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Chromosomal Mapping of a Gene Affecting Enterotoxin A Production in *Staphylococcus aureus*

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In a previous study, transformation demonstrated that a gene governing enterotoxin A production (*entA*$^+$) in *Staphylococcus aureus* strain S-6 was located on the chromosome between the *purB110* and *ilv-129* markers; in contrast, the *entA*$^+$ gene of strain FRI-196E was shown not to be located in the same position. In the current study, 54 enterotoxin A-producing strains of *S. aureus* were examined to locate the *entA*$^+$ gene. Conventional transformation procedures and a series of multiply marked derivatives of NCTC 8325 were used as recipients for chromosomal mapping. Of the 54 strains tested, 23 were found to contain the *entA*$^+$ gene at the original locus between the *purB110* and *ilv-129* markers. Twenty-seven strains could not be analyzed either because their DNA was genetically ineffective in transforming strain 8325 (23 strains), or Pur$^+$ Ilv$^+$ transformants could not be recovered (four strains). Four other strains contained an *entA*$^+$ gene that could not be located in any of the chromosomal linkage groups. A new insertion site for Tn*551* was located within the *hla*$^+$ gene involved in alpha-toxin production. It eliminated alpha-toxin production and was used to separate the *entA*$^+$ gene from the *hla*$^+$ marker in the *purB110-ilv-129* region. This segment of the chromosome is shown to consist of the *purB110*, *entA*$^+$, *hla*$^+$, and *ilv-129* markers in that order.

Staphylococcus aureus produces a number of extracellular toxins including the cytolytic or membrane-damaging toxins, leucocidin, exfoliative toxin, and the enterotoxins. There are at least five immunologically distinct enterotoxins, designated A, B, C, D, and E. These *S. aureus* enterotoxins are a major cause of food poisoning resulting from the ingestion of preformed enterotoxins from food. Recently, Bergdoll et al. (2) described a new enterotoxin, enterotoxin F, that is produced by strains of *S. aureus* isolated from cases of toxic-shock syndrome; it has been postulated that a causal relationship may exist between exterotoxin F and many of the symptoms of toxic shock. Staphylococcal enterotoxin A has been the toxin most frequently associated with food poisioning outbreaks caused by *S. aureus* (5).

A genetic determinant that affects enterotoxin A production (the *entA*$^+$ gene responsible for the EntA$^+$ phenotype) has been shown by Shafer and Iandolo (11) to be chromosomally located in two strains of *S. aureus*. One EntA$^+$ strain (FRI-100) contained no detectable plasmids, and a second strain (S-6) could be cured of its single plasmid species and still produce enterotoxin A. Pattee and Glatz (8) identified a chromosomal locus for the *entA*$^+$ gene in one strain of *S. aureus* (S-6) but were unable to find a locus for the *entA*$^+$ gene in a second strain (FRI-196E). The DNA from a third strain (FRI-100, a nontypable strain) failed to transform strain 8325, presumably because of restriction barriers, and thus could not be analyzed. The *entA*$^+$ gene in strain S-6 was located between the *purB110* and *ilv-129* markers of linkage group III in transformation analysis by using derivatives of NCTC 8325, a phage group III strain, as recipients. The relative locations of the *entA*$^+$ gene and a determinant for alpha-toxin production (*hla*$^+$) could not be ascertained in the study because of two minor classes of recombinants that could be explained by quadruple crossover events or by the possibility that either the *hla*$^+$ or *entA*$^+$ gene or both resides on transposable genetic elements.

The present study was initiated to determine the location of the *entA*$^+$ gene in a greater number of enterotoxin A-producing strains of *S. aureus*. The objectives were to evaluate the diversity of genomic sites of *entA*$^+$ in a greater number of strains and to distinguish between the *hla*$^+$ and *entA*$^+$ loci on the chromosome.

