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The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*

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Summary

***Tst*, the gene for toxic shock syndrome toxin-1 (TSST-1), is part of a 15.2 kb genetic element in *Staphylococcus aureus* that is absent in TSST-1-negative strains. The prototype, in RN4282, is flanked by a 17 nucleotide direct repeat and contains genes for a second possible superantigen toxin, a *Dichelobacter nodosus* VapE homologue and a putative integrase. It is readily transferred to a *recA*$^-$ recipient, and it always inserts into a unique chromosomal copy of the 17 nucleotide sequence in the same orientation. It is excised and circularized by staphylococcal phages ϕ13 and 80α and replicates during the growth of the latter, which transduces it at very high frequency. Because of its site and orientation specificity and because it lacks other identifiable phage-like genes, we consider it to be a pathogenicity island (PI) rather than a transposon or a defective phage. The *tst* element in RN4282, near *tyrB*, is designated SaPI1. That in RN3984 in the *trp* region is only partially homologous to SaPI1 and is excised by phage 80 but not by 80α. It is designated SaPI2. These PIs are the first in any Gram-positive species and the first for which mobility has been demonstrated. Their mobility may be responsible for the spread of TSST-1 production among *S. aureus* strains.**

Introduction

TSST-1 is a potent superantigen and is the most common cause of toxic shock syndrome. It is produced exclusively by *Staphylococcus aureus*, and approximately 20% of natural isolates are producers. The TSST-1 gene, *tst*, was cloned and sequenced by Kreiswirth *et al*. (1983) and later shown to be a chromosomal gene embedded in an accessory genetic element that contains conserved *tst* flanking sequences and is entirely absent from TSST-1-negative strains (Kreiswirth *et al*., 1983; 1989). *Tst* is located near *tyrB* in strain RN4282, the source strain for the original clone, which belongs to *agr* peptide group I (Ji *et al*., 1997), and within the *trp* locus in most other menstrual TSS isolates (Chu *et al*., 1988), which belong to *agr* peptide group III (Ji *et al*., 1997). It therefore seemed likely, at the time the present studies were initiated, that the *tst* element had inserted into two different sites and was therefore a transposon. We now find that the *tst* elements at the two different locations represent variant elements rather than different insertions of the same one (Lindsay *et al*., 1997), and we suggest that the *tst* element is not a transposon but rather conforms to the pathogenicity island (PI) paradigm (Hacker *et al*., 1997). The RN4282 element, now designated SaPI1, is 15.2 kb in length and is flanked by a directly repeated sequence of 17 nucleotides. In addition to *tst*, it contains two open reading frames (ORFs) that could encode pathogenicity factors, one in the family of superantigens (*ent*) and another homologous to VapE, encoded by the *vap* pathogenicity island of *Dichelobacter nodosus* (Billington *et al*., 1996). It also contains an ORF whose predicted product is a member of the integrase (Int) family of recombinases. It will be recalled that PIs are accessory genetic elements that range in size from 10 to 200 kb, contain one or more pathogenicity genes, are bordered by directly repeated sequences, can be deleted *en bloc* and may have integrase-like genes (Cheetham and Katz, 1995; Cheetham *et al*., 1995; Karaolis *et al*., 1998). PIs are widely assumed, but have not thus far been demonstrated, to be mobile.

In this paper, we present and describe the DNA sequence of SaPI1 and demonstrate its mobility, i.e. transfer intact to a *recA*$^-$ recipient. We show that it is mobilized at high efficiency by one particular staphylococcal generalized transducing phage, 80α, but not by most others, including the closely related phage, ϕ11. SaPI1 is induced to excise and replicate by 80α and to excise, but probably not to replicate, by ϕ13; related elements in other strains, SaPI2 etc., are induced to excise and replicate uniquely by phage 80. We also show that SaPI1 can integrate, but cannot excise, in the absence of a functional phage and that it interferes with the growth of 80α and ϕ13, whereas SaPI2

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interferes with the growth of phage 80. As SaPI1 can be transferred efficiently to *recA*$^-$ recipients by generalized transducing phages, it is the first PI showing mobility. It is also the first to be identified in any Gram-positive species. These remarkable properties suggest that *tst* is carried by a family of closely related PIs that interact in a highly specific way with certain staphylococcal phages and that this interaction may be responsible for the spread of TSST-1 among staphylococcal strains.

Results

Nucleotide sequence of SaPI1

In earlier studies, a 10 kb segment of the RN4282 chromosome, including *tst* and flanking regions, has been cloned and partially sequenced (Kreiswirth *et al*., 1984; Kreiswirth, 1986) and a derivative (SapI1 *tst*::tetM) constructed containing an insertion of *tetM* within the *tst* coding sequence (Sloane *et al*., 1991). The leftmost segment of the sequenced region hybridized with chromosomal DNA from *tst*$^-$ strains, indicating that the left junction (J_L) was contained within this region. The right junction (J_R), however, was not included, as a probe prepared from the rightmost segment of the cloned DNA hybridized with chromosomal DNA from *tst*$^+$ but not from *tst*$^-$ strains (Kreiswirth, 1986).

Identification and sequences of junctions

The present studies were initiated by defining the left and right junctions (J_L and J_R) between the inserted element and the flanking chromosomal DNA. We began by performing a polymerase chain reaction (PCR) using primers p134 (to the left of J_L)and p131(to the right of J_L) based on the known sequence in this region (Kreiswirth, 1986). This region was known to contain J_L, because its left end had previously been shown by Southern blot hybridization to be in common chromosomal DNA and its right end to be in element-specific DNA. With a sample of RN4282 (*tst*$^+$) chromosomal DNA as template, the expected 250 nucleotide product was produced. In a control PCR, using chromosomal DNA from RN450 (a *tst*-negative strain) as template, in which no product was expected, a 650 nucleotide product appeared. This 650 nt product, resulting from a misprimed PCR, was also generated with chromosomal DNA from other *tst*$^-$ strains and from RN3984, which has the *tst* element in a different location, whereas the 650 nt product was always replaced by the 250 in 80α SaPI1 *tst*::*tetM* transductants of RN450 (not shown). These results suggested that the 650 nucleotide product contained the chromosomal insertion site of SaPI1 in RN450 and therefore, presumably, in RN4282. The point of divergence between the sequences of these two PCR products defined the site of J_L. We next identified the right junction by means of an outward-directed PCR using primers p131 and JL43 (see below), and the resulting product containing att_{SaPI1} was cloned to pBluescript and sequenced. Comparison of the sequence of this PCR product with the sequences of the 650 nucleotide attachment site fragment and of J_L revealed a 17 nucleotide directly repeated sequence, TTATTTAGCAGGAATAA, that was present at each junction and also within the 650 nucleotide product at the site of divergence between the left and right ends of SaPI1 and the chromosomal sequence. This 17 nt sequence therefore represented the chromosomal attachment site, att_{SaPI1}, of the element in RN4282 and in RN450. The sequence of an 828 bp chromosomal region containing att_{SaPI1} has been deposited in GenBank, accession number U93687.

The remainder of the SaPI1 sequence, between J_R and the existing sequence to the right of *tst*, was determined by PCR walking. The sequence of SaPI1 has been deposited in GenBank, accession number U93688.

Sequence analysis

Fig. 1 shows a map of SaPI1 including certain restriction sites and some of the ORFs bigger than 65 codons. *Tst* is about 2 kb from the left junction. The right end contains an ORF with 47% amino acid sequence similarity to the integrase (Int) of staphylococcal bacteriophage L54a (Fig. 2), including the five amino acids thought to be important for integrase function (Carroll *et al*., 1995). This ORF shows significant homology with the integrases of the conjugative transposons Tn*5276* of *Lactococcus lactis* (Rauch and De

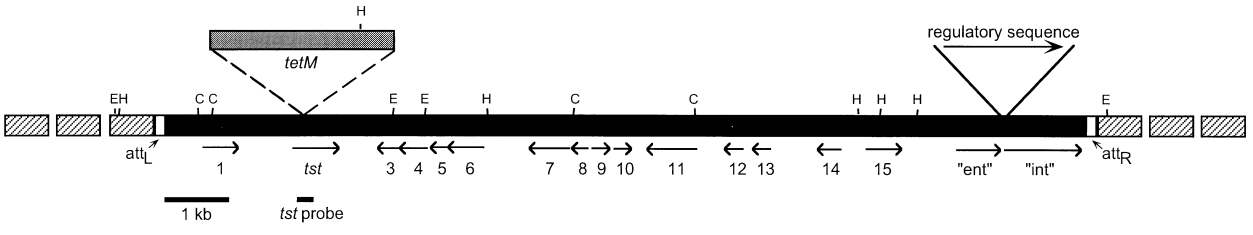

Fig. 1. Map of SaPI1 based on the sequence. The 15 233 bp element is shown as solid black, adjoining chromosomal regions as hatched, the 17 bp direct repeats at left and right junctions as open boxes labelled *att* and the *tetM* insertion in *tst* as shaded. ORFs with a predicted coding size of >65 amino acids including *tst*, 'int' (Fig. 3) and 'ent' (Fig. 4) are shown as arrows. C, *Cla*I; E, *Eco*RI; H, *Hin*dIII.

Vos, 1992), Tn*1545* (Poyart-Salmeron *et al*., 1990) and Tn*916* (Senghas *et al*., 1988), and also with that of the streptococcal bacteriophage T270, which is one of the phages that carries the gene for streptococcal erythrogenic toxin A (Yu and Ferretti, 1991). Immediately upstream of the putative *int* gene is a 46 bp sequence that is perfectly conserved among all of four staphylococcal phages that have been sequenced in this region, ϕ11, ϕ13, ϕ42 and L54a (Ye and Lee, 1989; Ye *et al*., 1990; Carroll *et al*., 1995). This region has been shown to be involved in the regulation of staphylococcal phage ϕ11 *int* by two ϕ11-encoded regulatory genes, *rinA* and *rinB* (Ye and Lee, 1993). We did not find *rinA* or *rinB* homologues in the sequence of the *tst* element. Downstream of the *int*-like gene is a second region of L54a homology, including direct and inverted repeats. Two of the four phages, ϕ11 and L54a, encode an excision (*xis*) function directly upstream of *int*; the other two do not, nor does the *tst* element contain any obvious *xis* homologue. The full extent of any sequence relation to the phages cannot be determined at present, as none of these phage sequences has been published. The sequences of ϕ11 and ϕ13 have been determined by Human Genome Sciences but are not available for proprietary reasons.

