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Staphylococcus Aureus Determined by DNA-Mediated Transformation

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GENE LINKAGES IN PHAGE GROUP 2 STAPHYLOCOCCUS AUREUS DETERMINED BY DNA-MEDIATED TRANSFORMATION

SCOTT M. MARTIN, STEVEN C. SHOHAM, MARGARET ALSUP, and MARVIN ROGOLSKY, Department of Biology and School of Medicine, University of Missouri at Kansas City, Kansas City, MO 64110

Abstract. A large pool of antibiotic resistant and auxotrophic mutants was isolated from the phage group 2 strains UT0002-19 and UT0017 of Staphylococcus aureus. Strain UT0002-19 has a chromosomal determinant for exfoliative toxin (ET), which causes "scalded skin syndrome" in man. Strain UT0017 is ET-negative. DNA-mediated transformation, which employed phage 80a to induce competence, was utilized to detect gene linkages in the two strains. Three linkage groups were identified on the strain UT0017 chromosome. The first linkage group was thy-4 — lys-5 — trp-21 — thr-4, the second was pyr-26 — nov 9 — his-3, and the third consisted of ilv-9 and pen-1. Two linkage groups were detected on the strain UT0002-19 chromosome. The first was thy-1 — lys-5 — trp-3 — thr-4 —ala-8, while the second consisted of nos-9 and his-3. A locus for ET synthesis could not be mapped.

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Staphylococcus aureus is a resident microbe on the integument and in the upper respiratory tract and gastrointestinal canal of man. Although the bacterium is often a harmless commensal, it also is an opportunistic pathogen capable of effecting a wide variety of infections in individuals or sites having a lowered host-resistance, especially in infants and the aged. A number of S. aureus strains that cause SSS may contain either chromosomal or extrachromosomal genes for ET synthesis, or both (Rogolsky et al 1976). The protein products specified by the chromosomal and plasmid loci are antigenically distinct (Wiley and Rogolsky 1977). The mapping of chromosomal genes for ET synthesis would provide important information about the molecular regulation of this toxin. Until recently, generalized transduction (Cavallo and Terranova 1955) was the sole means of investigating the genetic organization of the S. aureus chromosome. Although this technique has been useful for fine-structure analyses (Barnes et al 1971, Kloos and Pattee 1965, Pattee et al 1974, Proctor and Kloos 1970), it nonetheless is severely limited as a genetic tool because only a small fragment of DNA is transferred to the recipient. In 1972, it was reported that the phage group 3 strain S325 could become competent and undergo both transformation (Lindberg et al 1972) and transfection (Sjöström et al 1972). Strain S325 is the propagating strain for typing phage 47 (Novick and Bouanchaud 1971). The factors that affect competence in this strain are now

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well characterized (Rudin et al. 1974, Sjöström and Philipson 1974, Thompson and Pattee 1977). Strain 8325 is lysogenic for the temperate phage $11$. As it grows in broth, it liberates free phage that, in the presence of calcium cations, interact with recipient cells in some manner to mediate the movement of transforming DNA across the cell surface. Staphylococcal transformation has been used to define 3 distinct linkage groups on the strain 8325 chromosome (Kuhl et al. 1978, Pattee 1976, Pattee and Neveln 1975, Pattee et al. 1977).

Thompson and Pattee (1977) reported that phage 80a could be used as a helper phage to induce competence in the phage group 2 propagating strains, Ps3A, Ps3C, Ps55, and Ps71. This was a significant discovery, for with the exception of strain 8325, \textit{S. aureus} strains are not naturally competent. Furthermore, intergroup transformations are routinely unsuccessful. This inability to perform intergroup transformations in \textit{S. aureus} may be ascribed to restriction barriers, which are known to operate during transduction (Meijers and Stobberingh 1978), and which are the basis for phage group typing of staphylococci (Parker 1972). In the present study, phage 80a was used in DNA-mediated transformation to detect gene linkages on the phage group 2 staphylococcal chromosome by cotransformation frequencies. An attempt was also made to map the chromosomal determinant(s) for ET synthesis. Since the markers present in the phage group 3 strain 8325 were unavailable for mapping studies, a battery of antibiotic resistant and auxotrophic mutants was isolated from phage group 2 staphylococcal preparatory to performing the mapping work.

