Characterization and Expression Analysis of Staphylococcus aureus Pathogenicity Island 3

IMPLICATIONS FOR THE EVOLUTION OF STAPHYLOCOCCAL PATHOGENICITY ISLANDS*

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We describe the complete sequence of the 15.9-kb staphylococcal pathogenicity island 3 encoding staphylococcal enterotoxin serotypes B, K, and Q. The island, which meets the generally accepted definition of pathogenicity islands, contains 24 open reading frames potentially encoding proteins of more than 50 amino acids, including an apparently functional integrase. The element is bordered by two 17-bp direct repeats identical to those found flanking staphylococcal pathogenicity island 1. The island has extensive regions of homology to previously described pathogenicity islands, particularly staphylococcal pathogenicity islands 1 and 2. The expression of 22 of the 24 open reading frames contained on staphylococcal pathogenicity island 3 was detected either in vitro during growth in a laboratory medium or serum or in vivo in a rabbit model of toxic shock syndrome using DNA microarrays. The effect of oxygen tension on staphylococcal pathogenicity island 3 gene expression was also examined. By comparison with the known staphylococcal pathogenicity islands 3 gene expression context of gene expression described here, we propose a model of pathogenicity island origin and evolution involving specialized transduction events and addition, deletion, or recombination of pathogenicity island "modules."

Staphylococcus aureus is a leading etiologic agent of both nosocomial and community-acquired infections worldwide. These infections range from fairly benign cutaneous infections, such as furuncles, to potentially fatal diseases, including endocarditis and toxic shock syndrome (TSS)1 (reviewed in Ref. 1). The nucleotide sequence(s) reported in this paper has been submitted to the GenBank181 EBI Data Bank with accession number(s) AF410775.

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1 The abbreviations used are: TSS, toxic shock syndrome; SaPI, staphylococcal pathogenicity island; TSST-1, toxic shock syndrome toxin 1; ORF, open reading frame; TH, Todd-Hewitt; SE, staphylococcal enterotoxin.

It's ability to cause this range of disease is due in part to its elaboration of a vast array of both cell surface-associated and secreted virulence factors. Among the secreted factors are the pyrogenic toxin superantigens that have the ability to activate large populations (10–50%) of T lymphocytes in a manner specific to the variable region of the β-chain of the T-cell receptor (2). The ensuing massive cytokine release results in the symptoms of TSS, including fever, hypotension, rash, vomiting, diarrhea, multiple organ failure, disseminated intravascular coagulation, and desquamation. The staphylococcal enterotoxins (SEs), members of the superantigen family, are associated with both TSS and food poisoning and have proven emetic activities that appear to be separable from their superantigenic activity (3).

Most, if not all, staphylococcal superantigens are encoded by accessory genetic elements that are either mobile or appear to have been mobile at one time (reviewed in Ref. 4). These identified elements include plasmids, transposons, prophages, and the pathogenicity islands. The staphylococcal pathogenicity islands (SaPIs), of which five have, until recently, been described (SaPI1–4 and SaPIbov), are the first clearly defined pathogenicity islands in Gram-positive bacteria, and each encodes one or more of the staphylococcal superantigens (reviewed in Ref. 5). SEB, SEC, SEK, SEL, SEQ, and toxic shock syndrome toxin 1 (TSST-1) are known to be encoded by one or more of these phage-related elements (reviewed in Ref. 4). More recently, Kuroda et al. (6) identified six novel pathogenicity islands in the complete genomes of two S. aureus strains, N315 and Mu50, including three that carried tstH (encoding TSST-1), two that carried clusters of staphylococcal exotoxin-like proteins, and one, present in both strains, that carried clusters of serine proteases and enterotoxins.

These genomic loci meet the generally accepted requirements of the pathogenicity island subgroup of “genomic islands” as defined previously (7, 8). They are present in the genomes of many staphylococci but absent from closely related strains, they are relatively large genomic fragments (>15 kb), they differ in GC content from the rest of the chromosome, they are flanked by direct repeats likely generated upon insertion of the elements into the genome, some are associated with tRNA loci, and they possess genes coding for genetic mobility, including conserved integrases.

The prototypical staphylococcal pathogenicity island, SaPI1, was identified and characterized by Lindsay et al. (9) as the...
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Preliminary sequence data for the S. aureus COL strain was obtained from The Institute for Genomic Research website (www.tigr.org). A primer walking-based approach was used to amplify by PCR and sequence ~3 kb that spanned the unsequenced loci of SaPI3 in the COL genomic sequence data base. Automated sequencing using the ABI model 377 fluorescent sequencer was performed at the Advanced Genetic Analysis Center (University of Minnesota, St. Paul, MN).

Growth of S. aureus in vitro—50-ml cultures of S. aureus MN NJ were grown aerobically with shaking at 37 °C in either Todd-Hewitt (TH) broth (BC PharMingen) or rabbit serum (Inviron). Two independent cultures in each medium were grown in parallel, and samples were removed at the exponential, postexponential, and stationary phases of growth (2, 3, and 8 h after inoculation with an initial cell density of A660 nm = 0.1). Expression of SaPI3 ORFs and other virulence-associated genes was quantified using DNA microarrays.

