH64 (SaPIbov2 <i>bap::tetM</i> )	This study
JP2558	<i>S. epidermidis</i> CH83 (SaPIbov2 <i>bap::tetM</i> )	This study
JP2632	<i>S. epidermidis</i> CH829 (SaPIbov2 <i>bap::tetM</i> )	This study
JP2633	<i>S. epidermidis</i> CH830 (SaPIbov2 <i>bap::tetM</i> )	This study
JP2979	AK1 (SaPIbov2 <i>bap::tetM</i> )	This study
JP2980	AK1 (SaPIbov2 <i>bap::tetM Δint</i> )	This study
JP2997	JP2979 lysogenic for 80α	This study
JP2998	JP2979 lysogenic for φ11	This study
JP2999	JP2979 lysogenic for φ147	This study
JP3000	JP2980 lysogenic for 80α	This study
JP3001	JP2980 lysogenic for φ11	This study
JP3002	JP2980 lysogenic for φ147	This study
JP3245	JP2487(pCN51)	This study
JP3246	JP2487(pJP433)	This study
JP3247	JP2488(pCN51)	This study
JP3248	JP2488(pJP433)	This study
JP3249	JP2489(pCN51)	This study
JP3250	JP2489(pJP433)	This study
PS 53	Propagating strain for typing phage 53	10
PS 77	Propagating strain for typing phage 77	10
PS X2	Propagating strain for typing phage X2	10
RN27	RN450 lysogenic for 80α, φ13	14
RN4220	Restriction-defective derivative of RN450	14
RN450	NCTC8325 cured of φ11, φ12, and φ13	14
RN451	RN450 lysogenic for φ11	14
RN782	RN450 lysogenic for φ147	19
RN981	<i>recA</i> mutant of RN450	26

C)/Sip-14c (5'-CTCTCTTTCTGTTTAAAGCC-3') and Sip-15m (5'-GGCTTTA AACAGAAAGAGAGCATTGTTCTTTACTTTTGGAG-3')/IPR-2c (5'-TTGG TGGATTACCAGAAGACATGG-3'). Sip-14c and Sip-15m were complementary to enable the products of the first PCR to anneal. A second PCR was performed with primers Sip-13mB and Ipr-2c to obtain a single fragment. Specifically, 1 µl of each of the first PCR mixtures 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 BamHI/EcoRI sites of the shuttle plasmid pMAD (1), and the resulting plasmid, pJP378, was transformed into *S. aureus* RN4220 SaPIbov1 positive by electroporation. pMAD has a temperature-sensitive origin of replication and an erythromycin resistance gene. The plasmid was integrated into the chromosome through homologous recombination at the nonpermissive 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 tryptic soy agar containing X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) (150  $\mu$ g/ml). White colonies, which did not contain the pMAD plasmid, were tested to confirm replacement by DNA sequencing. Primers were obtained from Invitrogen Life Technologies.

An *int* clone was constructed by amplifying the *int* gene from *S. aureus* c104 with high-fidelity thermophilic DNA polymerase (Dynazyme Ext; Finnzymes) and with primers sip-16mB (5'-CGGGATCCCAATCCAATCAAACGCATGC G-3') and sip-17cE (5'-CGGAATTCGTCTTGAATACGTTTTGAATACG-3'). The PCR product was cloned into the BamHI/EcoRI sites of pCN51 (4), and the resulting plasmid, pJP433, was transformed by electroporation into *S. aureus* RN4220. Phage 80 $\alpha$  was used to transduce pJP433 from RN4220 to other *S. aureus* strains (15).

SaPIbov2 excision and circularization were analyzed using primers Ipl-1m and Ipr-2c (excision) and primers Ipl-16cB and Ipr-28m (circularization), as previously described (25).

**Induction of prophages.** Bacteria were grown in TSB to an optical density at 540 nm of 0.4 and induced by adding mitomycin C (MC) (2  $\mu$ g/ml). Cultures were grown at 32°C with slow shaking (80 rpm). Lysis usually occurred within 3 h. Samples were removed at various time points after phage induction, and standard sodium dodecyl sulfate minilyates were prepared and separated on 0.7% agarose gels, as previously described (11).

Procedures for preparation and analysis of phages lysates, lysogens, and transduction in *S. aureus* were performed essentially as previously described (15). Experiments were performed at least five times. Similar results were obtained in all cases.

**Real-time quantitative PCR.** Total *S. aureus* DNA was prepared as described above and analyzed by real-time quantitative PCR using an iCycler machine (Bio-Rad) and the LC-DNA master SYBR green I mixture (Bio-Rad). The SaPIbov2 DNA was amplified using primers bap-6m and bap-7c (5). The *gyrB* DNA was amplified as an endogenous control using primers *gyr-L* (5'-CACCA DTGTAACACACAGATA-3') and *gyr-U* (5'-TTATGGTGCTGGGCAAATA CA-3'). The level of SaPIbov2 DNA was normalized with respect to *gyr* DNA. Specificity was confirmed by determining melting curves and by electrophoresis of the final PCR products. In each experiment, all the reactions were performed in triplicate. The relative DNA levels in the different experiments were determined by using the  $2^{-\Delta\Delta Ct}$  method (12).