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial strains and phage.} Strain UTO002 is a phage group 2 (55/71) clinical isolate of \textit{S. aureus} that contains both chromosomal and plasmid genes for ET synthesis (Rogolsky et al. 1976). UTO002-19 is a strain of UTO002, which has been heat cured of a 56 S virulence plasmid that carries loci for the synthesis of ET and for a bacteriocin active against a number of gram-positive bacteria (Rogolsky et al. 1976, Warren et al. 1974). Strain UTO002-19 remains toxinogenic (Tox$^+$) because it still contains the chromosomal gene(s) for ET synthesis. Strain UTO017 is a phage group 2 (3B/3C/55/71) \textit{S. aureus} that is the propagating strain of strain UTO002-19.

\begin{table}[h]
\centering
\caption{Strains of Staphylococcus aureus that synthesize exfoliative toxin.}
\begin{tabular}{lll}
\hline
Strain & Genotype & Derivation** \\
\hline
UTO002-19 & pen-1 & Rogolsky et al. (1976) \\
MR11 & pen-1 & Single clone from UTO002-19 \\
MR2 & ery-3 & MR1 selected on Ery GP \\
MR3 & lin-3 & MR1 selected on Lin GP \\
MR5 & nov-1 & MR1 selected on Nov GP \\
MR6 & ole-3 & MR1 selected on Ole GP \\
MR7 & rif-1 & MR1 selected on Rif GP \\
MR8 & tet-4 & MR1 selected on Tet GP \\
MR9 & aly-8 & MR1 mutagenized with NTG + MecEN \\
MR10 & gly-1 & MR1 mutagenized with NTG \\
MR11 & his-3 & MR1 transformed with DNA from MR112 \\
& & nov-9 \\
MR12 & ivi-1 & MR1 mutagenized with NTG \\
MR18 & lys-5 & MR23 transformed with DNA from MR126 \\
MR19 & lys-7 & MR1 mutagenized with NTG \\
MR20 & met-1 & MR1 mutagenized with NTG + MecEN \\
MR21 & pur-6 & MR1 mutagenized with NTG \\
MR22 & thr-4 & MR23 transformed with DNA from MR135 \\
MR23 & thy-1 & MR1 selected with trimethoprim \\
MR24 & trp-3 & MR1 mutagenized with NTG \\
\hline
\end{tabular}
\end{table}

*The pen-1 marker that specifies $\beta$-lactamase production is indigenous to all derivatives of UTO002-19.

**GP, gradient plate; NTG, N-methyl-N'-nitro-N-nitrosoguanidine; MecEN, methicillin enrichment.

\textbf{Table 1}

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Genotype</th>
<th>Derivation**</th>
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<tbody>
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<td>UTO002-19</td>
<td>pen-1</td>
<td>Rogolsky et al. (1976)</td>
</tr>
<tr>
<td>MR11</td>
<td>pen-1</td>
<td>Single clone from UTO002-19</td>
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<td>aly-8</td>
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<td>gly-1</td>
<td>MR1 mutagenized with NTG</td>
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<tr>
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<td>his-3</td>
<td>MR1 transformed with DNA from MR112</td>
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<td>&amp; nov-9</td>
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</tr>
<tr>
<td>MR24</td>
<td>trp-3</td>
<td>MR1 mutagenized with NTG</td>
</tr>
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</table>

*The pen-1 marker that specifies $\beta$-lactamase production is indigenous to all derivatives of UTO002-19.

**GP, gradient plate; NTG, N-methyl-N'-nitro-N-nitrosoguanidine; MecEN, methicillin enrichment.
ing strain for typing phage 3B; it does not make ET (Tox~) (Rogolsky et al 1974). Strain UT0002-19 produces $\beta$-lactamase and is resistant to penicillin but not methicillin, whereas strain UT0017 is sensitive to both antibiotics. Auxotrophic and antibiotic resistant mutants from both strains were isolated or constructed as shown in tables 1 and 2. From each strain, a clone was selected that was sensitive to the antibiotics used in this study. These clones served as recipients for the transformation of antibiotic resistance markers and were the parental strains from which the auxotrophic mutants were derived.