For cultures exposed to altered oxygen levels, 1 ml of TH broth was inoculated with S. aureus MN NJ from an overnight culture to an initial cell density of A660 nm = 0.1 in a 35 x 12 mm-diameter polystyrene Petri dish (Nunc, Roskilde, Denmark). All cultures were placed into sealed, humidified Plexiglass cell culture chambers (20 x 26.75 cm, internal dimensions; Mishell-Dutton (14), flushed with gas mixtures containing either 1% oxygen (v/v) or 21% oxygen (v/v) balanced with nitrogen and 7% carbon dioxide (Praxair, St. Louis, MO), and sealed. Chambers were then ethanol sterilized at 37 °C with orbital shaking (~15 rpm) for the exponential, postexponential, and stationary phases of growth were removed at ~2, 3, and 8 h after inoculation, respectively, and expression of SaPI3 genes was quantified using DNA microarrays. We have previously determined that there is no significant difference in growth parameters (cell densities or timing of entry into the growth phases of interest) between cultures grown in 1% oxygen and 21% oxygen (v/v) balanced with nitrogen and 7% carbon dioxide (15, 16).

Immunization of Dutch-belted Rabbits—Two Dutch-belted rabbits were immunized with SEB, which is made in high concentrations (>10 μg/ml) by MN NJ grown in vitro. Rabbits were immunized by three subcutaneous injections at 2-week intervals, with each injection containing 25 μg of purified SEB resuspended in 0.5 ml of phosphate-buffered saline and emulsified in 0.5 ml of incomplete Freund’s adjuvant. Development of antibody to SEB was determined by enzyme-linked immunosorbent assay of serum samples taken 1 week after the final immunization. The two rabbits developed anti-SEB (IgG) titers of 1:5,120 and 1:10,240, respectively, as compared with preimmune titers of <1:20.

Subcutaneous Infection Model—Sterilized perforated hollow polyethylene balls were implanted subcutaneously in four Dutch-belted rabbits (17). Implantation of the polyethylene balls and subsequent healing created transudate-filled cavities in the rabbits with volumes of ~15 ml that contained few host cells, thus enabling the preparation of staphylococcal RNA relatively free of contaminating host RNA. 6 weeks after implantation of the polyethylene balls, ~109 colony-forming units of S. aureus MN NJ grown in TH medium were collected by centrifugation from the late exponential phase of growth (cell density of cultures was 6.7 x 106 colony-forming units/ml), resuspended in 2 ml of phosphate-buffered saline, and injected into the implanted polyethylene balls. Samples were removed from the inoculum culture before centrifugation for use in expression analysis by DNA microarrays. 2 ml of transudate containing S. aureus were then removed from the infection chambers at the indicated times after inoculation using a sterile syringe. S. aureus was enumerated by plating, and expression of SaPI3 genes was quantified using DNA microarrays.

RNA Preparation and DNA Microarrays—Analysis of staphylococcal gene expression in vitro and in vivo using DNA microarrays was performed as described elsewhere (www.agac.umn.edu/microarray/protocols/protocols.htm). In brief, a library of targets representing 68 genes from S. aureus MN NJ and MNS was constructed with primers designed to amplify fragments of ~300 bp of each gene from genomic DNA. Two successive rounds of PCR were performed to minimize genomic DNA contamination in the amplification products, and the final 100-μl reactions were checked for quality on agarose gels and purified with the QiAquick PCR Purification Kit (Qiagen, Valencia, CA). The purified products were printed in triplicate using a Total Array System robot (BioRobotics, Boston, MA). Pairs of RNA from MN NJ and MN8 was constructed with primers designed to amplify fragments of ~300 bp of each gene from genomic DNA. Two successive rounds of PCR were performed to minimize genomic DNA contamination in the amplification products, and the final 100-μl reactions were checked for quality on agarose gels and purified with the QiAquick PCR Purification Kit (Qiagen, Valencia, CA). The purified products were printed in triplicate using a Total Array System robot (BioRobotics, Boston, MA). Pairs of RNA from MN NJ and MN8 was constructed with primers designed to amplify fragments of ~300 bp of each gene from genomic DNA. Two successive rounds of PCR were performed to minimize genomic DNA contamination in the amplification products, and the final 100-μl reactions were checked for quality on agarose gels and purified with the QiAquick PCR Purification Kit (Qiagen, Valencia, CA). The purified products were printed on DNA microarrays.
is an isolate from a case of nonmenstrual TSS in which our laboratory has described the presence of two novel enterotoxins, SEK (12) and SEQ. The repeated 17-nucleotide sequences flanking SaPI3 were identical to the att sites of SaPI1 (5'-TTATTAGCAGGAAA-3') and thus might form the basis of pathogenicity island exclusion (i.e. the lack of TSST-1 and SEB in the same clinical isolates). In general, the identified SaPI3 genes form two apparent transcriptional blocks with ORFs 2–18 (including SEB) oriented toward the left of the island, whereas ORFs 19–24 (including SEK and SEQ) are oriented to the right (Fig. 1). The overall GC content of SaPI3 was 31.4%, somewhat lower than the 32.8–32.9% found for the whole genome of S. aureus (6).

We have identified genes in SaPI3 according to the following nomenclature: pathogenicity island ORF number (variant). Thus, the ninth ORF in SaPI3 is identified by the gene name sapi3_9. A mutant of this gene might be designated as sapi3_9Δ1.

Sequential genes are identified using a hyphen (e.g. sapi3_10-15 is used to identify all SaPI3 ORFs from 10 through 15). Upon determination of the gene's function, the name will be altered to reflect that function, such as sapi3_int (Exotoxin on the islands are identified according to their own nomenclature system.) If additional genes are identified on the island subsequent to its initial sequencing, those genes will be numbered sequential to those already identified, rather than renumbering all of the genes on the island. We have implemented this nomenclature system in our laboratory to promote systematic identification of SaPI genes, preclude confusion regarding identically numbered ORFs on different islands, and allow unambiguous assignation of expression data to SaPI genes.