Directly upstream of the 46 nt conserved segment is an ORF whose predicted product shows marked sequence similarity to the staphylococcal and streptococcal superantigen toxins (Fig. 3). Tests for expression of this protein and for its possible pathogenicity are in progress.

The predicted product of ORF 11 (Fig. 1) shows 26% sequence identity to *vapE*, a gene with unknown function present on the *vap* pathogenicity island (PI) of *Dichelobacter nodosus* (Cheetham and Katz, 1995). This PI also contains a bacteriophage-like *int*, has flanking direct repeats and is closely related to a plasmid that is present in some strains of *D. nodosus* (Billington *et al*., 1996). There was no significant homology to any protein in the PIR database among some 25 additional SaPI1 ORFs of more than 65 codons.

Genetic properties of SaPI1

Mobility. Using a derivative strain, RN6938, in which the staphylococcal *tetM* gene, determining tetracycline resistance (Nesin *et al*., 1990), had been inserted by homologous recombination into the *tst* coding sequence of RN4282 (Sloane *et al*., 1991), we have demonstrated the transfer of SaPI1 to two *recA*$^-$ strains of *S. aureus*, RN1030 and RN8645. The transduction frequency of the *tst*::*tetM* determinant in strain RN6938, using the standard generalized transducing phage ϕ11 (Novick, 1967), was about 10^{-7} pfu^{-1}, whereas that of the same marker inserted into the chromosomal *agr* locus in strain RN6911 (Novick *et al*., 1993) was $<10^{-10}$ pfu^{-1}. Furthermore, *recA*$^-$ *tst*::*tetM* transductants could be used in turn as donors in a second round of transduction, giving similar transduction frequencies with *recA*$^-$ and *recA*$^+$ recipients (data not shown). These results demonstrate that SaPI1 is capable of *recA*-independent mobility. The fact that ϕ11 transduced the *tetM* marker to a rec$^+$ recipient at similar frequencies (approximately 10^{-7} pfu^{-1}) whether integrated into SaPI1 or into *agr*, suggests that SaPI1 can be transduced to a *recA*$^+$ recipient as a conventional chromosomal marker, that is by means of homologous recombination between flanking chromosomal sequences. The ability of the *tetM* marker inserted into *tst* to be transferred to a *recA*$^-$ recipient by ϕ11 transduction confirms that SaPI1 is not a conventional transposon. Although a conventional transposon, such as Tn*551*, which belongs to the Tn*3* family (Khan and Novick, 1980), transposes at a frequency of about 10^{-5} per cell generation when located on a plasmid (Wyman *et al*., 1974), its transposition frequency is below the limit of detectability when it is introduced as part of a chromosomal fragment by a transducing phage. That is, its transposition cannot be detected in a cross between a chromosomal donor and a *recA*$^-$ recipient (Wyman *et al*., 1974) because the combined probabilities of transduction (approximately 10^{-7}) plus transposition (approximately 10^{-5}), that is, approximately 10^{-12} overall, make transposition events too rare to detect. From this, it is inferred that SaPI1 has a very high efficiency of integration following transfer by transduction and is similar in this respect to the site-specific transposon, Tn*554* (Murphy, 1988; Phillips and Novick, 1979).

Site-specificity. The junction sequences of SaPI1, plus the presence of one copy of the 17 nucleotide flanking repeat sequence at the known SaPI1 insertion site, suggested that the element would show strong insertional site specificity. Southern blot hybridization analysis of 39 *recA*$^-$ transductants, using a *tst* probe to hybridize with *Hin*dIII-digested chromosomal DNA, which would contain a J_L fragment including *tst* sequences, always revealed the same 5 kb fragment, indicating that SaPI1 insertion was always at the same site and in the same orientation as in the donor strain. Similarly, a PCR using primers p134 and p111 (see below) generated a constant 0.6 kb product. Typical Southern blot patterns using *Hin*dIII digests of chromosomal DNA hybridized with a *tst*-specific probe are shown in Fig. 4. Lanes 2–5 show the pattern seen with SaPI1 *tst*::*tetM* transductants of RN1030, and lane 8 shows the identical pattern seen with the donor strain, RN6938. In lanes 6 and 7 is the pattern seen with the native SaPI1 element. Band B in lanes 6 and 7, containing J_L, is replaced by bands C (5.0 kb, containing J_L) and D (3.5 kb, internal to SaPI1) in strains containing the *tst*::*tetM* derivative (*tetM* contains a *Hin*dIII site that is included in the probe; see Fig. 1). Bands B and C are,

A

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SaPI1 13766                                  →TAAAT CTATTAATTA TATTATAATC    13788
L54a  1421   ←CATTTCATCA CCTACTTTTT ATTTTATTAT ATCACATTTA GTAGCTAGTA    1372
ϕ11   1248   ←CATAACATCA CCTACTTTTT ATTTTATTAT ATCACATTTA GTACCTAGTA    1199
ϕ13   +3C    ←CATCTTATCA CCTACTTTTT ATTTTATTAT AACATATTTA GTACCTAGTA    -47C
ϕ42

SaPI1 13789 ATTATTTTTC GGGTAGCCCG CCTACCCTTA TTATTTTTTG CCAATTTTGA      13838
L54a  1371  CTAAAATCAC GGGTAGCCCG CCTACCCTTA TTATTTTTTG CCAATTTTGA      1322
ϕ11   1198  CTAAATTT-C GGGTAGCCCG CCTACCCTTA TTATTTTTTG CCAATTTTGA      1150
ϕ13   -48C  CTAAATTTT- GGGTAGCCCG CCTACCCTTA TTATTTTTTG CCAATTTTGA      -12B
ϕ42   1012A    →TAA-C GGGTAGCTCG CCTACCCTTA TTATTTTTTG CCAATTTTGA-     -12B

SaPI1 13839 GGAGGGAGAA GCAAAATG→
L54a  1321  GGAGGGATGT --AAAATG
ϕ11   1149  GGAGGGAGAA GCAAAATG→
ϕ13   -11B  GGAGGGAACC CATG→
ϕ42   -11B  GGAGGGAACG GATG→
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B

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                  v60       v70       v80       v90       v100      v110      v120
intSaPI1 LNERIEAKLNDKTPTTLKSLTFHAASDEWFQNYIKTSGSKRTTIKTKLSKLNTLKKFVDEDILINKITLS
         L:E:I..KLN:K::::LK:LTFHA  DEW:: .IKTSG K TT:..  :::::.:KK  .:::L:NKI. .
intL54a  LEEKIKEKLNNKSSSELKTLTFHALLDEWLEYHIKTSGFKVTTLDNLKTRIKNIKKNSSQNLLLNKIDTK
               ^10       ^20       ^30       ^40       ^50       ^60       ^70

                  v130      v140      v150      v160      v170      v180
intSaPI1 YAQQVFDEMDSKGYVYQVNKDALSIFKNVFEYTRRIYKLQDLEFLKDITLNKRIKSYDEV---KAKRNKY
         Y Q  ::E: S  Y  :  K. L: .K:.:.Y: ::Y: .: ::L:.:TL K: K: :::   .AK. :Y
intL54a  YMQTFINEL-SNVYSANQVKRQLGHMKEAIKYAVKFYNYPNEHILNSVTLPKKSKTIEDIEKEEAKMYNY
               ^80       ^90       ^100      ^110      ^120      ^130      ^140

           v190      v200      v210      v220      v230      v240      v250
intSaPI1 LELNEIQSIIKDINMKAQKMHSGIHKRFYLFVALMTEFQALNGMRIGEMLAIQNEDIDFDNKSLNINGTI
         LE::::  I :D: ::.::M:    : R  ::VA  .E QAL.GMRIGE:LA:Q .D:D:.NK::.INGTI
intL54a  LEMEQVIQI-RDFILNDNNMQ--YRAR--ILVAGAVEVQALTGMRIGELLALQVKDVDLKNKTIAINGTI
               ^150      ^160      ^170      ^180      ^190      ^200

           v260      v270      v280      v290      v300      v310      v320
intSaPI1 HWFHDESGGFGVKDTTKTESSYRTIGLSSRSCEILKKAILENKKDSKWNDGYLNRNFVFTNHKGNPMQTE
         H::. ::G FG KDTTKT.:S R.I:::SR  ::LKK :LENKK  :W: :Y::R.F:FT. :GNPMQ..
intL54a  HRIKCNAG-FGHKDTTKTAGSKRKIAINSRIANVLKKIMLENKKMQQWEPSYVDRGFIFTTCQGNPMQGS
           ^210      ^220      ^230      ^240      ^250      ^260      ^270

           v330      v340      v350      v360      v370      v380      v390
intSaPI1 RFNKILREAAKDVGIDKEVSSHILRHSHISLLSQQGVSLKAIMDRVGHSDHRTTLSIYSHVTEQMDKDMM
         R:NK L..AA..: I:K.V::H.LRH:HISLL:: .:SLKAIM.RVGH.D::TT:.:Y:HVTE:MD:::
intL54a  RINKRLSSAAESLNINKKVTTHTLRHTHISLLAEMNISLKAIMKRVGHRDEKTTIKVYTHVTEKMDRELE
           ^280      ^290      ^300      ^310      ^320      ^330      ^340

           v400
intSaPI1 NKLEQL
         :KLE:L
intL54a  QKLEKL
           ^350
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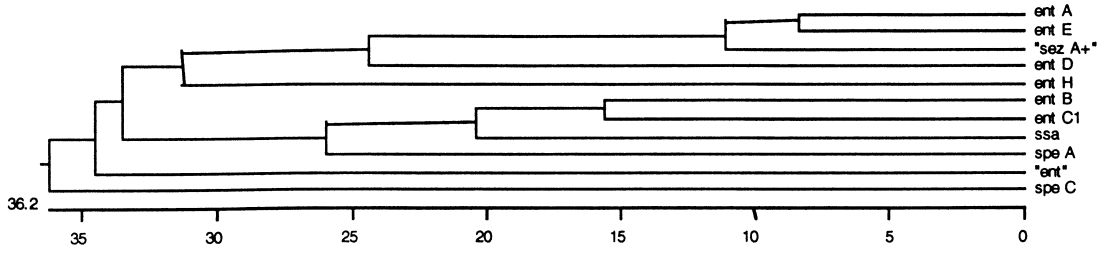