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TABLE 1. Designation, genotype, and origins of *S. aureus* strains[a]

Stock no.	Genotype	Origin and reference
ISP182	8325 *thy-101 thrB106 ilv-129 tmn-3110 purB110 pig-131*	(10)
ISP193	8325 *thy-101 thrB106 ilv-129 pig-131* Ω11[Chr::Tn*551*]	(8)
ISP467	8325 *thy-101 ala-126 pig-131* Ω34[Chr:Tn*551*] *rib-127 tmn-3106*	(8)
ISP479	8325-4 (pI258 *bla-401 mer-14 repA36*) *pig-131*	(7)
ISP483	8325 *uraA141 hisG15 nov-142 mec-4916 pig-131*	(8)
ISP484	8325 *thy-101 thrB106 ilv-129 tmn-3106 purB110 pig-131* Ω40[Chr::Tn*551*]	(8)
ISP546	8325-4 *pig-131 hla-316*::Tn*551*	ISP479 grown on Em at 43°C[b]
ISP730	8325 *thy-101 thrB106 ilv-129 tmn-3110 purB110 pig-131 hla316*::Tn*551*	ISP182 × ISP546 DNA[c]

[a] Several of these strains carry Tn*551*, a transposon conferring resistance to erythromycin (10).
[b] ISP479 grown at 43°C on brain heart infusion agar containing 20 μg of erythromycin per ml to rid the strain of its thermosensitive plasmid and to select for the incorporation of the Tn*551* transposon into the chromosome (7).
[c] Strain ISP182 transformed with DNA from ISP546.

MATERIALS AND METHODS

Bacteria. Several of the *S. aureus* strains used in this study, with their origins and genotypes, are given in Table 1. Also used in this study were 63 enterotoxin A-producing strains of *S. aureus* provided by Merlin S. Bergdoll (Food Research Institute, University of Wisconsin, Madison).

Culture media. Commercially available dehydrated culture media and various formulations of complete defined synthetic agar (6, 8–10) were used in this study. All media on which transformants were grown were supplemented with 5 μg each of adenine, guanine, cytosine, and uracil per ml and 20 μg of thymine per ml unless otherwise stated.

The enterotoxin assays were performed on N–Z amine agar which consisted of 3% N–Z amine NAK (Humko Sheffield Chemical Company, Norwich, N.Y.), 1% yeast extract, and 1.8% agar (Difco Laboratories, Detroit, Mich.). Hemolysin assays were performed either on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% (vol/vol) citrated sheep blood or on prepared Columbia agar with 5% sheep blood (Remel Regional Media Laboratories, Lexena, Kans.). The Columbia agar was used without the purine or pyrimidine supplements in studies with transformants.

Transformations and preparation of transforming DNA. The transformation procedures used and the methods of preparation of transforming DNA have been described (8, 9). Mapping of the *entA*$^+$ gene was done as described by Pattee and Glatz (8). The known linkage groups of *S. aureus* strain 8325 were divided into overlapping segments, with each segment being limited by readily scored or readily selected markers (Fig. 1). The analysis of these segments was then performed by transforming appropriate EntA$^-$ recipient strains with DNA from the EntA$^+$ FRI strains. Transformants that had acquired the donor markers at each end of the DNA segment in question were tested for acquisition of the EntA$^+$ characteristic. The transformation protocol followed is outlined in Table 2, and the genetic markers used in this study, along with their phenotypes and the compositions of the media used in their selection and scoring, are listed in Table 3. The Hla phenotype was assayed as described by Pattee and Glatz (8).

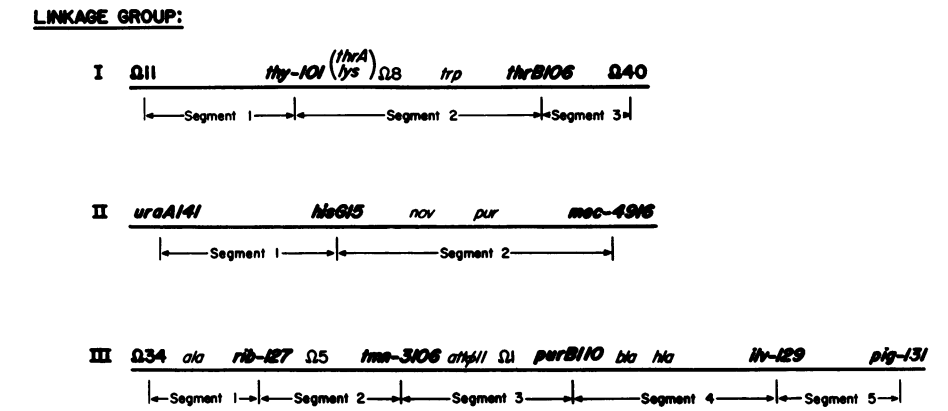

FIG. 1. The three known linkage groups of the *S. aureus* chromosome. The selected and scored markers used to screen these linkage groups for the presence of interior markers are shown in boldface print, and each linkage group is divided into segments as shown (modified from reference 8).