A comparison of SaPI3, SaPI1, and SaPIbov is shown in Fig. 1, and the corresponding ORFs are identified in Table I. The overall length of the three SaPIs is similar, although SaPI3 is larger on the 5' end by 1915 bp. SaPIbov also has a different att site than SaPI1 and SaPI3. Two core regions of high (>92% identity) homology between all three islands were identified (Fig. 1). The first core region includes nucleotides 2974–6709 of SaPI1, 3113–6929 of SaPI3, and 5100–8745 of SaPIbov. Within this core region, SaPI1 has an additional 100 bp not present in SaPIbov (nucleotides 5909–6009 in SaPI1), whereas
SaPI3 contains this 100-bp stretch as well as an additional 67 bp not present in either SaPI1 or SaPIbov (nucleotides 6063–6230). The second core region includes nucleotides 9380–10,284 of SaPI1, 9591–10,494 of SaPI3, and 11,419–12,429 of SaPIbov.

All three islands contain int genes adjacent to the attR sites, and SaPI3 contains an enterotoxin gene (seb) in the same position as tstH in SaPI1 and SaPIbov. SaPI1 and SaPI3 appear to be even more closely related. In addition to the shared elements among all three islands and the identical attachment sites in SaPI1 and SaPI3, these two islands are highly homologous (>95% identity) at the right end (nucleotides 10,285–11,046 in SaPI1 and 10,494–11,254 in SaPI3; nucleotides 12,248–15,250 in SaPI1 and 12,891–15,953 in SaPI3) (Fig. 1). The ear (sapi1_1 and sapi3_1) and sek genes are also in the same relative positions to each other and to the att sites of SaPI1 and SaPI3. Although the function of ear is unknown, several properties of the gene suggest that it may have an important function in the life cycle of S. aureus. Its position and predicted product are conserved (~75% identity at the amino acid level) among SaPI1, SaPI3, and SaPI4 (data not shown), it has the identical signal sequence as TSST-1, and it is secreted in abundant quantities by S. aureus RN4282. In addition to homology between SaPI1, SaPI3, and SaPIbov, the region encoding sapi3_3-9 and sapi3_15 shares extensive homology (~95% at the nucleotide level) to a matching region in SaPI1/SaPIbov, whereas sapi3_10-14 are >87% or more similar to regions presumably of phage origin in strain Mu50.

A locus of ~900 bp adjacent to the att site of SaPI3 was 95% identical to a phage 80a sequence adjacent to the putative phage amidase gene. However, this region of SaPI3 apparently does not encode for any protein. A ~1.6-kb region of SaPI3 with significant variance as compared with SaPI1 is found spanning the region between sapi3_17 and seq. Within this region is a stretch of ~500 nucleotides that is nearly identical to a sequence from the recently identified dSLT, a temperate S. aureus phage encoding the Panton-Valentine leukocidin. Immediately upstream of int in both SaPI1 and SaPI3 is a 46-bp sequence conserved among staphylococcal phages φ11, φ13, φ42, and L54a (18–20). This is the binding site for two phage φ11 proteins that regulate int expression, RinA and RinB (21).

In addition to the integrase and the noncoding regions with homology to phage DNA, several of the genes contained on SaPI3 suggest a mobile element of phage origin, perhaps a conglomeration of several phage elements (Table I). The terminase potentially encoded by sapi3_3 is homologous to the small subunit of identified terminases in the bacteriophages ρ15 (22) (52% similarity over 162 amino acids) and PBSX of Bacillus subtilis (23) (50% similarity over 102 amino acids). SaPI3 potentially encodes a product with high homology (54% similarity over 333 amino acids) to the virulence-associated protein (VapE) of Dichelobacter nodosus, a sheep pathogen (24). (Sapi1_11 is also a VapE homolog 9.) This has led to the supposition that this gene was acquired by S. aureus through horizontal transfer from D. nodosus during co-colonization or infection of sheep (5) because the D. nodosus vap genes appear to reside on an integrated bacteriophage (25, 26).

The predicted product of sapi3_18 is 75% similar over 58 amino acids to a putative cro-like repressor of Streptococcus thermophilus bacteriophage Sfi21 (27, 28). Even stronger homology is seen between the predicted product of sapi3_19 and the cl-like repressor of S. thermophilus phage SH21 (27, 28) (84% similarity over 67 amino acids) and Lactobacillus casei phage A2 repressor (29) (80% similarity over 76 amino acids). Sapi3_20 potentially encodes a glycoprotein similar to one found in bacteriophage A118 (30) (54% similarity over 123 amino acids) and is strongly similar to ORF 135 from the recently described S. aureus temperate phage dSLT carrying Panton-Valentine leukocidin (31) (85% similarity over 153 amino acids).