Fig. 3. Phylogenetic comparison of superantigen toxins. The tree was constructed with protein sequences of precursors using DNASTAR MEGALIGN, CLUSTAL method. Ent, staphylococcal enterotoxin; spe, streptococcal pyrogenic exotoxin; ssa, streptococcal superantigen; 'sez A+', silent variant of staphylococcal enterotoxin A; 'ent', enterotoxin-like sequence in SaPI1. Figures represent genetic distance as percentage of substitutions.

therefore, location specific; accordingly, the p134–p111 PCR product was sequenced for these same four transductants and, in all cases, the insertions were at the same nucleotide position as in the donor strain. Also shown in Fig. 4 (lanes 7 and 8) is the result of an experiment in which the *tst*::*tetM* derivative was transduced to RN8649, a *recA*$^-$ derivative of RN4282, which contains the native element. Here, the incoming copy precisely replaced the resident copy, generating a hybridization pattern (lane 8) indistinguishable from that seen with the transductants of a *tst*$^-$ recipient (lanes 2–5). This result is contrasted with the behaviour of Tn*554*, which is not replaced by an incoming copy and, instead, blocks insertion (Phillips and Novick, 1979).

Deletion. Pathogenicity islands typically undergo site-specific deletion involving their flanking directly repeated sequences. The length of the SaPI1 DR is more than sufficient to support the Rec-mediated precise excision seen with various mobile elements and commonly seen when DNA fragments are joined in novel combinations (Hahn and Dubnau, 1985; Ehrlich *et al*., 1991). Accordingly, we have attempted to demonstrate site-specific deletion of SaPI1 by replica plating but have failed to detect this in either an 80α lysogen or a non-lysogen in a screening of about 1500 colonies of each. As this failure may be a consequence of efficient re-integration of any excised copies of SaPI1, it is planned to repeat the deletion test with an *int* knockout, the construction of which is currently in progress.

Phage-induced excision of SaPI1. The presence of an *int*-like gene and the perfect direct flanking repeats suggested that the *tst* element might undergo excision and circularization. This possibility was supported by the Southern blot shown in Fig. 1. In lanes 10–13 are the hybridization patterns obtained with *Hin*dIII digests prepared from the DNA of *tst*::*tetM* transductants of strain RN27, a derivative of NCTC8325 that is lysogenic for phages 80α and φ13. These blotting patterns display an extra fragment of 7.8 kb (band A) that hybridized with the *tst*-specific probe. This band was seen only with RN27 and other 80α lysogens and is the expected size for a fragment that consists of the fused left and right ends of the *tst* element. Formally, this fragment could represent either a circle or a tandem head-to-tail dimer. This finding prompted us to examine intracellular DNA prepared from culture samples taken during the growth of phage 80α. Indeed, after UV induction of an 80α lysogen containing *tst*::*tetM*, a new DNA species appeared, migrating more rapidly than the bulk chromosomal and phage DNAs. A typical gel pattern demonstrating this new species is shown in Fig. 5 (left) in comparison with an 80α lysogen lacking the PI (centre) and with an SaPI1-containing non-lysogen (right). In Fig. 6C is the Southern blot hybridization pattern of the electropherogram shown in Fig. 6B. The new species hybridized strongly with a *tst*-specific probe, suggesting that it represents an excised form of SaPI1. It is notable that the bulk DNA also reacted strongly, suggesting that the excised PI was also present in a form similar to that of the chromosomal and replicating phage DNAs. Blotting of a similar sample with an 80α-specific probe revealed a strong signal in the bulk DNA but none corresponding to the excised SaPI1 (not shown). As shown in Fig. 6A (left),

Fig. 2. Sequence comparisons.
A. Conserved nucleotide sequences preceding the proposed *int* of SaPI1 and the staphylococcal bacteriophages (adapted from Carroll *et al*., 1995). Potential ribosome binding sites are underlined, and potential stem–loop structures are doubly underlined. ATG→ indicates the start codon of *int* in SaPI1, φ11, φ13 and φ42. ATG indicates the first potential start codon of L54a *int* (a second potential ribosome binding site and start codon are located about 150 bp downstream). →TAA indicates the termination codon of the proposed enterotoxin in SaPI1 and of ORFA in φ42. ←CAT indicates the start codon of *xis* in φ11 and L54a, and the start codon of ORFC in φ13.
B. Integrase alignment. The predicted SaPI1 integrase (403 amino acids) is aligned, using the Lipman–Pearson algorithm, with the 352 amino acid integrase from staphylococcal bacteriophage L54a (Ye and Lee, 1989), showing 47% sequence identity. The five amino acids thought to be important for integrase function (Carroll *et al*., 1995) are marked in bold.

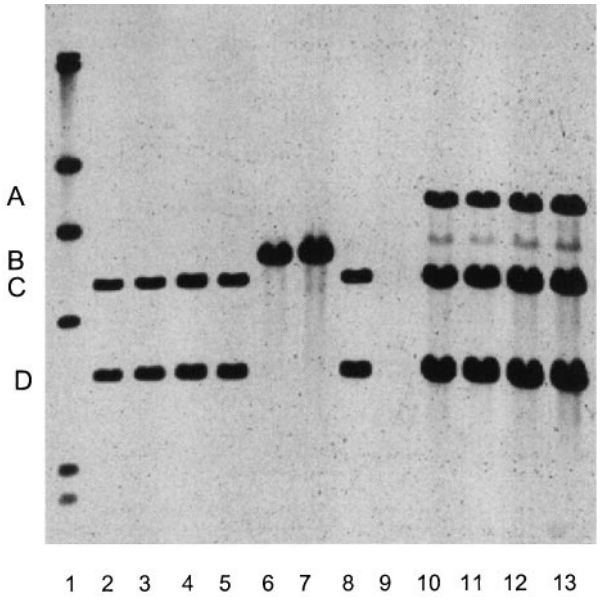


Fig. 4. Southern blot hybridization analysis of SaPI1. Genomic DNA was digested with *Hin*dIII, separated on 1% agarose, probed with a *tst*-specific probe that spans the *tetM* insertion site and detected using the direct-labelling ECL kit from Amersham. Lane 1, λ *Hin*dIII digest (23.1, 9.4, 6.6, 4.4, 2.3 and 2.0 kb); lanes 2–5, SaPI1 transductants of RN1030; lane 6, RN4282; lane 7, RN8649; lane 8, SAPI1 transductant of RN8649; lane 9, RN8645; lanes 10–13, SaPI transductants of RN8645. Band B (5.5 kb) is SaPI specific. Bands C (5.0 kb) and D (3.5 kb) are SaPI *tst*::*tetM* specific (see map, Fig. 1). Band A (7.8 kb) is the expected size for a fragment that consists of the left and right ends of the *tst* element, indicating the presence of an excised circle or a tandem dimer. We have no explanation for the weak band between A and B in lanes 10–13.

the SaPI1-specific band was seen after infection by 80α of an 80α-sensitive strain containing SaPI1 as well as after UV induction of a lysogen.

On dye–caesium separation of a lysate such as the 90 min sample shown in Fig. 5 (left), a species banding in the supercoil position was observed, isolated and digested with *Hin*dIII (not shown), yielding fragments of 7.5, 6.2 and 3.4 kb, corresponding to those predicted from the SaPI1 sequence for a circular form of the element. A PCR product prepared with this material as template and using outward-facing primers, p180 and p111, specific for the ends of the *tst* element, generated the predicted 553 bp product. This PCR product was sequenced by the Skirball Institute sequencing laboratory, and its sequence contained the two ends of the *tst* element plus a single copy of the 17 nucleotide attachment site: 5′ . . . ggcatttttatttaattgataTTA TTTAGCAGGAATAAttagccagattatcaagga . . . , confirming the identity of the supercoiled material as a circular form of the *tst* element and supporting the hypothesis that SaPI1 excises and integrates by the classical Campbell mechanism (Campbell, 1969).

These results delineate several stages in the life cycle of SaPI1, consistent with the proposal that it represents a pathogenicity island, and suggesting that it has a special relation with phage 80α involving its excision and circularization, phage-induced replication, encapsidation, transfer and integration. We next addressed the question of whether these stages can be studied separately so that their genetic determinants can be identified.