TABLE 2. Transformation protocol followed to identify enterotoxin A determinants within the known linkage groups of the chromosome of *S. aureus*

Stock no. of recipient strain[a]	Selected marker	Unselected phenotype scored	Linkage group/ segment examined[b]
ISP193	*thy*$^+$	Em^s	I/1
ISP467	*rib*$^+$	Em^s	III/1
		Tmn^s	III/2
ISP483	*his*$^+$	Ura$^+$	II/1
		Mec^s	II/2
ISP484	*thr*$^+$	Thy$^+$	I/2
		Em^s	I/3
	pur$^+$	Tmn^s	III/3
		Ilv$^+$	III/4
		Pig$^+$	III/4+5

[a] See Table 1 for genotypes.
[b] See Fig. 1.

The cotransfer frequency by transformation of two markers having individual transfer frequencies of A and B has been expressed as the estimated cotransfer frequency, which is AB/A or AB/B. Map distances between markers have been expressed as 1 − cotransfer frequency.

Enterotoxin assays. Gel double diffusion enterotoxin assays were performed by the method described by Pattee and Glatz (8), which employs an agar plate containing specific antiserum in a center well surrounded by spot inoculations of known enterotoxin producers and the *S. aureus* strains to be tested.

RESULTS

Analysis of the chromosomal segments for the *entA*$^+$ gene. To determine the location of the *entA*$^+$ gene, it was first necessary to find EntA$^+$ donor strains that were compatible with the ISP recipient strains in transformation experiments. A preliminary survey of the 63 EntA$^+$ FRI strains for their phage group revealed that 54 of the strains were either nontypable (11 strains), in phage group III (41 strains), or in combined phage groups (including group III, 2 strains). These strains were the most likely to be successful donors of the *entA*$^+$ gene in transformations using, as recipients, ISP strains that were multiply marked derivatives of NCTC 8325, a phage group III strain. Limited interstrain transformations have shown that linkage groups are highly preserved among phage group III strains and that good genetic compatibility among these strains is observed when competent recipient strains are used (12; Y. Limpa-Amara, M.S. thesis, Iowa State University, Ames, 1978). In contrast, genetic compatibility is poor between strains from different phage groups.

The 54 strains were first used as donors in transformation experiments with strain ISP484 as the recipient to determine if the *entA*$^+$ gene in these strains was located in segment 4 of linkage group III, between the *purB110* and *ilv-129* markers as in strain S-6. Twenty-seven of the donor strains could not be analyzed for the

TABLE 3. Genetic markers used in this study, their phenotypes, and the compositions of media used for their selection and scoring

Marker	Phenotype	Modification of complete defined synthetic agar for selection and scoring
Ω11, Ω34, Ω40[a]	Em^r (erythromycin resistance)	Addition of either 1 μg (for selection) or 10 μg (for scoring) of erythromycin per ml
hisG15	His$^-$ (histidine requirement)	Omission of histidine
hla-316::Tn*551*[a]	Hla$^-$ (no alpha toxin)	Scored as no hemolysis on sheep blood agar (see text)
	Em^r (erythromycin resistance)	See Em^r phenotype (above)
ilv-129	Ilv$^-$ (isoleucine-valine requirement)	Omission of isoleucine and leucine; valine reduced to 20 μg/ml; 1% (wt/vol) sodium pyruvate added
mec-4916	Mec^r (methicillin resistance)	Addition of 5% NaCl and 6.25 μg of methicillin per ml
pig-131	Pig$^-$ (inability to elaborate yellow pigment)	No modification necessary
purB110	Pur$^-$ (purine requirement)	Omission of adenine and guanine
uraA141	Ura$^-$ (uracil requirement)	Omission of cytosine and uracil
rib-127	Rib$^-$ (riboflavin requirement)	Omission of riboflavin
thrB106	Thr$^-$ (threonine requirement)	Omission of threonine
thy-101	Thy$^-$ (thymine requirement)	Omission of thymine
tmn-3106, *tmn-3110*	Tmn^r (tetracycline and minocycline resistance)	Addition of either 1 μg (for selection) or 3 μg (for scoring) of tetracycline per ml