## RESULTS

**Phages inducing SaPIbov2 replication and transfer.** In order to enable genetic manipulation, we have introduced selective markers into SaPI genomes. For this study, we introduced by standard allelic replacement the *tetM* marker into the *bap* gene of SaPIbov2 in strain c104, resulting in strain JP2025. Note that *bap* is within a transposon (25) for which we have been unable to demonstrate activity and therefore suggest that it is probably defective and that the inserted *tetM* gene can be safely used to monitor SaPIbov2 activity. Note that this transposon is the only region of SaPIbov2 that is certainly not involved in SaPI biology, since we have recently demonstrated for SaPIbov1 that the SaPI homologous region is involved in replication and/or transfer (unpublished results).

An important question for understanding SaPI mobility is the relationship between a temperate phage and a SaPI that it induces. This question has several parts: (i) how commonly is a SaPI induced by an endogenous prophage in its native host strain; (ii) how common, in general, are phages that can induce at least one SaPI; (iii) what are the specific mechanisms of SaPI transfer; and (iv) what is the specific phage gene(s) that is involved in induction of the SaPI ERP cycle? In this study, we address primarily the second and third questions. It is already known that phage 80 $\alpha$ , which is very closely related to phage 53 of the international typing set (A. Matthews and R. P. Novick, unpublished data) can induce several different SaPIs, including SaPI1, SaPI2, and SaPIbov1, whereas  $\phi$ 11 can induce SaPIbov1 but neither of the other two SaPIs (11, 23).

**Induction by 80 $\alpha$ .** In the first part of this study, we surveyed several temperate staphylococcal phages for the ability to induce SaPIbov2, using tetracycline resistance (Tc<sup>r</sup>) as a marker, starting with 80 $\alpha$ . To do this, strain JP2025 was infected with 80 $\alpha$ , SaPIbov2::*tetM* was transduced to RN27, an 80 $\alpha$  lysogen, and the resulting strain, JP2130, was induced with MC and tested for induction of the SaPIbov2 ERP cycle. Although the typical SaPI-specific band was not seen on a stained agarose gel containing cellular DNA isolated 90 min after MC induction, a Southern blot of this gel, obtained using a *bap*-specific probe, revealed dramatic amplification of the SaPIbov2 signal comigrating with the bulk (sheared chromosomal and phage) DNA (Fig. 1). Similar results were obtained using an *int*-specific probe (not shown). This amplification was confirmed using real-time quantitative PCR to determine the number of SaPIbov2 copies present in the bacteria after induction. In one representative experiment, after 90 min of exposure to MC the relative increase in the amount of DNA for wild-type strain JP2130 was 10.2-fold, whereas the relative increase for  $\Delta$ *int* strain JP2487 was 6-fold. The resulting lysate showed very-high-frequency SPST, with 5.7 transductants/PFU (Table 2); these results show that SaPIbov2 is strongly induced by 80 $\alpha$ . SaPIbov2 does not give rise to the typical SaPI band following induction because it lacks a gene (corresponding to SaPIbov1 *orf8*) required for the formation of specific small SaPI particles (24). Note that SaPIbov1 with a mutation in *orf8* very efficiently packages SaPIbov1 DNA in normal-size phage particles (24), and we assume that the same is true for SaPIbov2. Since RN27 is lysogenic for  $\phi$ 13 as well as for 80 $\alpha$ , we repeated the induction experiment with JP2523 (RN4220 80 $\alpha$ ), which contains no second prophage, and obtained similar results (Fig. 1 and Table 2), except that RN27 seemed to show a greater level of replicating SaPIbov2 DNA and a higher transduction frequency than the RN4220 derivative, possibly owing to the presence of  $\phi$ 13 in the former.

**Other phages.** The data on phage specificity of SaPI induction are rather limited. In particular, there is very little information on the prevalence of SaPI-mobilizing prophages among uncharacterized clinical strains. To begin an evaluation of the general prevalence of phages inducing SaPIbov2, we examined several of the phages from the international typing set, all of which have recently been sequenced (10), plus several of the typing phage propagating strains, which contain uncharacterized prophages and possibly SaPIs. We constructed lysogens of RN4220 or RN450 and then introduced SaPIbov2::*tetM* by transduction with 80 $\alpha$ . All of these derivative strains were tested for lysogeny with 80 $\alpha$  and found to be negative, and they were then tested with MC for the induction of SaPIbov2 replication and transfer. SaPIbov2 replication was tested by preparing whole-cell lysates of the lysogens and propagating strains before and 90 min after MC induction. These lysates were separated on agarose and Southern blotted with an SaPIbov2-specific probe. We interpret the results, shown in Fig. 1, as follows. If there was no clear difference in intensity between the two samples, we concluded that SaPIbov2 replication was not significantly induced by the resident prophage. If the induced sample showed greater intensity than the uninduced sample, we concluded that SaPIbov2 replication was induced and we determined the apparent degree of induction as shown