| Name        | Length (amino acids) | Predicted molecular mass (Da) | Corresponding ORFs in SaPI1 | Corresponding ORFs in SaPIbov | Description (homolog) | Expression detected in vitro | Expression detected in vivo | Expression detected in vivo |
|-------------|----------------------|-------------------------------|-------------------------------|-------------------------------|------------------------|-----------------------------|----------------------------|----------------------------|
| sapi3_1/ear | 152                  | 17,985                        | 1 (ear)                       | 1 (ear)                       | Enterotoxin (TstH)     | Y                           | Y                          | Y                          |
| seq         | 71                   | 7,184                         | seq                          | seq                          | Enterotoxin (TstH)     | Y                           | Y                          | Y                          |
| seb          | 87                   | 9,379                         | seq                          | seq                          | Enterotoxin (TstH)     | Y                           | Y                          | Y                          |
| sapi3_18    | 66                   | 6,575                         |                               |                               | (vapE)                 | Y                           | Y                          | Y                          |
| sapi3_19    | 110                  | 12,785                        |                               |                               | (cl-like repressor)    | Y                           | Y                          | Y                          |
| sapi3_20    | 152                  | 16,185                        |                               |                               | (ORF 153 of dSLT)      | Y                           | Y                          | Y                          |
| sapi3_21    | 143                  | 16,285                        |                               |                               | (ABC transporter)      | Y                           | Y                          | Y                          |
| sapi3_int   | 407                  | 47,695                        | int                          | int                          | Integrase              | Y                           | Y                          | Y                          |
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### TABLE II

Primers used to PCR-amplify SaPI3 microarray probes

| Gene name | Forward primer (5′-3′) | Reverse primer (5′-3′) |
|-----------|------------------------|------------------------|
| sapi3_1/ear | GTAGATTATCGGTACACGC | gatgggatctTAATTAGTTAGT |
| seh       | GAAATATTACTGCTCCGGA | gatgggatctCAGCTTTTTG |
| sapi3_3   | ATGAACAGATACCGGAAAGA | gatgggatctCAGCTTTTTG |
| sapi3_4   | GATCCCATGCTATGATGAAAACCAAAATAAAA | gatgggatctCAGCTTTTTG |
| sapi3_5   | CCATGGCATTAGTGGCGACCAC | gatgggatctCAGCTTTTTG |
| sapi3_6   | CCATGGCATTAGGAAACAAATGGCA | gatgggatctCAGCTTTTTG |
| sapi3_7   | AGCAATTAGTATATTATAAAAACA | gatgggatctCAGCTTTTTG |
| sapi3_8   | CCATGGCATTAGCGAAATCTGACGAA | gatgggatctCAGCTTTTTG |
| sapi3_9   | ATGATGCCCATGAAATTG | gatgggatctCAGCTTTTTG |
| sapi3_10  | CCATGGCATTAGTGAATGAAAGAAAATAATGAT | gatgggatctCAGCTTTTTG |
| sapi3_11  | CCATGGCATTAGTAATATATAATGAT | gatgggatctCAGCTTTTTG |
| sapi3_12  | TGGTAGTAGATGCGATGAG | gatgggatctCAGCTTTTTG |
| sapi3_13  | GTTATGATGTCCTGACAC | gatgggatctCAGCTTTTTG |
| sapi3_14  | cctgcgtATGAGAATATGATGAGAA | gatgggatctCAGCTTTTTG |
| sapi3_15  | cctgcgtATGAGAATACGAGGCAG | gatgggatctCAGCTTTTTG |
| sapi3_16  | cctgcgtATGAGAATACGAGGCAG | gatgggatctCAGCTTTTTG |
| sapi3_17  | cctgcgtATGAGAATACGAGGCAG | gatgggatctCAGCTTTTTG |
| sapi3_18  | cctgcgtATGAGAATACGAGGCAG | gatgggatctCAGCTTTTTG |
| sapi3_19  | cctgcgtATGAGAATACGAGGCAG | gatgggatctCAGCTTTTTG |
| sapi3_20  | cctgcgtATGAGAATACGAGGCAG | gatgggatctCAGCTTTTTG |
| sapi3_21  | cctgcgtATGAGAATACGAGGCAG | gatgggatctCAGCTTTTTG |
| sapi3_22  | cctgcgtATGAGAATACGAGGCAG | gatgggatctCAGCTTTTTG |
| sapi3_23  | cctgcgtATGAGAATACGAGGCAG | gatgggatctCAGCTTTTTG |
| sapi3_24  | cctgcgtATGAGAATACGAGGCAG | gatgggatctCAGCTTTTTG |
| seq       | ACGAAGAATACGAGGCAG | gatgggatctCAGCTTTTTG |
| seh       | AAAAGAATACGAGGCAG | gatgggatctCAGCTTTTTG |
| sapi3_int | ATTCGAAATACGAGGCAG | gatgggatctCAGCTTTTTG |

a The forward direction for each primer pair was determined by the orientation of the corresponding gene.
b Some forward primers incorporate a NcoI restriction site (lowercase characters).
c Reverse primers incorporate a BamHI restriction site (lowercase characters).

seq, or only in the maintenance and transfer of the island itself. We thus used DNA microarrays to examine the expression of all SaPI3 ORFs potentially encoding products over 50 amino acids in size to determine whether or not they were detectably expressed and whether their expression profiles were similar to those of the SaPI3-associated enterotoxins. DNA primers used to PCR-amplify probes for each ORF are listed in Table II.