[a] Specific chromosomal insertion sites for Tn*551*, a transposon originating from the pI258 plasmid and conferring resistance to erythromycin.

entA$^+$ gene in this segment because no Pur$^+$ Ilv$^+$ transformants were obtained in crosses involving these strains. Transformations with DNA from 23 of these strains (15 phage group III, 6 nontypable, and 2 miscellaneous) yielded no transformants when Pur$^+$ or Thy$^+$ transformants were selected, probably because of restriction barriers. Transformations with the four additional strains (two phage group III and two nontypable) produced Pur$^+$ transformants but no Pur$^+$ Ilv$^+$ double transformants. Of the remaining 27 strains tested, 23 (21 phage group III and 2 nontypable) possessed an *entA*$^+$ gene between the *purB110* and *ilv-129* markers, and 4 (3 phage group III and 1 nontypable) did not contain a demonstrable *entA*$^+$ gene in this region. In these experiments, six Pur$^+$ Ilv$^+$ transformants from each transformational cross were tested for the EntA phenotype (8). Because congression, the uptake of unlinked markers, is not seen in the *S. aureus* transformation system (9, 12), the cotransformation of markers indicates their physical proximity on the chromosome and signifies that all genetic material between these markers probably has been transformed as well. Therefore, almost all of the transformants that have acquired both the *pur*$^+$ and the *ilv*$^+$ markers also will have acquired all of the genetic material between these markers, and the testing of only six double transformants is sufficient to detect interior markers. In all of the transformations in this study in which the *entA*$^+$ gene was located between the *purB110* and *ilv-129* markers, at least five of the six transformants tested exhibited the EntA$^+$ phenotype.

The four strains (FRI-610, FRI-708, FRI-710, and FRI-1000) in which the *entA*$^+$ gene could not be detected in the *purB110-ilv-129* region also were tested for the presence of *entA*$^+$ in other segments of the staphylococcal chromosome in a series of transformations performed according to the protocol outlined in Table 2. None of the transformations with these strains produced Rib$^+$ Em^s transformants, so segment 1 of linkage group III could not be analyzed. Segment 5 in linkage group III could not be analyzed in FRI-610 because the lack of a strong Pig$^+$ phenotype in this strain prevented the production of easily identifiable Pur$^+$ Pig$^+$ transformants. Six isolates from all other possible classes of transformants were tested from strains FRI-610 and FRI-710. Extremely low transformation frequencies made it impossible to obtain six transformants in each class when donor DNA from FRI-708 and FRI-1000 was used. Table 4 shows the number of transformants examined for the *entA*$^+$ gene per segment for all four strains. None of the transformants obtained by utilizing DNA from these four strains were found to be EntA$^+$; therefore, the *entA*$^+$ gene could not be located in these four strains.

Analysis of the *purB110-entA*$^+$*-ilv-129* region. Determinants for beta-lactamase (*bla*$^+$) and alpha-toxin (*hla*$^+$) have been located, along with the *entA*$^+$ gene, between the *purB110* and *ilv-129* markers (1, 8). Pattee and Glatz (8) established that the *bla*$^+$ gene was located between the *purB110* and *entA*$^+$ markers but were unable to determine the relative positions of the *hla*$^+$ and *entA*$^+$ genes. Further investigation of the order of these markers was aided by using a newly isolated insertion of Tn*551*, a transposon that confers erythromycin resistance (Em^r) (16). This insertion is designated *hla-316*::Tn*551*; the evidence for this marker being an insertion of Tn*551* into the *hla*$^+$ gene is presented later in this section. Strain ISP730 (Pur$^-$ EntA$^-$ Hla$^-$ Em^r Ilv$^-$), which carries the *hla-316*::Tn*551* insertion (Em^r phenotype), was transformed with DNA from FRI-371 (Pur$^+$ EntA$^+$ Hla$^+$ Em^s Ilv$^+$). Original selection was for Pur$^+$ and Ilv$^+$ transformants. The Pur$^+$ transformants were tested for Ilv and Em, and the Ilv$^+$ transformants were tested for Pur and Em. The results of this transformation (Table 5) clearly placed *hla-316*::Tn*551* (Em^r) between the *purB110* and *ilv-129* markers. Whereas 223 of 310 Pur$^+$ transformants that remained Ilv$^-$ (class B) and 75 of