Phage specificity of excision. We have tested several TSS strains, including non-lysogens and those carrying prophages other than 80α, for UV-induced excision of SaPI1, either by simple electrophoretic analysis of sheared whole-cell lysates or by Southern blotting of gel electropherograms with a *tst*-specific probe. Thus far, we have been unable to detect any material that could represent an excised form of SaPI1 unless the strain was an 80α lysogen (not shown), from which we conclude that the element probably does not encode any *xis* function and,

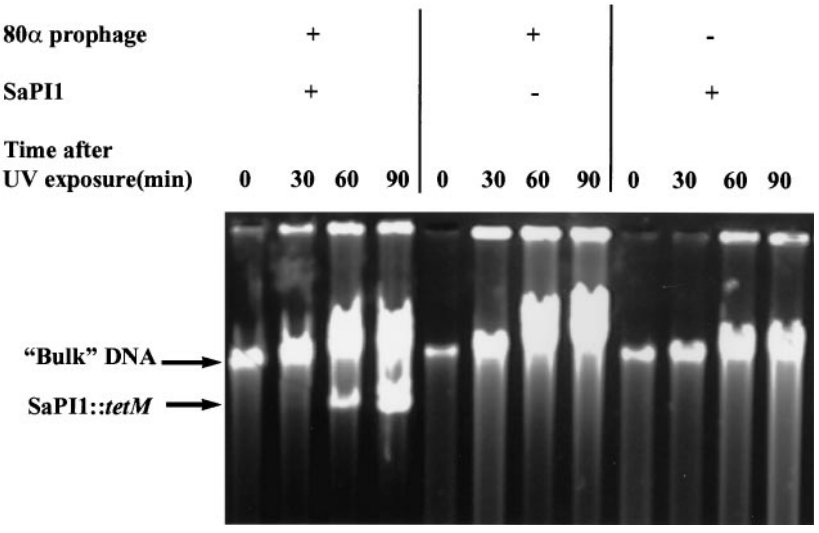
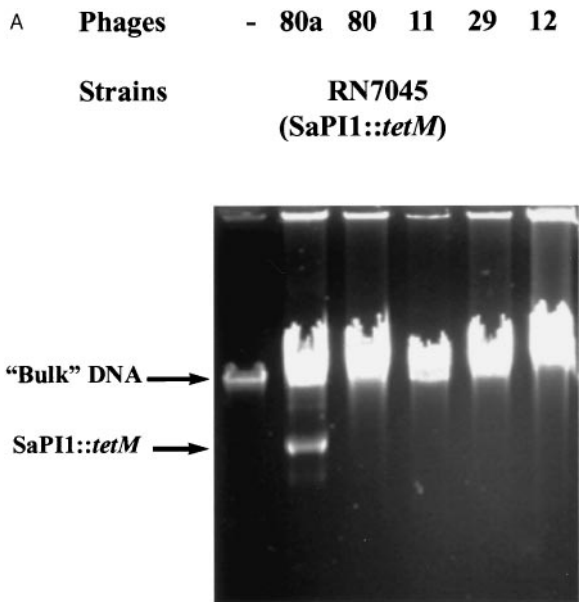
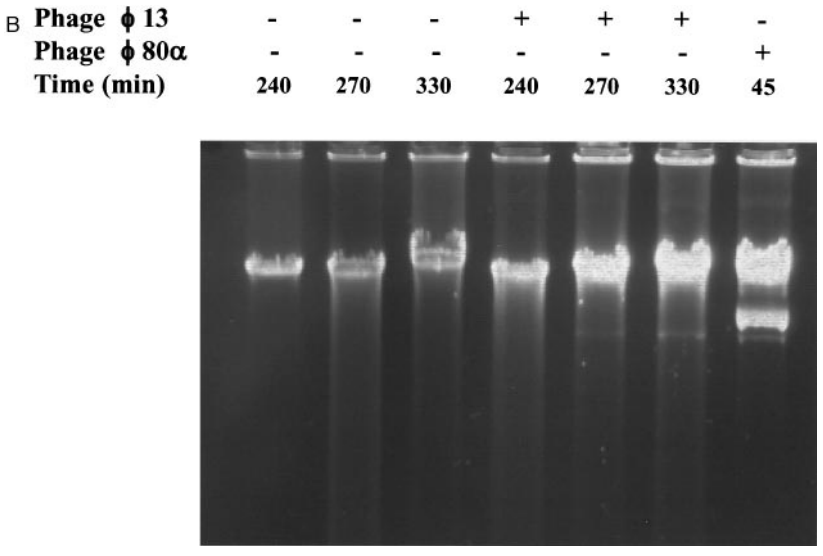


Fig. 5. Induction of SaPI1 excision by UV exposure. Bacterial cultures (RN8667, RN27 and RN7045) were exposed to UV light (70 J cm^{-2}), then incubated in broth at 32°C. Samples were removed at the indicated time points and used to prepare minilysates. Lysates were separated by agarose gel electrophoresis, stained with ethidium bromide and photographed.





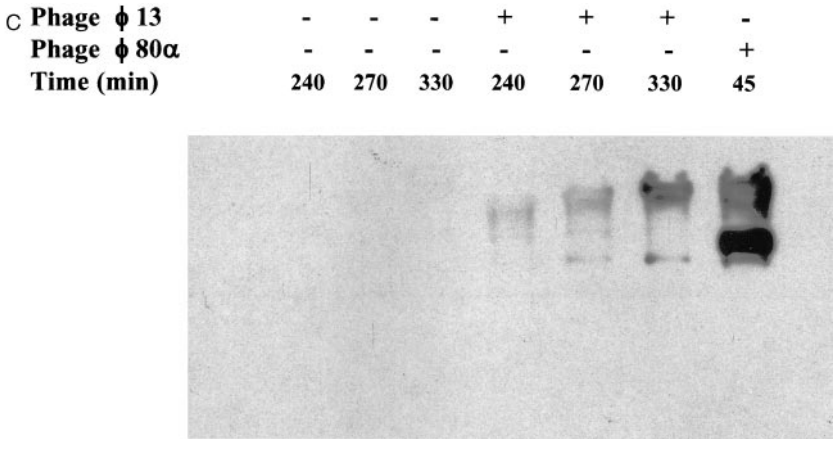


Fig. 6. Phage specificity of SaPI1 excision and replication. Bacteria were infected with phage at a multiplicity of 3:1. Cultures were grown at 32°C (A and B). Standard minilysates were prepared and analysed by agarose gel electrophoresis as in Fig. 5.
A. Several different phages were tested for their ability to excise SaPI1 from RN7045. Samples were removed 45 min after phage infection.
B. First six lanes, RN8685 uninfected (lanes 1–3) or infected with ϕ13 (lanes 4–6) was grown for the indicated times, and samples were taken for the preparation of minilysates. In lane 7 is a lysate of 80α-infected RN7045 taken 45 min after infection.
C. A blot-hybridization pattern of the gel shown in (B), using a *tst*-specific probe (a PCR product using primers p152 and p154; see Fig. 1).

therefore, that the excision of SaPI1 is phage induced and is highly phage-specific.

We note, however, that 80α is not a naturally occurring phage, having arisen as a rare plaque in an attempt to adapt staphylococcal typing phage 80 for growth on strain NCTC8325 (Novick, 1967). This strain is naturally lysogenic for three known phages, φ11, φ12 and φ13 (Novick, 1967), and is insensitive to phage 80. Phage 80α was later shown by Southern blot hybridization to be much more closely related to φ11 than to its progenitor, 80 (Stewart *et al*., 1985), suggesting that it is probably a recombinant between 80 and φ11; it is probably also a restriction–modification variant, as it does not plate on NCTC9789, the propagating strain for phage 80. We thus addressed two questions: (i) which phage(s) contributed the 80α genes that are involved in the excision, replication and transfer of SaPI1; and (ii) can other phages be shown to express one or more of these functions? Figure 6A and B shows a comparison of lysates obtained with several different phages, 80α, 80, φ11, 29, φ12 and φ13, infecting strain RN7045, a non-lysogenic derivative of NCTC8325, containing SaPI1 *tst*::*tetM*. As can be seen, in addition to 80α, φ13 generated a plasmid-like band that hybridized with a SaPI1-specific probe (Fig. 6B and C). As neither 80 nor φ11, which were presumed to have been the progenitors of 80α, caused SaPI1 excision, whereas φ13 did, this result suggests that φ13 is a third ancestor of 80α and that it contributed the gene that causes SaPI1 excision. The weakness of the φ13-induced band suggests that φ13 induces excision but not replication of SaPI1. It is interesting, as noted above, that neither φ13 nor its close relative, phage φ42, contains a *xis*-like reading frame at the location usually occupied by this gene (adjacent to *int*), whereas φ11 does (Carroll *et al*., 1995). Nevertheless, the lack of similarity of the SaPI1 core attachment site to those of φ11, φ13 or 80α (Carroll *et al*., 1995):

$att_{80\alpha}$:CTTCCCATGG
$att_{\phi 11}$: ACTTCCCATGG
$att_{\phi 13}$: TGTATCCAAACTGG
att_{SaPI1}: TTATTTAGCAGGAATAA

suggests that the sequence specificity of SaPI1excision/insertion is determined by the element itself and that φ13 must possess a non-sequence-specific *xis* function that it has contributed to 80α. Excision specificity is therefore likely to involve the interaction between *int* and *xis* rather than between *xis* and the attachment site sequence.

Replication. Densitometric analysis of the gel shown in Fig. 5, correcting for the reduced ethidium binding of supercoiled DNA, has shown that the material in the 80α-induced SaPI1-specific band corresponds to about 120 copies of the excised SaPI1 material per cell, just before the time of lysis. As there is only one chromosomal copy of the element, there cannot be more than four copies per cell during ordinary exponential growth (two chromosomes per cell, each partially replicated on average; Projan *et al*., 1983). Therefore, it is clear that the excised PI replicates, increasing at least 30-fold, during phage growth. The Southern blotting pattern shown in Fig. 6C, showing a great deal of SaPI1-specific material in association with the bulk DNA, suggests that the overall increase in SaPI1 DNA during the vegetative growth of 80α is probably much greater than 30-fold. We are unable to determine on the basis of the available data whether SaPI1 replicates autonomously or via reversible integration into the replicating phage DNA. If it were to replicate autonomously, it would have to possess a replication origin, which could be recognized either by an SaPI1-encoded initiator or by a phage-encoded one. Note in Fig. 6B and C) that φ13 induces a single SaPI1-specific band, whereas lysates of 80α-infected cells contain two – a strong upper band and a weak lower one that corresponds to the φ13-induced band. This result suggests that φ13 induces excision but not replication, whereas 80α induces both, and that the strong upper band corresponds to an 80α-induced replication product (whose structure has not yet been determined). As shown below, phage 80 appears to induce replication of a series of closely related SaPIs, and so we suggest that 80 is the source of the SaPI1 replication function expressed by 80α. Experiments to test these predictions are in progress.