Expression data for SaPI3 genes in various growth conditions are summarized in Table III. Because staphylococcal exotoxins are generally growth phase-regulated, we first examined the expression of SaPI3 genes, the cultures were exposed to microaerobic (1% O2 (v/v)) and aerobic (21% O2 (v/v)) growth conditions in 1-ml cultures of TH broth. There was no significant difference in growth parameters between the microaerobic and aerobic cultures because both cultures entered their respective growth phases (exponential, postexponential, and stationary) of interest at similar times. Also, differences in cell densities between the microaerobic and aerobic cultures were 2-fold or less in any given growth phase. In all, the expression of 18 of the 24 SaPI3 ORFs was detected. The expression of four, four, and three genes was significantly affected by growth in microaerobic conditions in the exponential, postexponential, and stationary phases of growth, respectively. The expression of seh was somewhat repressed in the exponential and postexponential phases of growth in aerobic conditions and was slightly up-regulated during stationary phase growth in aerobic conditions. The expression of no gene, however, was affected by more than approximately 2-fold.

To approximate in vivo conditions and determine those genes whose expression might be artificially enhanced by growth in rich laboratory medium, the expression of SaPI3 genes was examined during growth of MN NJ in TH broth versus rabbit serum. Effect of growth in serum was examined in the exponential, postexponential, and stationary phases of growth. Cells were quantified by both absorbance and dry weight. No difference in the time at which TH or serum cultures entered their respective growth phases of interest was observed. A600 nm values for S. aureus grown in TH medium were 0.83, 1.69, and 2.16 for the exponential, postexponential, and stationary phases of growth, respectively. A600 nm values for S. aureus grown in rabbit serum were 0.33, 0.97, and 1.27 for the same growth phases. As measured by dry weight, cell mass at 8 h was 8.0 mg/ml in TH medium and 8.3 mg/ml in rabbit serum. Quantification of S. aureus growth in serum in vivo is problematic because cells clump tightly and are resistant to dispersal, even by ultrasonication, thus preventing accurate counts by plating or absorbance. Alternatively, dry weight analysis is hampered by the tendency of S. aureus to bind serum components and thus add "artificial" cell mass. Thus, an effect of cell density on SaPI3 gene expression cannot be ruled out in these serum cultures. Taken as a whole, however, the data reflect the fact that growth in standard rich laboratory media may well enhance or repress expression of virulence-associated genes in a manner inconsistent with what occurs in vivo. In all, the expression of 19 of 24 SaPI3 ORFs was detected in these experiments. The expression of five, six, and three genes were affected by growth in serum as compared with TH

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3 J. M. Yarwood and P. M. Schlievert, unpublished observations.
### Table III

**Expression data for SaPI3 ORFs**

Bold and italic fonts are used to indicate those genes whose expression was significantly increased or decreased, respectively, in the test experimental condition (bold, italic font) as compared to the reference experimental condition (underline). Blank spaces indicated those ORFs whose expression was not detected under the growth conditions examined.

| Gene       | Growth phase | Microaerobic/aerobic | Serum/laboratory medium | In vivo/medium |
|------------|--------------|----------------------|-------------------------|----------------|
|            | P-exp/Stat  | Exp | P-exp | Stat | Exp | P-exp | Stat | N-Imm 2 h | Imm-2 h | Imm-8 h |
| sapi3_1 car | 1.7 ± 0.2\(^b\) | 1.1 ± 0.2 | -1.9 ± 0.1\(^b\) | 1.7 ± 0.1\(^b\) | 1.5 ± 0.1\(^b\) | -1.4 ± 0.3 | -3.3 ± 1.1\(^b\) | -2.0 ± 0.2\(^b\) | **3.4 ± 0.5\(^b\)** | **1.6 ± 0.1\(^c\)** | -1.4 ± 0.1\(^b\) |
| sec        | 6.4 ± 1.4\(^b\) | **8.2 ± 1.1\(^b\)** | -2.1 ± 0.2\(^b\) | -1.6 ± 0.2\(^b\) | **2.2 ± 0.5\(^b\)** | 1.3 ± 0.3 | -2.1 ± 0.1\(^b\) | -1.6 ± 0.2\(^b\) | **3.6 ± 0.2\(^b\)** | **1.8 ± 0.2\(^b\)** | -1.3 ± 0.1 |
| sapi3_3    | 1.3 ± 0.3 | 1.0 ± 0.1 | -1.3 ± 0.2 | 1.4 ± 0.1 | -1.1 ± 0.1 |
| sapi3_4    | 1.2 ± 0.2 | -1.1 ± <0.1 | 1.7 ± 0.3 | -1.4 ± 0.3 |
| sapi3_5    | 2.0 ± 0.5 | 1.2 ± 0.2 | 1.2 ± 0.1 | 1.1 ± 0.1 | 1.0 ± 0.2 | -1.1 ± 0.2 | 1.2 ± 0.1 | -1.5 ± 0.1 | 1.1 ± 0.2 | **1.3 ± 0.1\(^b\)** |
| sapi3_6    | 2.5 ± 0.5\(^b\) | **1.7 ± 0.3** | **1.2 ± 0.1**\(^b\) | 1.1 ± <0.1 | 1.5 ± 0.3 | -1.1 ± <0.1 |
| sapi3_11   | -1.2 ± 0.1 | -1.1 ± 0.1 | -1.2 ± 0.3 |
| sapi3_12   | -2.2 ± 0.3\(^b\) | 2.2 ± 0.5 | -1.2 ± 0.3 |
| sapi3_13   | 1.2 ± 0.1 | **1.7 ± 0.3**\(^b\) | 1.3 ± 0.3 | -1.1 ± 0.1 | -1.5 ± 0.1\(^b\) | -1.4 ± 0.1 | -1.2 ± 0.2 | -1.2 ± 0.1 |
| sapi3_14   | 1.3 ± 0.2 | 1.3 ± 0.2 | -1.3 ± 0.4 | -1.5 ± 0.2\(^b\) | -1.3 ± 0.1 | -1.3 ± 0.2 |
| sapi3_15   | 1.1 ± 0.1 | 1.2 ± 0.2 | 1.2 ± 0.1 | 1.1 ± 0.1 | 1.6 ± <0.1\(^b\) | -1.7 ± 0.1\(^b\) | -1.4 ± 0.2 | -1.5 ± 0.2\(^b\) | -1.6 ± 0.2\(^b\) | -3.7 ± 0.5\(^b\) |
| sapi3_19   | 1.8 ± 0.2\(^b\) | -2.4 ± 0.8\(^b\) | 1.6 ± 0.6 | -1.5 ± 0.1 | 1.0 ± 0.1 | 1.4 ± 0.2 | -1.5 ± 0.2 | -1.1 ± 0.1 | 1.0 ± 0.2 | -1.6 ± 0.2 | -1.3 ± 0.1 | -1.3 ± 0.2 |
| sapi3_20   | 1.5 ± 0.2\(^b\) | -2.7 ± 0.7\(^b\) | -1.1 ± 0.1 | -1.3 ± 0.1 | -1.2 ± 0.1 | **2.0 ± 0.2**\(^b\) | -1.4 ± 0.1 | -1.4 ± 0.2 | -1.3 ± 0.2 | -2.7 ± 0.7\(^b\) |
| sapi3_21   | -1.1 ± 0.2 | -1.5 ± 0.4 | 1.2 ± 0.1 | -1.2 ± 0.1 | **1.6 ± 0.1**\(^b\) | -2.2 ± 0.4\(^b\) | 1.1 ± 0.1 | -1.4 ± 0.2 | -1.4 ± 0.2 | -2.3 ± 0.5\(^b\) |
| seq        | -1.3 ± 0.3 | 1.2 ± 0.2 | 1.1 ± 0.1 | 1.4 ± 0.1 | -1.2 ± <0.1 | -1.4 ± 0.2 | -2.1 ± 0.5\(^b\) |
| sek        | 1.1 ± 0.1 | -1.3 ± 0.2 | -1.6 ± 0.1\(^b\) | 1.1 ± 0.1 | 1.6 ± <0.1\(^b\) | -1.7 ± 0.1\(^b\) | -1.4 ± 0.2 | -1.5 ± 0.2\(^b\) | -1.6 ± 0.2\(^b\) | -3.7 ± 0.5\(^b\) |
| sapi3_int  | -1.2 ± 0.1 | 1.6 ± 0.2\(^b\) | -1.3 ± 0.3 | 1.2 ± 0.1 | **2.4 ± 0.4**\(^b\) | -1.1 ± 0.2 | 1.9 ± 0.1 | -1.8 ± 0.1\(^b\) | -2.3 ± 0.2 \(^b\) | 