TABLE 4. Analysis for the *entA*$^+$ gene by transforming[a] EntA$^-$ strains with DNA from four EntA$^+$ FRI strains[b]

Donor (FRI no.)	No. of transformants examined for EntA$^+$ per linkage group/segment:									
	I/1	I/2	I/3	II/1	II/2	III/1	III/2	III/3	III/4	III/4 + 5
610	6	6	6	6	6	0	6	6	6	—[c]
708	4	1	0	0	6	0	1	1	6	1
710	6	6	6	6	6	0	6	6	6	6
1000	5	6	1	3	6	0	1	0	6	0

[a] See Table 2 for transformation protocol.

[b] Strains yielding no EntA$^+$ transformants in analysis of *purB110-ilv-129* region (segment III/4).

[c] —, FRI-610 lacked a strong Pig$^+$ phenotype, so segment III/4 + 5 could not be analyzed by using DNA from this strain.

TABLE 5. Transformation of strain ISP730[a] (Pur⁻ EntA⁻ Hla⁻ Em^r Ilv⁻) with DNA from strain FRI-371 (Pur⁺ EntA⁺ Hla⁺ Em^s Ilv⁺)

Phenotype (no. of transformants analyzed for unselected phenotypes)	No. (%) of recombinants detected in class[b]:						Frequency[c] of:	
	A	B	C	D	E	F	Reversion	Transformation
Pur⁺ (411)	87 (21.2)	223 (54.3)	101 (24.6)			0	8	320
Ilv⁺ (173)			16 (9.2)	75 (43.4)	82 (47.4)	0	<4	133

[a] See Table 1 for genotype.
[b] Class A, Pur⁺ Em^r Ilv⁻; B, Pur⁺ Em^s Ilv⁻; C, Pur⁺ Em^s Ilv⁺; D, Pur⁻ Em^s Ilv⁺; E, Pur⁻, Em^r Ilv⁺; F, Pur⁺ Em^r Ilv⁺.
[c] Expressed as transformants recovered per 10^9 colony-forming units; 1.3×10^9 colony-forming units and excess DNA were used per ml of the transformation suspension.

157 Ilv⁺ transformants that remained Pur⁻ (class D) had acquired the Em^s phenotype, all of the Pur⁺ Ilv⁺ transformants were Em^s regardless of whether the primary selection was for the Pur⁺ or the Ilv⁺ phenotype.

Representative transformants from classes A, B, and C were then analyzed for Hla by spot inoculations on 5% sheep blood agar plates. The results (Table 6) show a small but clear separation of the *entA*⁺ gene from the *hla*⁺ gene and *hla-316*::Tn*551*. Of 87 Pur⁺ transformants that remained Em^r and Ilv⁻ (class A), all 87 also remained Hla⁻, but 7 became EntA⁺. This places the *entA*⁺ gene between the *purB110* and *hla-316*::Tn*551* markers.

The results of this study also demonstrate that *hla-316*::Tn*551* is an insertion of Tn*551* into the *hla*⁺ gene. Although all 87 of the Pur⁺ Em^r Ilv⁻ transformants (class A) remained Hla⁻, 100 of 101 Pur⁺ Em^s Ilv⁻ transformants (class B) acquired the Hla⁺ phenotype when they lost the Em^r marker. The single Hla⁻ isolate in class B was also EntA⁻. Also consistent with the designation of the *hla* gene as *hla-316*::Tn*551* is the fact that ISP730 and ISP546, strains carrying the insertion, are Hla⁻, whereas their parent strains, ISP182 and ISP479, are Hla⁺. Figure 2 shows the relationship among the *purB110*, *entA*⁺, *hla*⁺, and *ilv-129* markers.

DISCUSSION

In 23 of 27 enterotoxin A-producing strains of *S. aureus* tested by transformation, the *entA*⁺ gene was located on the chromosome between the *purB110* and *ilv-129* markers (segment 4 of linkage group III). The *entA*⁺ gene could not be located in four EntA⁺ strains. It was impossible to analyze segment I of linkage group III in these four strains in the current study because of the inability to obtain Rib⁺ Em^s transformants, but Pattee and Glatz (8) were able to study this segment in strain FRI-196E and were unable to detect an *entA*⁺ gene there. It is possible that the *entA*⁺ gene in these four strains is located in a region of the chromosome that has not yet been mapped. The development of a more complete map of the *S. aureus* chromosome is necessary before this possibility can be fully tested.