Phage 80a is a serological group B phage that can be propagated on strain ISP8, a derivative of strain 8232 that lacks $\Phi11$, $\Phi12$, and $\Phi13$ prophage (Thompson and Pattee 1977). Both phage 80a and its propagating strain ISP8 were kindly supplied by P. A. Pattee. Dr. Pattee also provided strain ISP136 (Ps71-thy-136).

**Media.** All strains were routinely grown at 37 °C on heart infusion agar (HAI; Difco) that was fortified with 20 μg of thymine/ml. For certain auxotrophs of strain UT0017, it was also necessary to enrich HIA with the required growth factor (table 2). Heart infusion broth (Difco) was supplemented with thymine. The

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Strains of Staphylococcus aureus that do not synthesize exfoliative toxin.</th>
</tr>
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<tbody>
<tr>
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<td>ser-14</td>
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<td>trp-23</td>
</tr>
<tr>
<td>ISP136</td>
<td>thy-136</td>
</tr>
</tbody>
</table>

* All derivatives of UT0017 are sensitive to penicillin except for MR127, MR109, MR110, MR111, MR124, MR126, MR129, and MR135 were maintained on heart infusion agar that was fortified with the required growth factor. **PenEN, penicillin enrichment. Other abbreviations are the same as those used in table 1. †MR100 was selected for its high sensitivity to erythromycin, lincomycin, novobiocin, oleandomycin, rifampin, and tetracycline. ††Dr. P. A. Pattee, Department of Bacteriology, Iowa State University, Ames, Iowa.
trypticase soy broth (TSB; BBL) used for growing cells to competence was also fortified with thymine. TSB was sometimes additionally fortified with 2 μg each of adenine and guanine/ml (when growing Pur-"auxotrophs), or of cytosine and uracil/ml (when growing strain MR130) for the top agar used for titrations of phage 80a consisting of 3% TSB and 0.5% Bacto-agar (Difco), whereas bottom agar was trypticase soy agar (BBL) that contained 10 mM CaCl₂. Phage 80a was propagated on strain ISP8 in TSB that contained 2 mM CaCl₂. The complete, defined, synthetic (CDS) medium used for the isolation and transformation of auxotrophic markers was essentially identical to that described by Pattee and Neveln (1975) except that the concentration of methionine was adjusted to 30 μg/ml.

**Isolation of mutants.** Antibiotic resistant mutants were isolated by the gradient plate procedure of Szybalski and Bryson (1952). The Thy- auxotroph of strain UT0002-19 was isolated by selection with trimethoprim (Stacey et al. 1980). Most of the auxotrophic mutants were isolated by growing exponentially growing cells of strain UT0002-19 or strain UT0017 with 200 μg of N-methyl-N'-nitro-N-nitroso-guanidine (NTG)/ml in 20 mM citrate buffer (pH 5.0) and later replica plating the cells onto CDS agar from which a growth factor had been omitted. If enrichment with penicillin or methicillin were secondarily employed, the mutagenized cells were allowed to grow for 2 divisions in CDS broth that lacked a growth factor whose absence would prevent growth of the autotroph being sought. Then penicillin at 2 μg/ml (for strain UT0017), or methicillin at 6 μg/ml (for strain UT0002-19), was added to kill the dividing cells (Strominger et al. 1971), thus enriching for the non-growing auxotroph. The cells so treated were subsequently replica plated onto selective CDS agar to detect mutants.

**Isolation of transforming DNA.** Biologically active chromosomal DNA was isolated essentially by the procedure used for strain 8325 (Pattee and Neveln 1975). DNA was assayed by the colorimetric procedure of Setaro and Morely (1977).

**Transformation procedures.** Two transformation regimens, called the broth and plate methods, were utilized. The steps of these regimens, along with the media employed for selecting antibiotic resistant and prototrophic transformants, are detailed elsewhere (Martin et al. 1980).