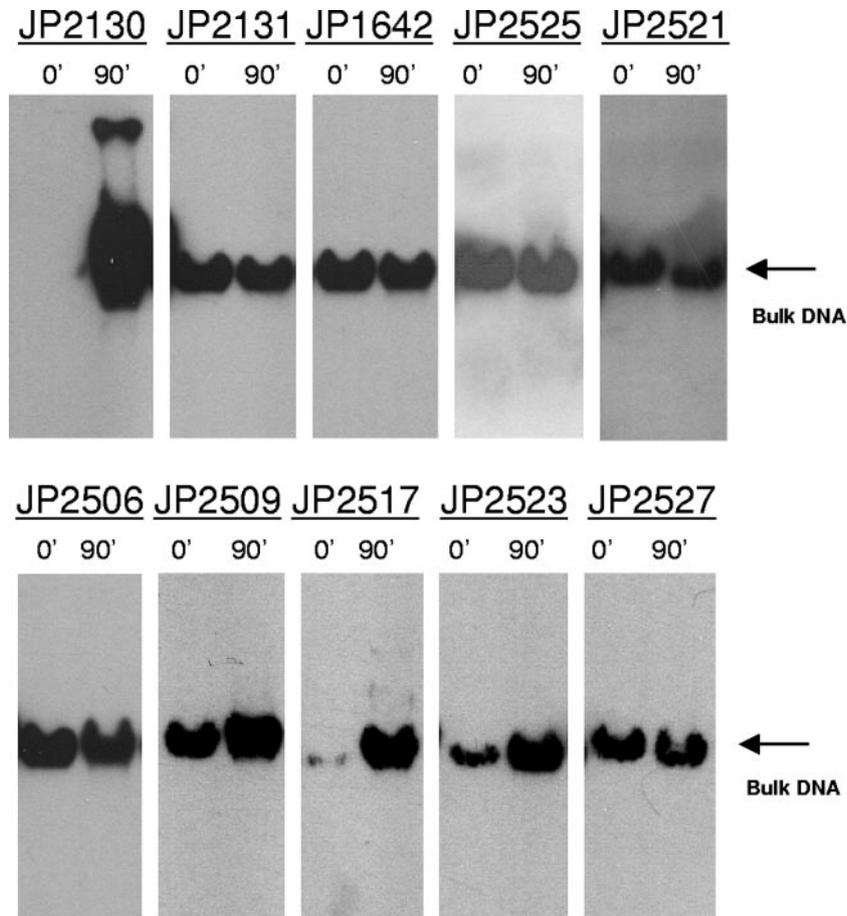


FIG. 1. Induction of SaPIbov2 replication by MC exposure. Bacterial cultures were exposed to MC and then incubated in broth at 32°C. Samples were removed at the indicated time points and used to prepare minilysates. Lysates were separated by agarose gel electrophoresis and transferred. The Southern blot hybridization pattern of the samples analyzed, obtained using a SaPIbov2-specific probe, is shown.

in Table 2. For most of the SaPIbov2-containing lysogens, SaPIbov2 replication was undetectable by this method.

Since all phages that have been found to promote SPST are known to be generalized transducing phages (14), we began with the assumption that SaPIbov2 transfer could occur either by SPST or by CGT and that the transfer frequencies would reveal clear differentiation. Instead, as shown in Table 2, there was a wide range of transfer frequencies, and all of the phages that did not induce SaPIbov2 replication transferred the SaPI with frequencies of  $>10^{-6}$ , which is higher than the frequencies usually seen with classical CGT (generally  $<10^{-7}$ ). We therefore considered the possibility that in strains where SaPIbov2 replication was not induced, the island was transferred by a mechanism distinct from CGT. To address this possibility, we analyzed the SaPIbov2-phage combinations for the effects of *recA* (required for CGT) and SaPIbov2 integrase (required for SPST).

**Effect of *recA*.** To determine whether these results simply represented higher-than-usual frequencies of CGT, we tested for the effects of *recA* by repeating the transduction experiments with a *recA* mutant strain, RN981, as a recipient. As shown in Table 2, there was a modest reduction in SPST to *recA* mutant strains compared to SPST to *recA*<sup>+</sup> lysates. Nevertheless, apparently significant *recA*-independent transfer was

observed with all of the lysates. The frequencies were then compared with the transduction frequencies for a marker known to require *recA*, an *ermB* insert in the aureolysin (*aur*) gene. The transduction of this marker to the *recA* mutant recipient was below the level of detection ( $<10^{-9}$  transductants/PFU) (Table 3). Even with  $\phi 11$  (JP2131), however, which does not induce SaPIbov2 replication, SaPIbov2 was readily transferred to *recA*, with a frequency between 60- and 300-fold lower than the frequency of transfer to *recA*<sup>+</sup> (Tables 2 and 3), suggesting that pure CGT is not seen in this system.