Encapsidation and transfer. In contrast to the standard transduction frequency for SaPI1 seen with φ11 (approximately 10^{-7} pfu^{-1}), that seen with 80α is exceptional high – usually of the same order of magnitude as the plaque-forming titre – presumably owing to an intimate relation between SaPI1 and phage 80α. Both phages, however, always transduce standard chromosomal markers at the same low frequency of about 10^{-7} pfu^{-1}. φ13, which is not a generalized transducing phage, does not transduce SapI1*tst*::*tetM* detectably. A summary of the transduction activities of different phages with SaPI1 is presented in Table 1. The relation between 80α and SaPI1 clearly involves replication, and it must also involve encapsidation, presumably by an explicit encapsidation signal. In view of earlier results from this and other laboratories (Lofdahl *et al*., 1981; Schmidt and Schmieger, 1984; Dyer *et al*., 1985; Novick *et al*., 1986) indicating that the incorporation of a segment of phage DNA into a plasmid greatly increases transduction frequency, we tested a series of SaPI1 transductants by Southern dot-blot hybridization for the presence of 80α phage DNA. As shown in Fig. 7, 18 or 19 of the 20 transductants tested contained phage sequences. The signals were much weaker than that obtained with DNA of RN27, an 80α lysogen, and none of the transductants were either immune to 80α or UV inducible, indicating that they did not contain the complete phage

Table 1. Interactions between phages and SaPIs.

Phage	Element	Excision	Replication[a]	Transduction (titre)[b]	Interference
φ11	SaPI1	−	ND	+ ($\approx 10^{-7}$)	−
φ12	SaPI1	−	ND	− ($< 10^{-9}$)	−
φ13	SaPI1	+	−	− ($< 10^{-8}$)	+
80	SaPI1	−	ND	ND	ND
80	SaPI2	+	+	+ (10^{-1}–10^{0})	+
80α	SaPI1	+	+	+ (10^{-1}–10^{0})	+
80α	SaPI2	−	ND	ND	ND
29	SaPI1	−	ND	ND	ND
52	SaPI2	+	+	ND	ND

ND, no data.
a. Replication could not be evaluated in the absence of an excision product.
b. Transductants pfu^{-1} using *tst*::*tetM* derivatives.

genome. We suggest that the encapsidation intermediate is an SaPI–80α recombinant and that the attached phage sequences are responsible for the high frequency of transduction. Interestingly, the observed region of sequence identity between SaPI1 and several staphylococcal phages (see Fig. 2) cannot be responsible for the high transduction frequency, as both φ11 and φ13 contain the same sequence, but φ11 does not transduce SaPI1 at an elevated frequency, and φ13 does not transduce it at all. Experiments are in progress to determine the nature of the co-transduced phage DNA, its relation to the SaPI1 element and the region of SaPI1 responsible for high-frequency transduction by 80α. A summary of the relation between the SaPI elements and several staphylococcal phages is presented in Table 1.

Is SaPI1 *a conjugative transposon?.* As conjugative transposons such as Tn*1545* and Tn*916* encode phage-like *int* and *xis* functions that are used for their excision and integration (Senghas *et al*., 1988; Poyart-Salmeron *et al*., 1990), we tested the possibility that SaPI1 is a conjugative transposon. However, using conditions similar to those used for *S. aureus* plasmid-determined mating (Archer and Johnston, 1983), we were unable to demonstrate intercell transfer of SaPI1 *tst*::*tetM* in the absence of a functional transducing phage, suggesting that SaPI1 is not a conjugative transposon. The possibility has not been eliminated, however, that SaPI1 is a conjugative element that cannot transfer autogenously owing to its inability to excise. Nor have we yet addressed the possibility of mobilization by a conjugative plasmid.

Integration. As SaPI1 contains an ORF whose predicted product corresponds to an integrase-like protein, and it is transduced with high frequency to a *recA*$^-$ recipient, where it integrates into the recipient chromosome at a specific site, we consider it likely that SaPI1 possesses a functional integration mechanism. To test for phage-independent integration, we have purified SaPI1 *tst*::*tetM*-specific supercoiled DNA from a dye–caesium gradient and used this DNA to transform protoplasts prepared from a non-lysogenic *recA*$^-$ recipient, RN450 *recA*$^-$. The efficiency of transformation to tetracycline resistance, about $10^4\ \mu g^{-1}$, was similar to that obtained with pure pT181 plasmid DNA using the same selection, and examination of several SaPI1 transformants revealed the same Southern blotting pattern and the same excision product after infection with 80α as seen with the donor strain (not shown).

Because the SaPI1 derivative that has been used in all experiments involving transfer contains an insertion of *tetM* in the *tst* gene, was initially transduced by phage 80α and is induced to excise by the same phage, and because transductants frequently contain 80α sequences (Fig. 7), we have considered the possibility that it permanently acquired a segment of phage DNA at some point,

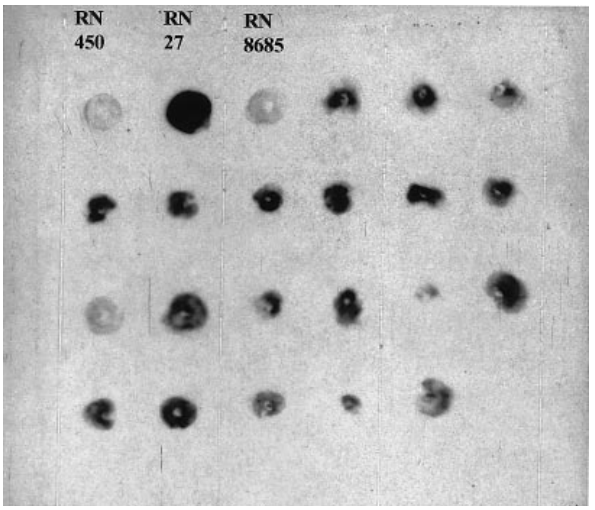


Fig. 7. Dot-blot hybridization analysis of 80α transductants of sap1*tst*::*tetM*. Samples (10 μl) of whole-cell minilysates were spotted on nylon membranes and hybridized with a peroxidase-labelled *tst*-specific probe. The blot was developed for chemiluminescence (Amersham ECL) and photographed. The three spots at the left in the top row correspond to RN450 (non-lysogenic, SaPI1-negative), RN27 (80α lysogen) and RN8685 (non-lysogenic, SaPI1 transformant) respectively.

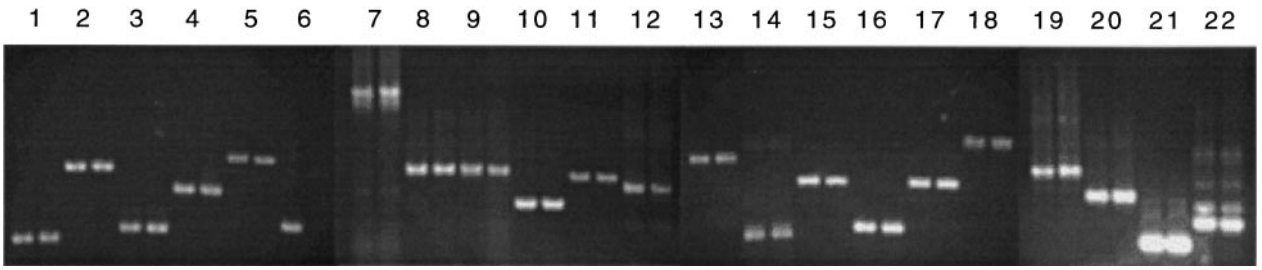


Fig. 8. PCR analysis of SaPI1 and SaPI1 *tst*::*tetM*. PCR reactions were performed under standard conditions using chromosomal DNA from RN4282 or RN7045 as template and primers as listed below. Each number refers to a pair of reactions in which the lefthand member used RN4282 DNA as template and the right, RN7045. Lanes 1, p134×p131; 2, p130×p97; 3, p96×p95; 4, p56×p40; 5, p78×p153; 6, p152×p154; 7, p155×p160; 8, p159×p57; 9, p41×JL4; 10, JL5×JL28; 11, JL45×JL34; 12, JL56×JL57; 13, JL26×JL59; 14, JL58×JL42; 15, JL33×JL61; 16, JL60×JL64; 17, JL65×JL30; 18, JL43×JL40; 19, JL32×JL31; 20, JL39×JL3; 21, JL2×p181; 22, JL6×p131. Reaction mixtures were separated by agarose gel electrophoresis, stained and photographed.

which could be responsible for some of its properties. This possibility was effectively ruled out by a point-to-point PCR comparison of the native SaPI1 with the *tetM* derivative, which had been through 80α-mediated transduction, using 22 pairs of primers covering the entire element, a subset of those that had been used for the sequencing of SaPI1 (see Fig. 8). The PCR products from the two forms, mostly 0.5–2 kb in length, were indistinguishable, except for the integrated *tetM* gene, which is in the region covered by primers p152 and p154 (lanes 6). It is noted also that 80α induces the excision of native SaPI1 from its original host strain, RN4282, as well as from RN8685, a transformant generated using supercoiled SaPI1 *tst*::*tetM* DNA, which does not contain detectable 80α sequences (not shown). These results indicate that the various manipulations have not resulted in the permanent incorporation of any detectable segment of 80α, confirming that the ability to integrate, as well as the other properties of SaPI1, are intrinsic to the element.

Phage interference

Phage-sensitive strains containing SaPI1 are insensitive to φ13 on spot testing and show a pattern with 80α that is typical of high-frequency lysogenization (turbid centre and clear periphery), such as that seen with coliphage lambda (see Fig. 9), However, the frequency of 80α lysogenization with this strain was < 1% (see below), i.e. no greater than that with strains lacking SaPI1. Rather, the observed pattern reflects relatively poor growth of the phage in comparison with 80α-sensitive strains lacking SaPI. Additionally, an indistinguishable 80α spot test pattern was seen with RN4282, the native strain from which the PI had originally been isolated, as well as with strains that had received the PI by transduction with φ11 or by transformation with SaPI1-specific DNA. Further, all of these strains were fully sensitive to φ11 (which does not plate on 80α lysogens) and were lysed by 80α or by φ13 in liquid culture, generating the usual SaPI1 excision product. These results suggest that SaPI1 carries a gene that causes weak interference with multiplication of 80α and φ13, but not with multiplication of the closely related phage, φ11.