**Scoring unselected markers.** Cotransformation of an unselected marker within a class of transformants was usually determined by individually streaking 100-200 recombinants onto 1/16 plate sectors of appropriate media to test for linkage. For checking cotransformation of 2 auxotrophic markers, transformants were transferred from the CDS agar on which they were selected to medium identical in composition but also now selective for the marker to be scored. Occasionally, certain transformants would not grow on these doubly auxotrophic media, thereby necessitating the use of CDS agar that lacked a single growth factor to check for linkage. Cotransformation of an antibiotic resistance marker was examined by streaking the recombinants onto HIA that contained the pertinent antibiotic. In order to investigate the cotransformation of a Tox + or Tox- focus with another genetic marker, transformants were first streaked onto 1/16 sectors of HIA and incubated overnight at 37 °C in a candlejar anaerobic chamber. A slight CO₂ tension seems to enhance the elaboration of ET (Kapral 1975). Cells from each recombinant clone were suspended in 0.5 ml of 0.85% NaCl, and 0.05 ml of this suspension (about 5 x 10⁸ colony-forming units) was inoculated subcutaneously into a 1 to 3 day old neonatal mouse. Control animals were injected with the wild-type UT0002-19 strain. The next day, the mice were examined for a positive Nikolsky sign (Melish and Glasgow 1970), which was indicated by breaking and peeling of the skin at the site of inoculation after gently stroking the affected area. In all linkage checks, the cotransformation frequency was calculated as (A/B x 100%), where A was the number of double transformants among the total number of transformants (B) examined. Map units were equivalent to (100 - % cotransformation).

**RESULTS**

**Mutants isolated in this study.** All of the antibiotic resistance markers used in this study were isolated as spontaneous mutations in the wild-type strains by using gradient plates (tables 1 and 2). Using this method, ery, ole, nov, and rif markers were also isolated in the ET producing strains, 47 and 55 (Arbuthnott and Billcliffe 1976). It later was discovered that the ery and ole markers in these 2 strains, as well as the comparable markers in strains UT0002-19 and UT0017, were cross-resistant.

Trimethoprim was used specifically to isolate the spontaneous thy-1 marker in strain UT0002-19 (table 1). Trimethoprim selection also was employed to isolate Thy- mutants of the propagating strains, Ps54, Ps85, and Ps95 (Parker 1972), as well as to rid strain ISP136 (table 2) of Thy⁺ revertants. The Thy- auxotroph of strain UT0017 was isolated by NTG mutagenesis, followed by penicillin enrichment (table 2). A Thy⁺ auxotroph of strain UT0017 could not be isolated by trimethoprim selection because this strain would not grow in effective concentrations of the drug.

Most of the auxotrophic markers used in this study were isolated by NTG mutagenesis both with and without enrichment by penicillin or methicillin (tables 1 and 2). Isolation of nutritional
mutants in phage group 2 staphylococci was very difficult, for reversion frequencies were extremely high. Indeed, it is conceivable that some of the auxotrophs used in this study may be lost after a future reversion to the wild-type state. Originally, stationary phase cells that had grown overnight on HIA slants were treated with NTG, but this method proved unsatisfactory. After mutagenesis, a number of half-sectioned and quarter-sectioned colonies were observed, which suggested the presence of multiple chromosomes in some of the old cells (Hayes 1960). No auxotrophs could be isolated from sectored colonies, and this phenomenon was never encountered when the mutagenesis protocol was adapted to using log phase cells. The isolation of NTG-induced auxotrophs was increased when the cells were enriched with penicillin or methicillin prior to replica plating. The data also suggested a preferential recovery of Ilv\textsuperscript{−} mutants after antibiotic enrichment.

Not all stable auxotrophs could be transformed. Some of the nontransformable strains might serve as competence mutants whose study could elucidate the mechanics of staphylococcal transformation. Other auxotrophs were fully capable of incorporating a nov marker by transformation but not a nutritional locus. Another intriguing observation was that sister clones may differ in competency. Mutant MR129\textsuperscript{pur-3} (table 2) was competent, but 3 of its sister clones were not, although all 4 were derived from the same parental auxotroph.