**Effect of integrase.** As expected, and consistent with the SaPI1 results (P. Barry and R. P. Novick, submitted for publication), an integrase mutation ( $\Delta int$ ) caused a  $10^3$ - to  $10^6$ -fold reduction in SaPIbov2 transfer to the *recA*<sup>+</sup> recipient for each of the phages tested (Table 2), including the intermediate frequency nonreplicative transfer, as well as standard SPST. Also as expected, the  $\Delta int$  mutation blocked excision and circularization, either spontaneously or following MC induction of a lysogen, as revealed by a PCR test for the circular form of SaPIbov2 (Fig. 2). Remarkably, the  $\Delta int$  mutant showed only a modest decrease in replication following MC induction, as revealed by real-time quantitative PCR (see above), suggesting replication in situ.

As also shown in Table 2, transfer of SaPIbov2  $\Delta int$  to the

TABLE 2. Transduction frequencies for MC-induced SaPIbov2-containing lysogens<sup>a</sup>

Donor strain	SaPIbov2 derivative	Prophage <sup>b</sup>	SaPI replication	Phage titer <sup>c</sup>	SaPI transfer titer (Tc <sup>r</sup> transductants/ml) <sup>d</sup>		SaPI transfer frequency (Tc <sup>r</sup> transductants/PFU) <sup>e</sup>		<i>recA/recA<sup>+</sup></i> ratio
					RN4420	RN981	RN4420	RN981	
JP2130	Wild type	80α, φ13	+++	2.1 × 10 <sup>8</sup>	1.2 × 10 <sup>9</sup>	1.0 × 10 <sup>8</sup>	5.7	0.47	0.08
JP2487	Δ <i>int</i>	80α, φ13	++	1.6 × 10 <sup>8</sup>	2.4 × 10 <sup>3</sup>	<1	1.5 × 10 <sup>-5</sup>	<6.2 × 10 <sup>-9</sup>	
JP2131	Wild type	φ11	-	4.6 × 10 <sup>9</sup>	9.4 × 10 <sup>3</sup>	30	2.0 × 10 <sup>-6</sup>	6.5 × 10 <sup>-9</sup>	0.003
JP2488	Δ <i>int</i>	φ11		8.0 × 10 <sup>9</sup>	20	<1	2.5 × 10 <sup>-9</sup>	<1.2 × 10 <sup>-10</sup>	
JP1642	Wild type	φ147	-	1.4 × 10 <sup>9</sup>	3.0 × 10 <sup>4</sup>	7.0 × 10 <sup>3</sup>	2.1 × 10 <sup>-5</sup>	5.0 × 10 <sup>-6</sup>	0.24
JP2489	Δ <i>int</i>	φ147		1.3 × 10 <sup>9</sup>	740	<1	5.7 × 10 <sup>-7</sup>	<7.7 × 10 <sup>-10</sup>	
JP2525	Wild type	φ55	-	7.1 × 10 <sup>8</sup>	1.9 × 10 <sup>3</sup>	30	2.6 × 10 <sup>-6</sup>	4.2 × 10 <sup>-8</sup>	0.016
JP2526	Δ <i>int</i>	φ55		5.5 × 10 <sup>8</sup>	<1	<1	<1.8 × 10 <sup>-9</sup>	<1.8 × 10 <sup>-9</sup>	
JP2521	Wild type	φ69	-	6.3 × 10 <sup>7</sup>	7.0 × 10 <sup>4</sup>	3.9 × 10 <sup>3</sup>	1.1 × 10 <sup>-3</sup>	6.2 × 10 <sup>-5</sup>	0.056
JP2522	Δ <i>int</i>	φ69		8.8 × 10 <sup>6</sup>	20	<1	2.2 × 10 <sup>-6</sup>	<1.1 × 10 <sup>-7</sup>	
JP2523	Wild type	80α	+++	2.4 × 10 <sup>8</sup>	9.0 × 10 <sup>7</sup>	1.2 × 10 <sup>7</sup>	0.37	0.05	0.14
JP2524	Δ <i>int</i>	80α		1.2 × 10 <sup>8</sup>	3.5 × 10 <sup>4</sup>	<1	2.9 × 10 <sup>-4</sup>	<8.3 × 10 <sup>-9</sup>	
JP2527	Wild type	φ85	-	4.8 × 10 <sup>9</sup>	1.8 × 10 <sup>3</sup>	50	3.7 × 10 <sup>-7</sup>	1.0 × 10 <sup>-8</sup>	0.027
JP2528	Δ <i>int</i>	φ85		6.4 × 10 <sup>9</sup>	<1	<1	<1.6 × 10 <sup>-10</sup>	<1.6 × 10 <sup>-10</sup>	
JP2506	Wild type	UC	-	9.0 × 10 <sup>9</sup>	130	<1	1.4 × 10 <sup>-8</sup>	<1.1 × 10 <sup>-10</sup>	<0.0077
JP2509	Wild type	UC	+	5.7 × 10 <sup>6</sup>	1.2 × 10 <sup>3</sup>	80	2.1 × 10 <sup>-4</sup>	1.4 × 10 <sup>-5</sup>	0.007
JP2517	Wild type	UC	+++	1.0 × 10 <sup>10</sup>	8.2 × 10 <sup>5</sup>	2.6 × 10 <sup>5</sup>	8.2 × 10 <sup>-5</sup>	2.6 × 10 <sup>-5</sup>	0.32

<sup>a</sup> The data are data from a representative experiment.