The observed interference by SaPI1 with 80α propagation was quantified by a one-step phage growth analysis, as shown in Fig. 10. In this experiment, the average burst size of 80α on the SaPI1-containing strain (7) was about 1% of that observed with a strain lacking SaPI1 (600). The possibility that this apparent difference in burst size was a reflection of a lower rate of infection of the SaPI1-containing strain was ruled out by determining the frequency of infective centres produced by 80α infecting an SaPI1-containing strain in comparison with a strain lacking the element. In this experiment, using a multiplicity of about 3 pfu cell^{-1}, approximately the same number of infective centres were produced by both strains; interestingly,

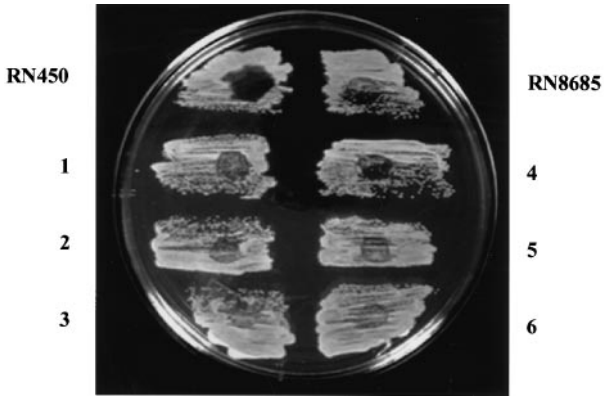


Fig. 9. Spot testing of 80α on SaPI1-containing strains.
A. Equal inocula from fresh overnight plates were spread on GL agar. Phage (4×10^6 pfu) was spotted in 10 μl of phage buffer. Plates were incubated overnight at 32°C and photographed. Numbers 1–6 represent independent RN450 transductants containing SaPI1 *tst*::*tetM*.

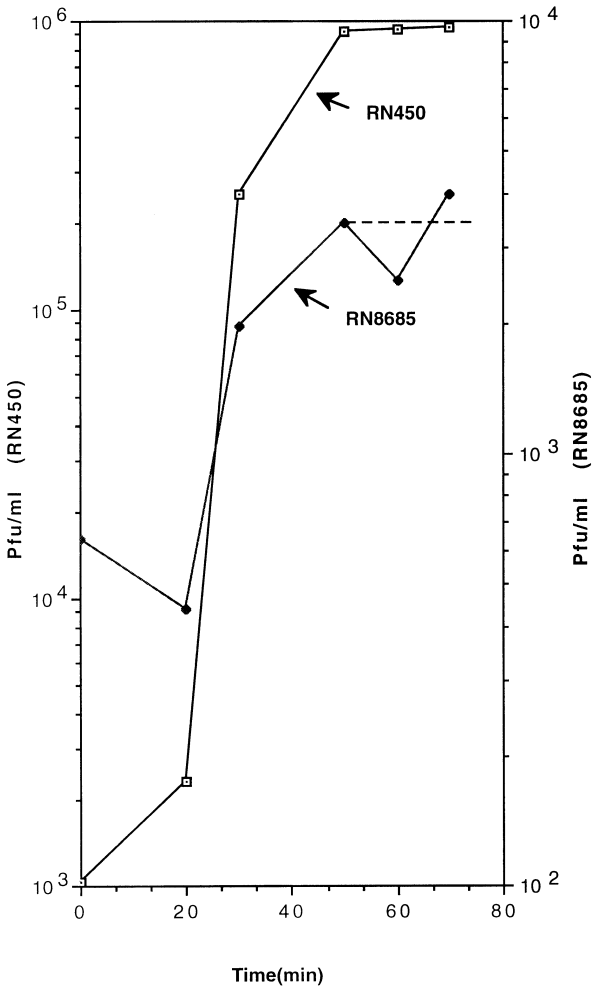


Fig. 10. One-step growth curves for 80α. Cultures of RN450 or RN8685 were infected with 80α at a multiplicity of 0.1, centrifuged to remove unadsorbed phage, then diluted to give approximately 10^4 infected cells ml^{-1} and incubated with slow shaking at 32°C. Samples were removed at the indicated times and phage titres determined using RN450 as indicator.

the frequency of survivors of phage infection in this experiment was about 3% with the SaPI1 strain compared with 0.2% with the strain lacking SaPI1. These survivors were tested for lysogeny by UV irradiation (70 J cm^{-2}) of agar plates containing about 300 colonies. The irradiated plates were then overlaid with RN450, 10^8 ml^{-1} in phage top agar, and incubated overnight at 32°C. None of the colonies generated phage active on RN450 in this test. Additionally, 10 survivor colonies were picked for each strain and tested for 80α immunity by spot testing and for lysogeny by UV induction and spotting on RN450. For both strains, none of the 10 survivors tested was immune or lysogenic, suggesting that, in a small proportion of cells, either the phage failed to infect or the infection was abortive.

SapI variants

It will be recalled that, in most TSS strains, *tst* is located within the *trp* locus, whereas in RN4282, it is located near *tyrB* (Chu *et al*., 1988). One of the former, RN3984, is the prototype of human menstrual TSS strains; as its ends do not hybridize with probes specific for the ends of SaPI1 (Lindsay *et al*., 1997), we have concluded that it does not represent a second-site insertion of SaPI1, but rather may represent a variant SaPI. We next addressed the question of whether such naturally occurring TSS strains contain prophages capable of inducing the excision and replication of SaPI1-like elements. Of 30 natural TSS isolates tested by UV induction under our standard conditions (70 J cm^{-2}), three underwent lysis at the usual time for staphylococcal lysogens (approximately 2 h after irradiation), but none of the 30 showed any SaPI1-like band at 90 min after UV, the time when the 80α-induced band is strongest (not shown). Assuming that *tst* is generally carried by SaPI1-like elements, it appears that the ability to excise and replicate these is infrequent among prophages inhabiting natural TSS strains.

Although neither 80α nor φ11 (not shown) induced excision of an SaPI1-like molecule from RN3984, phage 80 did induce the appearance of such a band, as did typing phage 52 (not shown). To confirm that the observed band represented a *tst*-containing element, we made use of RN8652, a derivative of RN3984 in which *tetM* had been inserted into *tst* (B. Kreiswirth, J. Lindsay and R. Novick, unpublished data). This strain showed an 80-induced excision product that was larger than that seen with RN3984 by about 3 kb, corresponding to the *tetM* insertion, and showed the same high frequency of tetracycline resistance transduction with 80 as that seen with SaPI1 and 80α (see Table 1). We have therefore designated the RN3984 version SaPI2. We next tested the same set of naturally occurring TSS strains that were tested by UV induction (above) and found that, in all of seven that were sensitive to 80, an SaPI2-like band was generated during growth of the phage (see Fig. 11). None of these strains was sensitive to 80α, and so this phage could not be tested. A possible explanation for these remarkable findings is as follows: the *tst* elements in RN4282, from which SaPI1 was cloned and sequenced, and RN3984 have been mapped at different locations in the staphylococcal chromosome (Chu *et al*., 1988). The latter, which is the prototypical menstrual TSS strain and is representative of the 30 strains used in the tests described above, has the element in or near the *trp* operon and is associated with tryptophan auxotrophy, whereas in the former it is near the *tyrB* locus. Southern blotting experiments have shown that the two *tst* elements are different, having a common central region including *tst* and flanking sequences, but different ends that do not cross-hybridize with end-specific

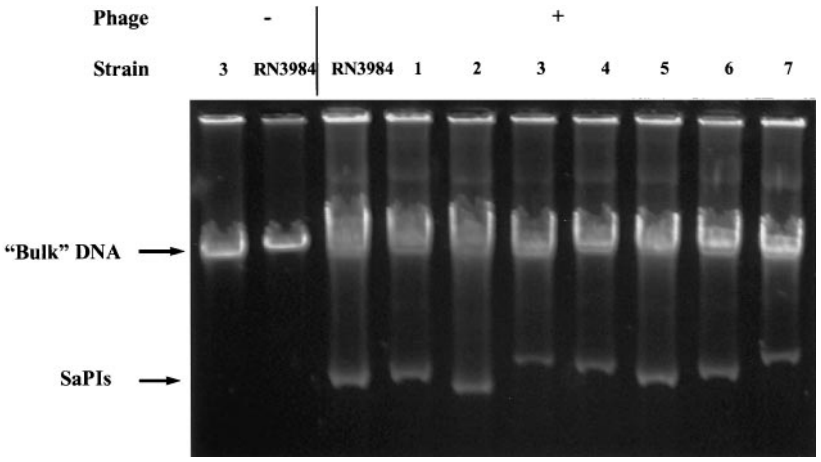


Fig. 11. Excision of SaPI-like elements in naturally occurring $TSST^+$ strains by phage 80. Superinfection was performed as described in Fig. 6. Samples were removed 45 min after phage infection and analysed for SaPI-like bands as described. Numbers 1–7 represent naturally occurring menstrual TSS isolates sensitive to phage 80.

probes (Lindsay *et al*., 1997). Their differential responses to 80 and 80α infection suggests that the PI–chromosomal junctions are different and are differentially recognized by the two phages, which must therefore differ in their excision specificities.

Discussion

As already noted, we began this study thinking that the *tst* element would turn out to be a high-frequency, site-specific transposon, similar to Tn*554*. We suggest that the *tst* element in RN4282 is not a transposon, because it always inserts into a single chromosomal site and always in the same orientation, unlike any known transposon (the *tst* element in RN3984 represents a variant element rather than a second-site insertion), and because it generates a 17 nt direct repeat, matching its attachment site, which is considerably longer than those generated by transposons (0–9 bp) (Berg and Howe, 1989). We suggest also that the *tst* element is not a defective phage, even though it has several features shared by bacteriophages, including a putative *int* gene and a conserved regulatory sequence, a phage-like *att* site, a putative phage-like insertion–excision mechanism and a specific functional interaction with a known bacteriophage. However, with the exception of *int*, its ORFs are not detectably homologous to phage genes in GenBank, including those from *E. coli*, *Bacillus subtilis* and *Streptococcus pyogenes*. And the presence of an *int* homologue and a circular excision product is not specific for phage-like units, because circular intermediates generated by site-specific integration/excision occur with a diversity of accessory genetic elements, including conjugative transposons (Senghas *et al*., 1988; Poyart-Salmeron *et al*., 1990) and possibly certain PIs (Cheetham *et al*., 1995; Karaolis *et al*., 1998). At the same time, the prototypical RN4282 element fulfils three of the four presently accepted criteria for pathogenicity islands: it encodes one known virulence factor and two other probable ones, is dispensable (entirely absent in TSST-1-negative strains) and is flanked by a directly repeated sequence. Although we have not observed site-specific deletions directly, the element is mobile and moves as a discrete unit with virtually absolute site specificity and, given the length of its flanking direct repeat, it is strongly predicted to undergo site-specific deletion. Its base composition, however, unlike that of many PIs, does not differ from that of the staphylococcal genome. Interestingly, the G + C content of PIs is often lower than that of the surrounding chromosome, often approaching 35%, which corresponds to that of *S. aureus* DNA.