\(^a\) Exp, exponential phase growth; P-exp, postexponential phase growth; Stat, stationary phase growth; N-Imm, nonimmune; Imm, immune.

\(^b\) \(p < 0.05\), Student's \(t\) test.

\(^c\) \(p < 0.01\), Student's \(t\) test.
medium in the exponential, postexponential, and stationary phases of growth, respectively, although no gene was affected by more than 3.3-fold. In general, expression of genes on either end of the island was more consistently detected than sapi3_7-17.

Finally, we examined the expression of SaPI3 genes during incubation of MN NJ in vivo in a rabbit model of TSS using subcutaneous hollow polyethylene infection chambers (17, 36). The expression of SaPI3 genes in vivo was compared with that in the inoculum, which was harvested from cultures in the late exponential phase of growth and used to infect both nonimmune rabbits and rabbits immunized with SEB. All animals developed symptoms consistent with TSS, including hypotension, respiratory distress, and obvious discomfort in the vicinity of the infection chamber. The SEB-immunized rabbits experienced a delayed onset of symptoms and were euthanized 22 h after inoculation, whereas the nonimmune rabbits died several hours after inoculation. Cell densities recovered from the infection chambers did not vary more than 0.22 log units from the cell density of the inoculum before concentration (6.7 × 10⁸ colony-forming units/ml), thus we were able to effectively eliminate the potential effects of cell density and growth phase on gene expression in vivo. Using this subcutaneous infection model, we were previously able to demonstrate up to 18-fold changes in virulence-associated gene expression between cells in the inoculum and cells in vivo (36). The expression of 13 of 24 SaPI3 ORFs was detected in vivo. Sapi3_1 (ear) and seb were significantly up-regulated during incubation in vivo in the non-immune rabbits as compared with the inoculum and were affected very little by incubation in the immune animals. The expression of sek and seq was only significantly affected by incubation of MN NJ in the immune animals at 8 h, in which case they were repressed, together with sapi3_20, sapi3_21, and sapi3_24.

To determine whether any of the genes contained on SaPI3 might act in the regulation of or be co-regulated with the enterotoxins carried by the island, the genes were clustered according to the similarity of their expression profiles (Fig. 2). The expression profiles of the secreted toxin α-hemolysin (hla) and the surface molecule protein A (spa) in these experimental conditions are provided as representative virulence factors. To our knowledge, the presence and expression of hla and spa are not affected by the presence of SaPI3 or any of the other described pathogenicity islands. Three primary clusters of SaPI3 genes were observed. The expression profile of seb matched most closely with that of hla and not any of the other SaPI3 genes. In contrast, the expression of sek and seq was found to be most similar to that of the surrounding genes (sapi3_19-21 and sapi3_24). This clustering of sapi3_19-24 in their expression profiles is suggestive because the direction of transcription of these genes is opposite that of nearly all of the remaining genes (sapi3_2-18) on SaPI3. Finally, those genes centrally located in the island (sapi3_3-18, with the exception of sapi3_6), whose expression was detected only in a limited number of growth conditions, form a third group with variable expression patterns. Although it was consistently detected,
sapi3_1 (ear) did not group with any of the other genes examined.