<sup>b</sup> UC, uncharacterized.

<sup>c</sup> Number of phage particles/ml of induced culture, using RN4220 as the recipient strain.

<sup>d</sup> Number of SaPIbov2 particles/ml of induced culture, using RN4220 or RN981 (*recA* mutant) as the recipient strain.

<sup>e</sup> SaPIbov2 transfer titer divided by phage titer.

*recA* mutant recipient could not be detected with any of the phages tested, indicating that *int* is required for even the low-level residual transfer to the *recA* mutant recipient observed with phages φ11, φ55, and φ85 and therefore that pure CGT can account for only a fraction of the SaPIbov2 transductants obtained with nonmobilizing phages. Since *int* is required for SaPI integration into the recipient chromosome, we suggest that one possible explanation for these results is that phages unable to induce the SaPIbov2 ERP cycle encapsidate and transfer chromosomal fragments containing the SaPI as they would for any other chromosomal locus. In a *recA* mutant

recipient, any gene thus transferred would be lost; with a fragment containing SaPIbov2, however, the integrase would be expressed and could catalyze excision of the element and insertion into its specific *att<sub>C</sub>* site. Alternatively, spontaneously excised SaPIbov2 circular monomers (25) could be the substrate for encapsidation and, following transfer, could circularize and integrate. Note that in the first mechanism proposed *int* would be necessary only in the recipient strain, while in the second *int* would be necessary in both the donor and recipient strains.

**Int complementation.** To distinguish between these two possibilities, we cloned SaPIbov2 *int* into pCN51, generating plas-

TABLE 3. Transfer of SaPIbov2-*tetM* and chromosomal *aur::ermB*<sup>a</sup>

Prophage	SaPIbov2	MC-induced phage titer <sup>b</sup>	SaPIbov2 titer (Tc <sup>r</sup> ) <sup>c</sup>		<i>aur::ermB</i> titer (ErmB) <sup>d</sup>	
			RN4220	RN981	RN4220	RN981
80α	Wild type	1.4 × 10 <sup>9</sup>	1.3 × 10 <sup>9</sup>	6.8 × 10 <sup>8</sup>	840	0
80α	Δ <i>int</i>	3 × 10 <sup>8</sup>	1.4 × 10 <sup>3</sup>	0	200	0
φ11	Wild type	3.2 × 10 <sup>9</sup>	5.2 × 10 <sup>3</sup>	90	220	0
φ11	Δ <i>int</i>	8.3 × 10 <sup>9</sup>	60	0	110	0
φ147	Wild type	4.4 × 10 <sup>9</sup>	2.6 × 10 <sup>3</sup>	1.9 × 10 <sup>3</sup>	460	0
φ147	Δ <i>int</i>	6.3 × 10 <sup>9</sup>	560	0	730	0

<sup>a</sup> The data are data from a representative experiment.

<sup>b</sup> Number of phage particles/ml of induced culture, using RN4220 as the recipient strain.

<sup>c</sup> Number of SaPIbov2 particles/ml of induced culture, using RN4220 or RN981 (*recA* mutant) as the recipient strain.

<sup>d</sup> Number of ErmB-resistant transductants/ml of induced culture, using RN4220 or RN981 (*recA* mutant) as the recipient strain.

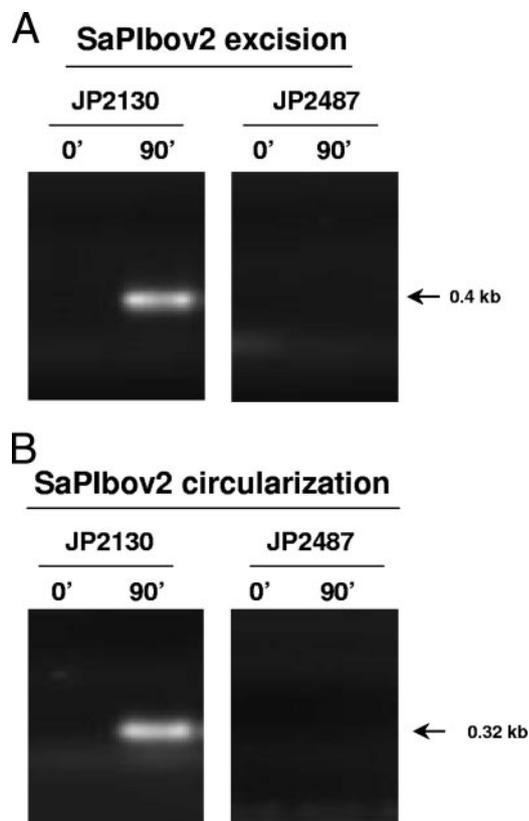


FIG. 2. Precise excision and circularization of SaPIbov2 mediated by the *Int* protein. (A) Detection of *Int* mediated SaPIbov2 excision and formation of *att<sub>B</sub>*. DNA from MC-induced strains were extracted (at 0 and 90 min) and PCR amplified using specific primers Ipl-1m and Ipr-2c recognizing the flanking sequence of the SaPIbov2 island. (B) Detection of *int*-mediated SaPIbov2 circularization. Samples obtained as previously described were PCR amplified with a pair of primers used divergently at both termini of the SaPIbov2 island (primers Ipl-16cB and Ipr-28m). JP2130 is a wild-type strain, and JP2487 is an *int* mutant strain.