Features of the transformation routine. Transformation frequencies, regardless of whether the broth method or plate method were used, fell within the range of 10\textsuperscript{−6} to 10\textsuperscript{−4}. However, derivatives of strain UT0017 typically exhibited a plate method transformation frequency of 1 or 2 magnitudes lower than the broth method frequency. Most derivatives of strain UT0002-19 transformed well by the plate method; exceptions were MR20\textsuperscript{met-1} and MR22\textsuperscript{thr-4}. Strain 8325 could not be transformed by the plate method. The choice of TSB for growing the cells to competence in the broth method was critical. Cells grown either in heart infusion broth or CDS broth did not become competent.

Two important steps in the transformation procedure were adsorption of the recipient strain with phage 80\textalpha, and presence of Ca\textsuperscript{2+} during the incubation with transforming DNA. If either of these steps were omitted, no transformation occurred. The optimal multiplicity of infection (M.O.I.) of phage 80\textalpha to induce competence was found to be 2 to 4 phage per cell for mutant MR23\textsuperscript{thy-1}. Inclusion of rabbit serum during the phage adsorption step doubled the number of Nov\textsuperscript{r} recombinants in strains MR1 and MR100 transformed with heterologous DNA.

Gene linkages detected by DNA-mediated transformation. Two linkage groups were identified on the strain UT0002-19 chromosome by both homologous and heterologous crosses (figure 1a). Linkage group 1 was thy-1—lys-5—trp-3—thr-4—ala-8. Linkage group 2 consisted of nov-9 and his-3, which exhibited a 31\% cotransformation frequency when DNA from mutant MR112 was incorporated into strain MR1 (table 1). The lys-5 and...
Ihr-4 markers were originally transformed into strain UT0002-19 from mutants of strain UT0017 (table 1).

Three linkage groups were identified on the chromosome of strain UT0017 (figure 1b). Linkage group 1 was thy-4—lys-5—trp-21—thr-4, linkage group 2 was pyr-26—nov-9—his-3, and the third linkage group consisted of ilv-9 and pen-1. The latter 2 markers had a cotransformation frequency of 3.4%. All of these linkages were detected by homologous crosses except for ilv-9—pen-1, which involved transfer of the pen-1 marker from mutant MR8 into mutant MR115 (table 2).

The linkage groups detected on the chromosomes of strain UT0002-19 and UT0017 corresponded closely to linkage groups 1, 2, and 3 that have been delineated on the strain S325 chromosome (Pattee and Neveln 1975, Pattee et al 1977). A his marker maps between pyr and nov markers in strain S325, whereas this arrangement could not be confirmed or precluded in strain UT0017. The pyr-26 marker in strain UT0017 was highly reversible, so it was difficult to utilize strain MR130pyr-26 as a donor and recipient in transformation experiments. Since the markers used in this study were isolated and mapped independently in staphylococci that belonged to a different phage group than strain S325, these results indicate that there probably are many similarities in gene order on the chromosomes of all S. aureus strains.

Undetectable linkages. A 0% cotransformation was interpreted as representing no linkage between the markers in question. Many of the linkage checks performed in this study yielded negative data (tables 3, 4). It is curious that pen-1 was linked only to ilv-9 and not to any of the other ilv markers isolated in strain UT0017 (table 4), which suggests that ilv-9 is a different locus from the others.

Nearly 3100 neonatal mice were individually inoculated with recombinant clones in order to check for linkage of a genetic locus for ET synthesis with either an antibiotic resistance marker or an auxotrophic marker. If ET synthesis is controlled by a single genetic locus in strain UT0002-19, then this Tox+ determinant should cotransform with markers to which it is linked in crosses that utilize strain UT0017 as a recipient. On the other hand, if multiple, unlinked genes for ET synthesis exist, then cotransformation of the Tox+ genotype with a linked chromosomal marker might not be possible. Therefore, donor DNA from the ET-negative strain UT0017 was transformed into the Tox+ UT0002-19.