Although the resolution of these array experiments did not allow a conclusive determination that any of the SaPI3 genes are co-transcribed, the proximity of several ORFs to one another and the clustering of their expression profiles are suggestive. For instance, sapi3_4 and sapi3_5, which are separated by only 2 nucleotides, cluster together, as do sapi3_19 and sapi3_20, separated by 11 nucleotides. Interestingly, sapi3_21 and seq, separated by 11 nucleotides, also have similar expression profiles. Genes sapi3_9-11 form a set of overlapping or nearly overlapping ORFs, but their expression was not detected in a sufficient number of conditions to make a conclusion regarding their possible co-transcription. Genes sapi3_14-18 also form a set of overlapping or nearly overlapping reading frames; however, only sapi3_15 and sapi3_16, which overlap by 13 nucleotides, cluster in their expression profiles. The presence of co-transcribed genes would not be unexpected if the pathogenicity island was indeed of phage origin. Co-transcribed genes are common in phage genomes because numerous products, such as structural elements, are encoded by polycistronic transcripts to achieve coordinate temporal regulation at critical points within the phage lytic cycle. The presence of several stem-loop structures that may serve as rho-independent transcription terminators is indicated in Fig. 1. The placement of these structures is consistent with those genes that are known or likely to be independently transcribed, such as seb, sapi3_1 (ear), sek, and int, as well as with those genes that may well be co-transcribed, such as sapi3_14-18.

In all, we were able to detect the expression of 22 of the 24 SaPI3 ORFs by microarray analysis (Table I). 13 ORFs were detectably expressed in vivo and in vitro, 9 ORFs were detectably expressed in vitro only, and 2 ORFs were not detectably expressed in any growth condition examined. The expression of genes toward either end of SaPI3 was more consistently detected than that of genes contained in the central region of the island. Consistent with a role for these islands in dissemination of staphylococcal enterotoxins, the expression of the toxins seb, sek, and seq was consistently detected under multiple growth conditions.

DISCUSSION

In the work presented here, we have described a novel pathogenicity island, SaPI3, in S. aureus strains COL and MN NJ. We propose that the newly identified island meets the consensus definitions of a pathogenicity island (7), including the presence of demonstrated virulence genes on the island (seb, sek, and seq), the lack of the island in closely related strains, the occupation of a relatively large genomic region (~16 kb), a lower GC content than the overall S. aureus genome (31.4% versus 32.8%), the presence of flanking direct repeats, and the presence of mobility factors (integrase). Many of the genes contained on the island are homologous to genes contained on described bacteriophages, suggesting that this SaPI1, like others described previously, is of bacteriophage origin. Furthermore, we have detected the expression of 22 of the 24 genes contained on the island, suggesting that many of them may be active in the maintenance of the island or in regulation of the associated enterotoxins.

Several explanations for the origin of the staphylococcal pathogenicity islands are possible. A large number of pathogenicity islands may circulate through the combined gene pool of numerous bacterial species with which staphylococci are transiently associated, in or on its human and animal hosts, and from which staphylococci acquires these elements through horizontal transfer. However, this hypothesis seems unlikely because superantigen production has been identified in a very limited number of bacterial species, suggesting that these elements are not in general circulation. The possibility cannot be eliminated completely, however, until the genomes of currently unidentified bacterial associates of staphylococci are characterized. A second hypothesis suggests that exotoxins are carried primarily by other genetic elements, such as plasmids and phage, and have integrated into sites of high recombination frequency in one or more ancestral strains. These recombination events might be mediated by homologous genes in the accessory genetic elements and the pathogenicity island, giving rise to the exotoxin-carrying islands. However, this hypothesis does not account for the origin of superantigen genes on transmissible elements, the apparently exclusive presence of certain superantigen genes on only pathogenicity islands, and the likely role for the demonstrably mobile pathogenicity islands in dissemination and evolution of superantigens. A third hypothesis, which we favor, combines certain elements of the first two, with the addition of the assumption of a common ancestral genetic element to SaPI1–4, SaPIbov, and SaPIm1/SaPIm1 and the continual generation of unique toxins and islands through modular recombination events.