mid pJP433, in which *int* is driven by a Cd-inducible promoter (4). As this promoter has considerable basal activity, cadmium induction is usually not required. As shown in Table 4, when a *recA* mutant recipient was used, *int* was required in the recipient but not in the donor for *recA*-independent SaPIbov2 transfer by phages such as  $\phi$ 11 and  $\phi$ 147 that do not induce the ERP cycle and do not induce SaPIbov2 excision and circularization (not shown). This result clearly supports the model shown in Fig. 3 and

TABLE 4. Complementation of the SaPIbov2  $\Delta int$  mutant<sup>a</sup>

SaPIbov2 $\Delta int$ strain	Donor strain		Recipient strain titer <sup>b</sup>		
	Prophage	Plasmid	Empty	pCN51	pCN51- <i>int</i>
JP3247	$\phi$ 11	pCN51	0	0	580
JP3248	$\phi$ 11	pCN51- <i>int</i>	0	0	110
JP3249	$\phi$ 147	pCN51	0	0	590
JP3250	$\phi$ 147	pCN51- <i>int</i>	0	0	790

<sup>a</sup> The data are data from a representative experiment.

<sup>b</sup> Number of SaPIbov2 particles/ml of induced culture, using RN981 (*recA* mutant) as the recipient strain.

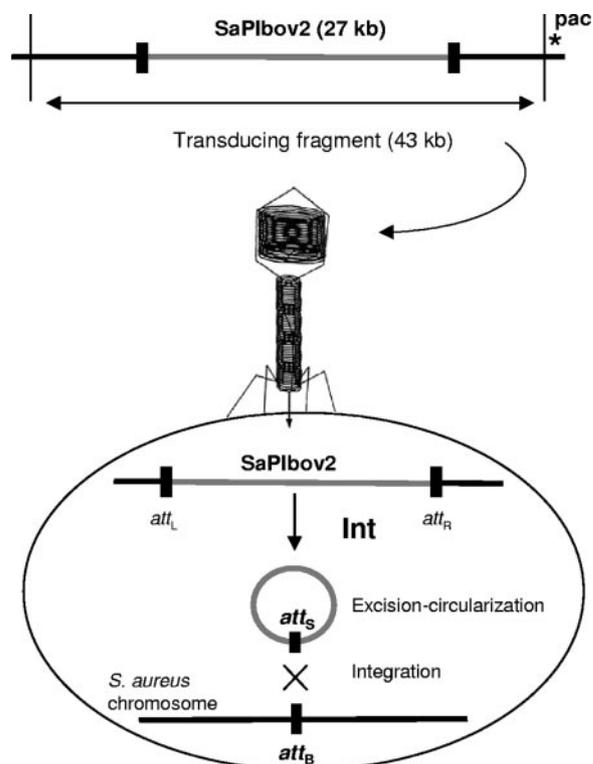


FIG. 3. *Int*-mediated mechanism involved in SaPIbov2 transfer.

effectively rules out the possibility that excised circular molecules in the donor represent the substrate for this transfer. This mode of low-frequency transfer is referred to replication-independent SaPI transfer. It was noted that the frequencies of SaPIbov2 transfer by this intermediate mechanism vary over a very wide range ( $10^{-6}$  to  $10^{-3}$ ) with the set of generalized transducing phages studied, as shown in Table 2. This could simply reflect differences in the intrinsic transducing activity of these phages, a possibility that has not been critically analyzed.

**Transspecies transfer of SaPIbov2.** It is generally accepted that phage-mediated transduction is the most important mechanism of horizontal gene transfer in the staphylococci. Accordingly, accessory genes occurring in different staphylococcal strains and species are usually assumed to have been acquired by transduction. A case in point is *bap*, which we have previously identified in a variety of different staphylococcal species, including *S. epidermidis*, *S. chromogenes*, *S. xylosus*, *S. simulans*, and *S. hyicus* (22). We noted that *bap* is not present in the sequenced genomes of any of the staphylococci, including *S. epidermidis*, indicating that it is a variable gene. Given the rather common occurrence of *S. aureus* phages capable of mobilizing SaPIbov2, it seemed possible that transspecific transduction of the island might occur, which would represent a theoretical demonstration of transspecific gene transfer. As shown in Table 5, we were able to demonstrate transduction of SaPIbov2 to *S. xylosus* and to several *S. epidermidis* strains at frequencies that were about 0.1% of the frequency of transduction to RN4220. Interestingly, it was not possible to transfer the SaPIbov2  $\Delta int$  island to the coagulase-negative staphylococci, which indicates that the transfer was *int* mediated. In