### Table 3

<table>
<thead>
<tr>
<th>Recipient strain</th>
<th>Recipient genotype</th>
<th>Selected trait</th>
<th>Cotransformation data</th>
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<td>Linked traits</td>
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</table>

*Both heterologous and homologous crosses were performed.
recipient to detect linkage between a Tox- marker and either a gene for nutritional biosynthesis or antibiotic resistance. In this type of cross, one would predict that synthesis of ET would be blocked in the affected recipient. All efforts to map either a Tox+ or Tox- locus were unsuccessful. In crosses that utilized strain UT0017 as recipient, a Tox+ marker could not be linked to \textit{ery}-3, \textit{lin}-3, \textit{nov}-1, \textit{ole}-3, \textit{pen}-1, \textit{rif}-1, or \textit{tet}-4 resistance markers, nor to \textit{ala}-8, \textit{his}-3, \textit{ilv}-9, \textit{lys}-5, \textit{phe}-4, \textit{pur}-3, \textit{ser}-14, \textit{thr}-4, or \textit{trp}-21 nutritional loci (table 4). Also, a Tox+ marker could not be linked to the \textit{thy}-136 locus in strain ISP136 (table 4). In reciprocal crosses that utilized strain UT0017 as recipient, a Tox+ marker from strain UT0017 could not be linked to \textit{ery}-1, \textit{nov}-9, \textit{ole}-1, \textit{rif}-10, or \textit{tet}-3 resistance markers, nor to \textit{ala}-8, \textit{ile}-1, \textit{lys}-7, \textit{pur}-6, \textit{thr}-4, or \textit{thy}-1 auxotrophic loci (table 3).

**DISCUSSION**

Competence in strain MR23thy-1 was optimally effected by an M.O.I. of 2 to 4 of phage 80a. Thompson and Pattee (1977) reported that an M.O.I. of 5 to 8 of phage 55 was optimal in conferring competence to a nonlysogenic derivative of strain S825. Rudin et al (1974) reported that transformation in strain S825 occurred only if one of the divalent cations, Mg$^{2+}$, Ba$^{2+}$, or Ca$^{2+}$, were present during the incubation with DNA. In the present study, it was found that Ca$^{2+}$ was absolutely essential to promote transformation in phage group 2 staphylococci. The mode of action of Ca$^{2+}$, of
rabbit serum, and of TSB in promoting competence in phage group 2 staphylococci remains elusive. The transformation frequencies obtained in this study were several orders of magnitude lower than those observed in the well-characterized systems of *Bacillus subtilis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*, but they are comparable with those obtained by Thompson and Pattee (1977), who used phage 80α to induce competence in homologous crosses of the phage group 2 propagating strains.

Cotransformation of genetic markers in *S. aureus* probably indicates true genetic linkages. Congestion did not appear to occur in strain 8325 (Pattee and Neveln 1975, Thompson and Pattee 1977), and transformation frequencies are so low that it is unlikely that more than one piece of DNA gets incorporated into a recipient cell. Genetic mapping in *S. aureus* was hampered by the fact that two-point reciprocal crosses did not yield additive map distances (figure 1). This peculiarity probably occurred because markers differed in the efficiency of their integration. In some reciprocal crosses, cotransformation of loosely linked markers cannot be detected. This is the reason why some of the map distances depicted in figure 1 do not have 2-way arrows. The phenomenon of nonadditive map distances has also been observed in strain 8025 (Kuhl et al. 1978, Pattee 1976, Pattee and Neveln 1975, Pattee et al. 1977). It is possible that some of the negative data depicted in tables 3 and 4 represent loosely linked markers whose linkage could not be detected. Since neither Tox+ nor Tox− loci could be mapped in this study, these genes must be distantly separated from loci that specify antibiotic resistance or govern the biosynthesis of various growth factors. Another possibility is that a Tox+ or Tox− marker may have been loosely linked to a marker selected in a given cross, but that this ET locus reverted before the phenotype could be detected in the mouse bioassay. The high reversion frequency of staphylococcal markers has already been noted.

The antibiotic resistance and auxotrophic markers isolated in this study can now be used for additional mapping studies. The negative experiments depicted in tables 3 and 4 need not be repeated. There are a number of products besides ET that are synthesized by *S. aureus*, which are related to pathogenicity such as enzymes, cytolysins, leukocidin, enterotoxin, and cellular antigens. Perhaps the genes that encode for these products will be less elusive to map than that for ET synthesis. The mapping of genes that contribute to pathogenesis would provide an important foundation for understanding how staphylococci cause disease.

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LITERATURE CITED


