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Genetic Mapping in Phage Group 2 *Staphylococcus aureus*

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Two regimens for transformation have been devised for the phage group 2 strains UT0002-19 and UT0017 of *Staphylococcus aureus*. Strain UT0002-19 produces exfoliative toxin, which is responsible for the clinical manifestations of the staphylococcal scalded skin syndrome, whereas strain UT0017 does not produce exfoliative toxin. A large pool of antibiotic-resistant and auxotrophic mutants from strains UT0002-19 and UT0017 were isolated by using (i) antibiotic gradient plates, (ii) trimethoprim selection, and (iii) nitrosoguanidine mutagenesis, which sometimes was coupled by enrichment with penicillin or methicillin. Transformation frequencies of genetic markers in mutant strains ranged from 10^{-6} to 10^{-8}. Three genetic 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, a genetic determinant for β-lactamase synthesis. Two linkage groups were detected on the strain UT0002-19 chromosome. The first was defined as *thy*-1-*lys*-5-*trp*-3-*thr*-4-*ala*-8, whereas the second consisted of *nov*-9 and *his*-3 markers. A chromosomal locus that controlled synthesis of exfoliative toxin could not be mapped.

Staphylococcal exfoliative toxin (ET) is responsible for an overlapping spectrum of clinical manifestations called the staphylococcal scalded skin syndrome (11). The disease is characterized by a scarlitiniform rash, fluid-filled bullae, and a generalized exfoliation which is characterized by intraepidermal cleavage through the stratum granulosum (11). Neonatal humans are extremely susceptible to ET which is produced by phage groups 1, 2, and 3 staphylococci (11). The inoculation of either ET or ET-producing staphylococci into the skin of neonatal mice produces a syndrome of events which simulate those seen in the human. Between 12 and 16 h after injection of ET-producing staphylococci into the skin of a newborn mouse, the epidermis becomes irreversibly wrinkled after gentle stroking and can be displaced with slight pressure (11). This phenomenon is called a positive Nikolsky sign.

For the past several years our laboratory has been investigating the genetic control of ET synthesis. It was found that phage group 2 strains can contain either chromosomal gene(s), extrachromosomal gene(s), or both types of genes for ET synthesis (13, 23). The extrachromosomal gene(s) were housed on a large 56S virulence plasmid (13, 22). The product of the chromosomal determinant for ET synthesis has been termed ET A, which is antigenically distinct from the ET product (ET B) of the extrachromosomal locus (3, 11, 23).

The mapping of chromosomal determinants for ET synthesis would provide important insights about the molecular regulation of this toxin. Genetic studies of *S. aureus* have been facilitated with the discovery of deoxyribonucleic acid (DNA)-mediated transformation for the phage group 3 strain 8325 (6, 17). The factors that affect the competence of strain 8325 are now well characterized (15, 16, 20). This strain is lysogenic for the temperate phage φ11. As it grows in broth, it liberates free phage, which in the presence of calcium cations interact with recipient cells in some manner to mediate the movement of exogenous DNA across the cell surface. Staphylococcal transformation has been used to define three distinct linkage groups on the phage group 3 chromosomal and to study the transfer of plasmids and the integration of translocation elements (4, 5, 8-10).

In 1977, Thompson and Pattee (20) reported that 80α could be used as a helper phage to induce competence in phage group 2 strains. It appears that the competence-conferring activity of both φ11 and 80α may be due to an intact protein of the mature virion (20; N. E. Thompson and P. A. Pattee, personal communication). A "pilot protein" in the virion may aid in the transport of transforming DNA across the cell surface.

The major objectives of this study were to

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define genetic linkage groups on the phage group 2 chromosome and to map the genetic determinant for ET A synthesis in relation to one of these defined linkage groups.

MATERIALS AND METHODS

Bacterial strains and phage. Strain UT0002 is a phage group 2 (55/71) clinical isolate of *S. aureus* (14). It contains both chromosomal and plasmid genes for ET synthesis. UT0002-19 is a substrain of strain UT0002 that has been heat cured of a high-molecular-weight, 56S virulence plasmid that carries genetic determinants for the synthesis of ET B (13). Strain UT0002-19 remains toxinogenic (Tox+), since it still contains the chromosomal gene(s) for ET A production. Strain UT0017 is a phage group 2 (3B/3C/55/71) strain that serves as the propagating strain for typing phage 3B; it does not produce ET (14). Strain UT0002-19 synthesizes β-lactamase and is resistant to penicillin at 5 μg/ml, but it is sensitive to methicillin. Strain UT0017 is sensitive to both antibiotics. Antibiotic-resistant and auxotrophic mutants from both strains were isolated or constructed as indicated in Tables 1 and 2. From each strain, a clone was isolated that was highly 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 for all of the auxotrophic mutants.