The PIs of *E. coli* and *Salmonella typhimurium* are found in virulent strains and are thought to represent mobile genetic elements: they are flanked by directly repeated sequences, can be deleted in their entirety and often have an overall base composition that differs from that of the surrounding chromosomal DNA (McDaniel *et al*., 1995; Mills *et al*., 1995; Shea *et al*., 1996). However, mobility has not been observed, and no obvious mobility mechanism has been identified. As noted, at least two accessory genetic elements, the *vap* element of *Dichelobacter nodosus*, described as a PI, and the recently described *Vibrio cholerae* PI (Karaolis *et al*., 1998) encode an Int homologue. The *D. nodosus* PI may be related to SaPI1, particularly considering the homology between *vapE* and ORF11 of SaPI1, and is very similar to a plasmid found in some *D. nodosus* strains (Cheetham and Katz, 1995; Cheetham *et al*., 1995; Billington *et al*., 1996), suggesting that the *vap* element may be mobile.

Therefore, SaPI1 and SaPI2 are the first PIs for which mobility has been demonstrated. We hypothesize that this mobility is dependent on the SaPI1 *int*-like gene and involves the classic Campbell mechanism of excision and circularization followed by integration into the target genome. In support of this hypothesis, we have recently cloned

a 3.5 kb fragment containing the SaPI1 *int* and *att* determinants and found the resulting plasmid to integrate with 100% efficiency into the standard SaPI1 chromosomal insertion site and to remain stably integrated despite the presence of the attached cloning vector, a functional staphylococcal plasmid replicon (A. Ruzin and R. Novick, unpublished data).

Thus far, we have detected SaPI transfer only by phage-mediated transduction; as the circular form would have a molecular size of 15 or 18 kb, depending on whether the 3 kb *tetM* fragment has been inserted, and as typical staphylococcal transducing phages have genomes of approximately 45 kb (Stewart *et al*., 1985), the PI could be packaged as part of a 45 kb chromosomal transducing fragment or, if excised, as a PI–phage recombinant or a PI multimer. Thus, the encapsidation mechanism may be a reflection of whether SaPI1 replicates autonomously as a plasmid or passively upon (reversible) integration into the phage genome. We have determined previously that staphylococcal transducing phages, such as ϕ11 and 80α, encapsidate plasmids as tandem multimers, consisting of as many copies of the plasmid as are required to form a phage headful (Novick *et al*., 1986). These are resolved into monomers in the recipient cell after transfer. If the plasmid contains a fragment of the transducing phage genome, the transduction frequency is greatly elevated owing to efficient encapsidation, but the overall mechanism remains the same (Novick *et al*., 1986). One consequence of this mechanism is that transductants generated at low multiplicity are never lysogenic for the transducing phage. With P22, a plasmid containing a phage segment is also transduced at a very high frequency but, in this case, a phage–plasmid co-integrate is formed, replicates along with the phage DNA and is encapsidated as such (Schmidt and Schmieger, 1984). The resulting transductants are generally lysogenic for P22; if the plasmid–phage recombinant dissociates (by reversal of the recombination event by which it was formed), the plasmid recovers its autonomy. If dissociation does not occur, the plasmid becomes a stable or semi-stable component of the integrated prophage. If the plasmid is above a certain size, however, the lysogens will be defective, as part of the phage genome will have to be left out of the transducing particles. Given the headful packaging mechanism, the phage genomes in such defective lysogens will represent partial circular permutations, i.e. different transductants will contain different subsections of the phage genome. Our results suggest that SaPI1 transduction by 80α does not adhere precisely to either of these paradigms – the native SaPI1 does not contain any detectable segment of 80α, and the transductants usually contain 80α sequences but are not defective lysogens. We propose, therefore, that SaPI1 recombines reversibly with the 80α genome during growth of the phage and that this recombination is responsible for the high transduction frequency and possibly also for phage-induced replication of the element. An analysis of the structure of the replicating DNA and of the DNA content of the transducing phage particles and transductants is in progress.

One major unanswered question is how to explain certain transduction results. As SaPI1 does not contain any ORF with homology to phage Xis proteins and is evidently incapable of excision from its chromosomal site in the absence of certain specific phages, we are puzzled by the ability of the PI to insert into the resident chromosome after transduction, especially by ϕ11, to a *recA*$^-$ strain and by its ability to displace a resident copy in a *recA*$^-$ recipient. Both of these findings imply that SaPI1 can be specifically excised from a chromosomal transducing fragment. Perhaps the SaPI1 *int* product can catalyse excision at a low frequency from a transducing fragment, although it appears to be unable to do so from a chromosomal location.

The high-frequency, phage-dependent mechanism outlined for SaPI1 transfer is likely to be responsible for the horizontal spread of *tst* among clinical isolates of *S. aureus. S. aureus* bacteriophages are known to be extremely species specific, which may account for the absence of *tst* among other staphylococcal species (Kreiswirth *et al*., 1987).

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 2. RN450 is a derivative of a naturally occurring strain NCTC8325, cured of its three known phages, ϕ11, ϕ12 and ϕ13 (Novick, 1967). RN27 is a derivative of NCTC8325, lysogenic for phages ϕ13 and 80α, but not for ϕ11 or ϕ12. RN4282 and RN3984 are naturally occurring TSS strains and are the original sources of SaPI1 and SaPI2 respectively. RN6938 is a derivative of RN4282 with *tetM* inserted in *tst* in SaPI1. RN7045 is a derivative of 8325-4, obtained by transduction of SaPI1 *tst*::*tetM* from RN6938 with phage 80α. RN8667 was obtained by transduction of SaPI1 *tst*::*tetM* from RN6938 into RN27 with 80α. RN8652 is a derivative of RN3984 with *tetM* inserted in the *tst* gene of SaPI2. RN8685 is RN450 transformed with CsCl-purified supercoiled SaPI1 *tst*::*tetM*. Transductants 1–6 (see Fig. 9) were produced using RN450 as a recipient and RN8667 as a donor at low phage multiplicity (approximately 10^{-5}).

Inocula were prepared by overnight growth at 37°C on GL agar medium with antibiotics as appropriate. Cultures for phage studies and for DNA isolation were prepared by growth in CY + GP broth at 37°C (Novick, 1991) with shaking (240 r.p.m.). Overnight cultures in CY + GP were used for certain experiments. Procedures for the preparation and analysis of phage lysates were essentially as described previously (Novick, 1991), with minor modifications.

Table 2. Strains used in this study.

Strain	Relevant genotype, derivation	Source or reference
NCTC8325	Propagating strain for typing phage 47	
NCTC9789	Propagating strain for typing phage 80	
RN450	NCTC8325 cured of ϕ11, ϕ12 and ϕ13	Novick (1967)
RN27	RN450 lysogenic for 80α, ϕ13	Novick (1967)
RN1030	*recA*$^-$ mutant of RN450	Wyman *et al*. (1974)
KB103	RN4220 *recA*::*ermC*	Bayles *et al*. (1994)
RN4282	Clinical isolate, TSST-1$^+$	Kreiswirth *et al*. (1983)
RN3984	Clinical isolate, TSST-1$^+$	Chu *et al*. (1988)
RN6938	RN4282 (SaPI1*tst*::*tetM*)	Sloane *et al*. (1991)
RN8652	RN3984 (SaPI2*tst*:*tetM*)	This study
RN7045	RN450 (SaPI1*tst*::*tetM*), 80α transductant from RN6938	Sloane *et al*. (1991)
RN6911	RN6390B *agr*::*tetM*	Novick *et al*. (1993)
RN8645	RN27 *recA*::*ermC*, 80α transduction from KB103	This study
RN8649	RN4282 *recA*::*ermC*, 80α transduction from KB103	This study
RN8646	RN8645 (SaPI1*tst*::*tetM*) 80α transduction from RN6938	This study
RN8650	RN8649 (SaPI1*tst*::*tetM*) 80α transduction from RN6938	This study
RN8667	RN27(SaPI1*tst*::*tetM*), 80α transduction from RN6938	This study
RN8685	RN450 transformed with CsCl-purified, supercoiled SaPI1*tst*::*tetM* DNA	This study
RN8465 (1)[a]	Clinical TSS isolate	P. Schlievert, personal communication
RN5946 (2)	Clinical TSS isolate	P. Schlievert, personal communication
RN5905 (3)	Clinical TSS isolate	P. Schlievert, personal communication
RN5947 (4)	Clinical TSS isolate	P. Schlievert, personal communication
RN5951 (5)	Clinical TSS isolate	P. Schlievert, personal communication
RN5877 (6)	Clinical TSS isolate	P. Schlievert, personal communication
RN5944 (7)	Clinical TSS isolate	P. Schlievert, personal communication

a. Numbers refer to strains shown in Fig. 11.

UV induction of prophages

Bacteria were grown in CY + GP broth to $OD_{650} = 0.15$, centrifuged, resuspended in phage buffer at a density of $OD_{650} = 0.05$, exposed to UV light (70 J cm^{-2}), diluted twofold with CY broth and grown at 32°C with slow shaking (80 r.p.m.). Lysis usually occurred within 2 h. Samples were removed at various time points, and standard SDS minilysates were prepared and separated on 0.7% agarose gels.