TABLE 5. Transduction of SaPIbov2 to coagulase-negative staphylococci<sup>a</sup>

Recipient strain	Transduction (titer)
<i>S. aureus</i> RN4220.....	$2.6 \times 10^9$
<i>S. xylosus</i> C482.....	$1.8 \times 10^6$
<i>S. epidermidis</i> CH829.....	$3.9 \times 10^5$
<i>S. epidermidis</i> CH830.....	$1.2 \times 10^6$
<i>S. epidermidis</i> CH64.....	$8.4 \times 10^5$
<i>S. epidermidis</i> CH83.....	$1.3 \times 10^6$
<i>S. epidermidis</i> CH332.....	$4.4 \times 10^3$

<sup>a</sup> The data are data from a representative experiment. JP2130 was used as a SaPIbov2 donor strain.

*S. aureus*, the 18-bp SaPIbov2 *att* gene is at the 3' end of the GMP synthase (*gmpS*) gene (25). To determine whether SaPIbov2 integrates at the same site in the *S. epidermidis* or *S. xylosus* chromosome, JP1522 (a SaPIbov2-containing *S. xylosus* strain) and JP2632, 2633, 2557, and 2558 (SaPIbov2-containing *S. epidermidis* strains) were analyzed by PCR using primers Ipr-13mP (specific for SaPIbov2) and Ipr2c (specific for the *gmpS* gene [25]), designed to amplify the right junction fragment of SaPIbov2. In all these strains, a 0.7-kb band comprising the right junction of SaPIbov2 and having the same mobility as the corresponding product from RN4220 containing SaPIbov2 (Fig. 4) was obtained. Sequence analysis of these PCR products confirmed the site-specific localization of SaPIbov2 in the *att*<sub>C</sub> site of the *S. xylosus* and *S. epidermidis* strains analyzed. It is additionally interesting that a BLAST search for the SaPIbov2 *att*<sub>C</sub> site revealed that each of the *S. epidermidis* sequenced genomes and the *S. saprophyticus* genome contain one copy of the site, at the 3' end of the *gmpS* gene, but no other copy, indicating that the site is unoccupied. It is also unoccupied in all of the sequenced *S. aureus* genomes (with the exception of the genome of RF122 [the native host for SaPIbov1]) and in all 43 toxic shock strains that we have analyzed independently (21).

## DISCUSSION

In this study, we demonstrated mobilization and high-frequency transfer of a fourth SaPI prototype, SaPIbov2, the largest of the known elements in this class, the second such element from a veterinary source, and a close relative of the first element, SaPIbov1. SaPIbov2 and SaPIbov1 both use the same chromosomal *att*<sub>C</sub> site, at the 3' end of *gmpS*, a site that is not used by any of the other known SaPIs, all of which are from human isolates, including those in a set of 43 toxic shock syndrome isolates that we have recently characterized (21). SaPIbov2 shows the known classical SaPI ERP cycle upon SOS induction of a coresident 80α prophage, except that the typical SaPI-specific monomer band is not seen in an agarose gel. This is because SaPIbov2 completely lacks one of the genes, corresponding to SaPIbov1 ORF8, that we have shown to be required for the formation of the typical small-headed SaPI particles (24). Since this band is derived from disruption of mature SaPI particles during the preparation of DNA samples for electrophoretic analysis (24), this is not surprising because the 27-kb SaPIbov2 genome is too large to be encapsidated in such particles. This finding raises a number of interesting questions. First, since SaPIbov2 is about 12 kb larger than any of

the 16 other known SaPIs (16), owing to the insertion of a 12-kb transposon, did it “lose” the ORF8 homolog after acquisition of the transposon, or did the transposon insert into a SaPI genome that was already missing this gene? Since SaPIbov2 is stably maintained as a chromosomal element, selection for transferability could account for the evolutionary loss of the ORF8 homolog. Alternatively, since at least two of the known SaPIs, SaPI5 and SaPImw2, are missing all or part of the capsid morphogenetic system (16), the transposon could have inserted into an element lacking this system. Second, since SaPIbov2 is transferred at the same high frequency as other SaPIs and possesses the typical phage terminase small subunit required for SaPI-specific packaging, it must package its DNA in standard phage heads, as an ORF8 mutant of SaPIbov1 does (24). What, then, is the biological advantage of SaPI-specific capsid morphogenesis? One possibility is that since the small-headed SaPI particles can encapsidate only about one-third of the inducing phage genome, coinfection with a SaPI-containing particle and a small-headed particle containing phage DNA will result only in SaPI transfer. Third, why is there no SaPIbov2-specific band in lysates of cells in which SaPIbov2 is replicating? Presumably this material would consist of ~40 kb, representing about 1.5 SaPIbov2 genomes, and would comigrate with the bulk DNA in the gel. Experiments to demonstrate it are in progress.