Several features of the enterotoxin-encoding pathogenicity islands identified thus far suggest a common ancestral genetic element and that these islands have arisen in part through specialized transduction and recombination events. The overall layout of the islands, even specific genes, is similar, with exotoxins encoded on either end of the islands (the location of intH is nearly identical in SaPIn1/SaPIm1, SaPI1, and SaPIbov), and the exotoxin genes on the right end of the islands consistently located upstream of the integrase genes. Toward the center of the islands lie multiple genes of apparently phage origin. Furthermore, the region in SaPI3 between nucleotides 3113 and 6929 and nucleotides 9591 and 10,494 is nearly identical to regions in all of these islands. Thus, we propose a generalized model for the origin and evolution of staphylococcal pathogenicity islands (Fig. 3). The presence of the exotoxin genes on the left ends of the islands can be explained by a specialized transduction event involving an ancestral, exotoxin-free bacteriophage in either Staphylococcus or a bacterial species with which Staphylococcus has subsequently engaged in horizontal gene transfer. In this model, a bacteriophage element excised from the chromosome adjacent to a superantigen locus and through a mis-recombination event gained the enterotoxin while either simultaneously or subsequently losing the gen locus and through a mis-recombination event gained the enterotoxin while either simultaneously or subsequently losing a segment of phage DNA necessary for complete phage function (such as xis). It thus became dependent upon wild-type helper phage for excision, packaging, and/or mobilization, as observed with SaPI1. Indeed, the expression of seb, which is known to be regulated by the staphylococcal accessory gene regulator (agr) in vitro, did not correlate with the expression of any other gene contained on the island but rather correlated with the agr-regulated gene hla, which is not associated with SaPI3. This supports the hypothesis that these enterotoxins were incorporated into an existing phage.

The presence of exotoxins on the right end of the island, inside the integrase, may instead be due to addition, deletion, or mutual crossover of large pathogenicity island fragments or “modules.” The hypothesis of modular recombination events perhaps mediated by key regions of sequence homology is supported by both the presence of long stretches of homology between distinct regions of the islands and the expression data described in this work. Extensive regions of very high similarity (>95% identity), such as that between SaPI3 and SaPI1, are abruptly interrupted by regions with very little homology between the islands. Some of these regions appear to be derived from phages such as Panton-Valentine leukocidin-converting...
phage ϕSLT. Furthermore, SaPI1 and SaPI3 appear to have lost the sel/sek/sec3 module present on SaPIN1/SaPIN1 and SaPIbov, or, alternatively, SaPIN1/SaPIN1 and SaPIbov have gained these elements after diverging from a common ancestor. In addition, we found that we could consistently detect expression of genes on either end of the island, particularly sapi3_19-24. However, the “core” bacteriophage genes were much more difficult to detect and may only be strongly expressed upon mobilization of the island. Although most of these central genes have no definite homolog, they may well encode proteins involved in the structural components of the original phage. In phage λ, for instance, genes encoding head and tail components are located together at the end of the prophage opposite the int and xis genes (37). Thus, these genes may only be expressed when the pathogenicity island is mobilized. Alternatively, the promoter regions for these genes may be defective or even absent, preventing the expression of these genes at any time and requiring the structural components of a helper phage for transfer. The observation, then, that sapi3_19-24 are transcribed opposite to sapi3_3-18 and cluster in their expression

profiles implies co-regulation of these genes and the addition of this enterotoxin module to the already existing pathogenicity island. Interestingly, the DNA sequence containing sapi3_3-17 is highly conserved between SaPI1 and SaPI3 and, to a lesser extent, SaPIbov. It is not yet clear whether the size and type of these modules might be constrained by recombination events that require conserved stretches of DNA sequences in the phage genomes or are more or less randomly generated. The possibility also cannot be excluded that staphylococcal pathogenicity islands have arisen in part as a result of horizontal transfer from another species. Indeed, streptococcal pyrogenic exotoxin A, a phage-encoded superantigen, appears to be more closely related to staphylococcal enterotoxins SEB, SEC3, and SEG, than other streptococcal superantigens (4).

We also propose that the promiscuity of these islands and their tendency to undergo recombination events underlie the evolutionary divergence of staphylococcal superantigens. The presence of an ancestral island in multiple staphylococcal lineages undergoing separate genetic events (e.g., point mutations, genetic rearrangements, and insertion of other mobile genetic
elements) and evolutionary pressures in various communities and hosts would lead to a great diversity of exotoxin genes as well as pathogenicity islands (Fig. 3). Further recombination events upon mixing of these various staphylococcal lineages would lead to even greater diversity among the pathogenicity islands and perhaps other mobile genetic elements as well. However, the evolutionary fitness that superantigenic toxins confer to the recipient strains is not yet completely understood. It is clear that these superantigens have significant immunodepressive effects, including inducing anergy and deletion of a large population of T cells (38–40) as well as preventing the development of antibody to the toxin itself (41–44). There is also evidence that superantigens destroy endothelial cells (45) and that they may exclude neutrophils from infection foci (46–48). Thus, superantigens likely confer immunological protection to their host strains and perhaps prevent clearance of those strains from the human host.

The demonstrated mobility of SaPI and the presence of apparently functional integrase in all of these islands (5, 9, 10) provide further support for the hypothesis that they remain a significant part of the evolutionary scheme of S. aureus and will likely give rise to new enterotoxins and pathogenicity islands. The mobility of SaPIs has led to speculation that these islands represent relatively “young,” recently acquired genetic elements, as opposed to islands that likely entered their host organisms millions of years ago and have become relatively stable. Development of new superantigens and pathogenicity islands and the presence of the SaPIs become apparent functional integrases in all of these islands (5, 9, 10) and that they may enter from neutrophils from infection foci (46–48). Thus, superantigens likely confer immunological protection to their host strains and perhaps prevent clearance of those strains from the human host.

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As additional S. aureus genome sequences and SaPIs become available and are associated with various staphylococcal lineages and infection types, it may become easier to identify a common ancestor of staphylococcal pathogenicity islands and superantigens. Regardless, continued research into the mechanisms of superantigen and SaPI evolution and acquisition will allow more accurate epidemiological investigations as well as a greater understanding of which potential therapeutic interventions might be viable, particularly in the development of toxoid vaccines.

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