The possibility of a plasmid-borne *entA*⁺ genes merits attention. Preliminary evidence not detailed here has indicated that only one of the four strains with unlocated *entA*⁺ genes possesses detectable plasmid DNA. A single plasmid band appeared in agarose gel electrophoresis of cleared lysates only of strain FRI-1000. This observation argues against the possibility of the *entA*⁺ gene being plasmid-associated in at least three of the four strains. In addition, Pattee and Glatz (8) noted that both EntA⁺ and spontaneous EntA⁻ isolates of strain FRI-196E contained a single identical plasmid species. Shafer and Iandolo (11) ruled out the involvement of plasmids in enterotoxin A production by strains FRI-100 and S-6. Thus, the evidence continues to support the notion that enterotoxin A production is controlled by chromosomal genes.

S. Khan and R. Novick (Plasmid 3:237–238, 1980) and E. Israeli, E. Kaufmann, and A. Shafferman (Israel J. Med. Sci. 16:470) have

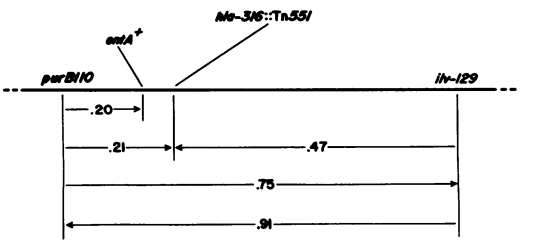

FIG. 2. Linkage relationships among the *purB110*, *entA*⁺, *hla-316*::Tn*551*, and *ilv-129* markers. Numerical values are 1 − cotransfer frequency calculated from the transformation frequencies. Arrows point from the selected to the unselected markers.

TABLE 6. Analysis of the *entA*$^+$ and *hla*$^+$ determinants in Pur$^+$ transformants obtained by transforming[a] strain ISP730[b] (Pur$^-$ EntA$^-$ Hla$^-$ Em^r Ilv$^-$) with DNA from strain FRI-371 (Pur$^+$ EntA$^+$ Hla$^+$ Em^s Ilv$^+$)

Transformant class	No. tested	No. (%) of transformants exhibiting the phenotype:			
		EntA$^+$	EntA$^-$	Hla$^+$	Hla$^-$
A (Pur$^+$ Em^r Ilv$^-$)	87	7 (8%)	80 (92%)	0 (0%)	87 (100%)
B (Pur$^+$ Em^s Ilv$^-$)	101	100 (99%)	1 (1%)	100 (99%)	1 (1%)
C (Pur$^+$ Em^s Ilv$^+$)	100	100 (100%)	0 (0%)	96 (96%)	4 (4%)

[a] See Table 5 for description of the transformation.
[b] See Table 1 for genotype.

recently reported that a plasmid, pSN2, containing a determinant for enterotoxin B production does not contain the structural gene for enterotoxin B. Dyer and Iandolo (3) speculated that pSN2 has a regulatory role in enterotoxin B expression. Similarly, the structural and regulatory genes for enterotoxin A might be widely separated on the chromosome in the FRI strains that failed to produce EntA$^+$ transformants. The transformants may have obtained either a structural or a regulatory gene for enterotoxin A production from the EntA$^+$ donor strains but did not obtain both genes and thus remained EntA$^-$.

Pattee and Glatz (8) were unable to determine the relative positions of the *entA*$^+$ and *hla*$^+$ genes. These genes were shown to be clearly separable in the current study through transformation experiments with a strain containing *hla-316*::Tn*551*. The separation of these two genes was determined by the occurrence of a class of recombinants (class A, Table 6) containing a substantial percentage (8%) of EntA$^+$ isolates but no Hla$^+$ isolates.

The question of whether the *entA*$^+$ and *hla*$^+$ genes reside on transposable elements remains unresolved. The variability previously reported for the Hla (1, 4, 8) and EntA (8) characteristics and the fact that strains belonging to linkage group III carry insertion sites for a number of genetic elements (10) support this possibility. However, this study shows that, at least in the majority of strains, the *entA*$^+$ gene is found in a consistent location on the *S. aureus* chromosome.

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