Among the known phages tested, only 80α could induce the SaPIbov2 ERP cycle. Examination of several uncharacterized strains to which SaPIbov2 had been transferred suggested that at least one strain, the propagating strain for typing phage X2, contained a prophage that could induce the SaPIbov2 ERP cycle. Experiments to characterize this phage are in progress. *recA*-independent transfer was observed with all five of the noninducing phages and with at least one of the uncharacterized phages carried by PS77, whereas *recA*-independent transfer was not detectable with a standard chromosomal gene. This suggested that there was a SaPI-specific transfer mechanism that was not dependent on the replication-induced formation of the multimeric substrates required for the standard headful packaging mechanism. One possibility was that the spontaneously excised circular SaPIbov2 monomers, previously shown to be present (25), could have been encapsidated and transferred. This was unlikely because such monomers would be too

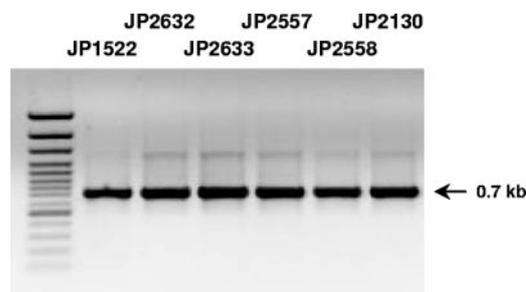


FIG. 4. Integration-specific PCR: PCR analysis using primers Ipr-13mP and Ipr-2c to test the site-specific integration of the SaPIbov2 island in the coagulase-negative staphylococci mediated by Int activity. JP1522 is an *S. xylosus* SaPIbov2-positive strain, JP2632, JP2633, JP2557, and JP2558 are *S. epidermidis* SaPIbov2-positive strains, and JP2130 is an *S. aureus* positive control.

TABLE 6. Mechanisms of phage-mediated SaPI transfer

Transfer mechanism <sup>a</sup>	SaPI replication	RecA requirement <sup>b</sup>	Int requirement <sup>c</sup>	
			Donor	Recipient
SPST	+	–	+	+
RIST	–	–	–	+
CGT	–	+	–	–

<sup>a</sup> RIST, replication-independent SaPI transfer.

<sup>b</sup> RecA requirement in the recipient strain.

<sup>c</sup> Int requirement for SaPI transfer in the donor and/or recipient strain.

small to fill a phage head and, therefore, some secondary mechanism, such as recombination between two monomeric circles, would have been required. A second possibility was that phage-genome-size chromosomal fragments containing the intact SaPIbov2 genome were generated by the usual mechanism for the formation of generalized transducing fragments and that following transfer, SaPIbov2 was excised, circularized, and inserted into its *att<sub>C</sub>* site by the SaPIbov2-encoded integrase, as shown in Fig. 3. In the first mechanism, a functional *int* (and possibly also a functional *recA*) would have to be present in the donor strain, whereas in the second scenario, *int* would be required only in the recipient. Using an *int*-defective mutant of SaPIbov2, we demonstrated that *int* was required for replication-independent transfer as well as for classical SPST. The key finding was that a cloned *int* was required in the recipient but not in the donor for the replication-independent transfer of SaPIbov2, strongly supporting the second of the possibilities described above. We note, incidentally, that *int* is predictably required in both the donor and the recipient for classical SPST (P. Barry and R. P. Novick, submitted); an experiment to confirm this with SaPIbov2 could not be interpreted owing to the occurrence of SaPI-plasmid recombinants, and correction of this problem is currently in progress. We note also that the replication-independent transfer of SaPIbov2 is decreased to a greater or lesser extent with a *recA* mutant recipient, suggesting that CGT accounts for some of the transductants and therefore that the *int*-dependent mechanism is less than 100% efficient. We have no explanation at present for the apparently different frequencies of *recA*- and *int*-dependent transfer observed with different phages (Table 2). The three different SaPIbov2 transfer mechanisms are listed in Table 6.

In addition to studies with *S. aureus*, we investigated SaPIbov2 transfer to several non-*S. aureus* staphylococci with particular reference to the possible source of the SaPIbov2-carried *bap*. *Bap* is a very large adhesin involved in biofilm formation and contributes significantly to bovine mastitis. Its presence within a well-defined transposon in SaPIbov2 suggests strongly that it was acquired by SaPIbov2 via transposition. Since *bap* has been demonstrated in non-*S. aureus* staphylococci (22), it was possible that one such species was the source of the SaPIbov2 ortholog. Although *bap* was not contained either within a SaPI or within a transposon in any of the species that were examined (22), we demonstrated in this study that SaPIbov2 could be readily transferred to each of these species, suggesting at least the possibility that intergeneric SaPI transfer may have been involved in the acquisition by *S. aureus* of the *bap* determinant. It remains to be determined, however, whether any of

these species contains a prophage capable of transferring SaPIbov2 or other genetic elements to *S. aureus*.

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