Analysis of phage-infected cells

Bacteria were grown in broth to $OD_{650} = 0.15$, centrifuged, resuspended in CY broth–phage buffer (1:1) at a density of $OD_{650} = 0.05$ and UV induced or infected with phage at a multiplicity of 3:1. Cultures were grown at 32°C with slow shaking (80 r.p.m.), samples were removed at various time points after phage infection, whole-cell minilysates were prepared and separated by agarose gel (0.7%) electrophoresis, then stained and photographed. Samples were also diluted and plated for viable counts to determine the frequency of bacterial survivors.

One-step phage growth curves

Bacteria were infected at a multiplicity of 1:10, centrifuged to remove unadsorbed phage, diluted to give about 10^4 infected cells ml^{-1}, then incubated at 32°C in a 1:1 mixture of CY broth and phage buffer. Samples were diluted and plated at various time points, using RN450 as indicator.

Transduction

For the preparation of phage lysates, early exponential phase cells were washed in CY + GP, then infected at a multiplicity of 0.05. Lysis took 2–5 h. Lysates were centrifuged, then sterilized by passage through a 0.45 μm Millipore filter. Transduction mixtures consisted of approximately 10^9 mid-exponential phase cells mixed with phage in phage buffer + 20 mM $CaCl_2$. For the transduction of phage-sensitive recipient strains, sodium citrate (0.15 mM) was added to the agar.

Phage spot tests

Cells from fresh overnight plates were resuspended in CY + GP broth at approximately 10^{10} cfu ml^{-1}, and 10 μl was patched onto GL agar. Different amounts of phage were spotted in 10 μl of phage buffer. Plates were incubated overnight at 32°C and photographed.

Preparation and analysis of genomic DNA

Cells from 25 ml of an overnight culture in CY + GP broth were washed three times in 30 mM Tris, 50 mM NaCl, 5 mM EDTA, pH 7.6, resuspended in 5 ml of the same buffer and lysed using 350 μg of lysostaphin. NaCl to 2 M and guanidine hydrochloride to 7 M were added, and the mixture was incubated at 55°C for 1.5 h until all the crystals had dissolved. The mixture was layered onto a 5 ml CsCl step gradient (2.5 ml of 2.85 M over 2.5 ml of 5.7 M, made up in 20 mM Tris, 20 mM EDTA) and centrifuged in a Beckman SW-40 rotor at 38 000 r.p.m. for 20 h at 15°C. The DNA was collected from the interface and dialysed against three changes of 10 mM Tris, 10 mM EDTA.

PCR reactions

PCR reactions were performed with a Perkin-Elmer 9600

thermocycler in a volume of 100 μl using the reaction buffer supplied by the manufacturer. Deoxynucleotide triphosphates were used at 50 μM. Cycling times were according to the properties of the primer pairs. Ordinarily, reactions were carried out for 35 cycles with an annealing temperature of 55°C. For outward-directed PCR, genomic DNA was digested with *Mbo*I, and fragments of approximately 1.5 kb were purified by gel electrophoresis and ligated in a volume of 1 ml for use as template.

Sequencing

Sequencing was performed by the Skirball Microchemistry Facility, using the Sanger method with an IBI automatic sequencer. For sequencing of SaPI1, PCR products were prepared using Expand Taq (Boehringer Mannheim) with an extension temperature of 68°C and primers containing a 5′ *Mbo*I site. PCR products were digested with *Mbo*I, and the fragments were cloned to pBluescript for sequencing. All sequences were confirmed in both directions using small PCR products amplified directly from genomic preparations, and all alignments were confirmed by Southern blotting.

Oligonucleotides

The following synthetic oligonucleotides were used for specific experiments (aside from the general sequencing primers):

p40 5′-CGCGATGAAAAAACGCCCAACT-3′ (reverse) SaPI1551-535
p41 5′-TCCCCTTTACACTTTAATTCGT-3′ (forward) SaPI14405-4426
p56 5′-GGTGGAGAAAATACCATAGAAGGA-3′ (forward) SaPI1 51-73
p57 5′-CGAATTAAAGTGTAAAGGGGAT-3′ (reverse) SapI15271-5250
p78 5′-GGAGTTGGGCGTTTTTTCATC-3′ (forward) SapI11379-1399
p95 5′-TCCTTCTATGGTATTTTCTCCACC-3′ (reverse) SapI1919-896
p96 5′-GTAATCGATTGCAAATAAG-3′ (forward) SapI1629-647
p97 5′-CTTATTTGCAATCGATTAC-3′ (reverse) SapI1647-629
p111 5′-GGCAAGAGGCCATATATCTG (reverse) SaPI1326-307
p130 5′-AGCAGGGATAATTAGCCAG-3′ (forward) SapI115213-8
p131 5′-CTGGCTAATTATCCCTGCT (reverse) SapI1 8-15213
p134 5′-CATCATTAACATTGAGGG (forward) chromosomal DNA approximately 0.5 kb from J_L
p152 5′-ATCGTAAGCCCTTTGTTG (forward) SaPI12130-2147
p154 5′-TGGATATAAGTTCCTTCGC (reverse) SaPI12464-2446
p155 5′-GCGAAGGAACTTATATCCA-3′ (forward) SaPI12446-2463
p159 5′-CACCCCTATAACAGAGCCCACC-3′ (forward) SapI14384-4406
p160 5′-GCCATACGAACACAATAACAAGTTG-3′ (reverse) SapI14706-4682
p180 5′-CTCACGTTACTGAACAGATGG-3′ (forward) SapI115010-15030
p181 5′-CCATCTGTTCAGTAACGTGAG-3′ (reverse) SapI115030-15010
JL2 5′-GGATTCGGTGTAAAAGATACCAC-3′ (forward) SapI11469-14671
JL3 5′-GTGGTATCTTTTACACCGAATCC 3′ (reverse) SapI114671-14649
JL4 5′-GACACAAGAGGATTACAAGG-3′ (reverse) SapI16125-6106
JL5 5′-CCTTGTAATCCTCTTGTGTC-3′ (forward) SapI16106-6125
JL6 5′-GGATTGAGTAGCAGAAGTTGCG-3′ (forward) SapI114700-14721
JL26 5′-AACAATGCTTTATCCGTCGG-3′ (forward) SapI18096-8115
JL28 5′-TTGAAACACGAGCAGGCACG-3′ (reverse) SapI16663-6644
JL30 5′-CATTTTGAGTAAGTAGCC-3′ (reverse) SapI111845-11828
JL31 5′-GTGGGTCTTTGTACTTCTCG-3′ (reverse) SapI113926-13907
JL32 5′-GTTTTACACCGGAACTGG-3′ (forward) SapI112897-12914
JL33 5′-GTCATCTGATTCTGTCGCC-3′ (forward) SapI110081-10099
JL34 5′-ACAGTGAGACAGTGAGACAG-3′ (reverse) SapI17467-7448
JL39 5′-CGAGAAGTACAAAGACCCAC-3′ (forward) SapI113907-13926
JL40 5′-GCTTGACCAGTTCCGGTG-3′ (reverse) SapI112920–12903
JL42 5′-GGCGACAGAATCAGATGAC-3′ (reverse) SapI110099-10081
JL43 5′-CTTAAATTGGCTACTTACTC-3′ (forward) SapI111820-11839
JL45 5′-AGTAACGTGCCTGCTCGTG-3′ (forward) SapI16639-6657
JL56 5′-TCTGTCTCACTGTCTCAC-3′ (forward) SapI17447-7464
JL57 5′-CTAACACCAGTTGTTGTGC-3′ (reverse) SapI18175-8157
JL58 5′-AATGCTTGTGTAGTGGGCAG-3′ (reverse) SapI18988-9007
JL59 5′-TTCATGCCCACTACACAAGCA-3′ (reverse) SapI19009-8990
JL60 5′-GTACTCCTCCGAATCGCGG-3′ (forward) SapI110769-10787
JL61 5′-CCGCGATTCGAGGAGTAC-3′ (reverse) SapI110787-10769
JL64 5′-GCACGGTAACTGGTATTTCCAG-3′ (reverse) SapI111167-11146
JL65 5′-CTGGAAATACCAGTTACCGTGC-3′ (forward) SapI111146-11167

Southern blot hybridization

*Hin*dIII digests of chromosomal DNA or unfractionated whole-cell minilysates were separated on 0.7% agarose gel, transferred to nylon (Hybond) membrane with a vacuum blotter and hybridized overnight with a peroxidase-labelled (ECL;

Amersham) *tst*-specific probe. For dot-blot hybridization, unfractionated minilysates (10 μl) were spotted directly on the membrane. The probe was a PCR product using the cloned *tst* gene as template and primers p152 and p154 spanning a 300 bp portion of *tst*. Membranes were washed and exposed to Kodak X-OMAT film. For Southern blotting, restriction digests separated on agarose were transferred to Hybond-N+ nylon (Amersham) with PCR products as probes.

Isolation of SaPI1 DNA

RN8667 was grown in 30 ml of broth to $OD_{650} = 0.19$, mitomycin C was added to 0.5 μg ml^{-1}, cells were incubated for 40 min under the same conditions, pelleted and resuspended in 30 ml of 1:1 mixture of broth and phage buffer. Cell suspension was incubated at 37°C with slow shaking (80 r.p.m.) for 100 min, pelleted, resuspended in 3 ml of TES buffer (20 mM Tris, 50 mM NaCl, 10 mM EDTA, pH 7.5), mixed with 3 ml of EtOH–acetone (1:1) and incubated for 15 min on ice. Water (30 ml) was then added and incubation continued for another 15 min. Cells were pelleted, resuspended in 2 ml of TES, treated with lysostaphin (50 μg ml^{-1}) and RNase A (500 μg ml^{-1}) at 37°C for 45 min and then with proteinase K (3 mg ml^{-1}) for another 45 min. The resulting lysis mixture was layered onto two Quick-Seal tubes each filled with 4 ml of CsCl solution (10.4 g of CsCl dissolved in 8.4 ml of TES) mixed with 1.3 ml of EtBr (10 μg ml^{-1}). The tubes were sealed and centrifuged in a Beckman TLA 100.4 rotor at 80 000 r.p.m. for 20 h at 22°C. Supercoiled DNA was visualized by UV light, collected with a needle, extracted with *n*-butanol and dialysed against three changes of TE buffer (pH 7.8).

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