This study utilized two ET-producing strains provided by J. P. Arbuthnott that were typed by phage groups 1 and 3, as well as by the miscellaneous phage 95. Strain 47 (29/54/85/95) was resistant to 1 μg of penicillin per ml, whereas strain 55 (80/54/85/95) was sensitive to this drug. E. E. Stobberingh supplied two restriction-deficient mutants that were sensitive to all group 1, 2, and 3 phage. These were strains 3A-R1 and 80C-R3. They did not make ET.

Phage 80α is a serological group B phage that can be propagated on strain ISP8, a derivative of the phage group 3 strain 8325 (20). Both 80α and its propagating strain ISP8 were kindly supplied by P. A. Pattee. Several genetic variants that belonged to phage groups 2 and 3 (strains ISP1, ISP5, ISP133, ISP134, ISP135, and ISP136) were also provided by P. A. Pattee.

Culture media and chemicals. All strains were routinely maintained and subcultured on heart infusion agar (HIA; Difco Laboratories, Detroit, Mich.) fortified with 20 μg of thymine per ml. Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.) was also fortified with 20 μg of thymine per ml. When used for growing Pur^- and Pyr^- auxotrophs to competence, TSB was additionally fortified with purines (adenine and guanine) or pyrimidines (cytosine and uracil), respectively, each at 5 μg/ml. The top agar used for titrations of 80α consisted of 3% TSB and 0.5% agar (Difco), whereas bottom agar was Trypticase soy agar (BBL Microbiology Systems) that contained 10 mM $CaCl_2$.

The complete, defined, synthetic (CDS) medium employed for the isolation and transformation of auxotrophic markers was identical to that described by Pattee and Neveln (9), with the exception that the concentration of methionine was adjusted to 30 mg/liter.

Erythromycin, novobiocin (sodium salt), oleandomycin phosphate, penicillin G (potassium salt), tetracycline hydrochloride, trimethoprim, and 2-(2′-carboxyphenyl)benzoyl-6-aminopenicillanic acid (CBAP) were obtained from Sigma Chemical Co., St. Louis, Mo., as were all the amino acids, vitamins, purines, and pyrimidines. Methicillin (Staphcillin) came from Bristol Laboratories, Syracuse, N.Y. Lincomycin hydrochloride (Lincocin) was a gift of The Upjohn Co., Kansas City, Mo. Rifampin, pronase (B grade), and salmon sperm DNA (sodium salt) came from Calbiochem, La Jolla, Calif. *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) and diaminobenzoic acid dihydrochloride were purchased from the Aldrich Chemical Co., Milwaukee, Wisc. Lysostaphin came from Schwarz/Mann, Orangeburg, N.Y. Sterile rabbit serum was obtained from Granite Diagnostics, Burlington, N.C.

Mice. Female mice (inbred Swiss Webster strain), approximately 14 days pregnant, were purchased from Sasco, Inc., Omaha, Nebr.) Suckling mice, 1 to 3 days old, were used for the bioassay of ET.

Isolation of mutants. Mutants of strains UT0002-19 and UT0017 were isolated by three methods: (i) antibiotic gradient plates; (ii) trimethoprim selection; and (iii) NTG mutagenesis. The latter could be enhanced by enrichment with penicillin for strain UT0017 or methicillin for strain UT0002-19. Mutants were stored at −55°C in 1-dram (ca. 1.77 g) vials that contained 1 ml of a filter-sterilized mixture of 10% dimethyl sulfoxide and 0.5% glucose. The antibiotic concentration in the top agar used in preparing the gradient plates was usually about 10 times greater than the inhibitory concentration for the strains. Trimethoprim selection is specific for the isolation of Thy^- auxotrophs (18). It was used to obtain the *thy*-1 marker in strain UT0002-19. Most of the auxotrophic mutants were isolated by incubating (60 min at 37°C) log-phase cells of either strain with a final concentration of 200 μg of NTG per ml in 20 mM citrate buffer (pH 5.0) and replica plating the treated 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 two divisions in CDS broth which lacked a growth factor whose absence would prevent the desired auxotroph from growing. 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, thus enriching for the nongrowing auxotroph. The remaining cells were summarily collected and replica plated onto CDS agar that lacked the required growth factor to screen for the desired auxotroph.

Preparation of transforming DNA. Biologically active chromosomal DNA was isolated by the procedure used for strain 8325 (6, 9). Samples typically contained 250 μg of DNA per ml and maintained biological activity for at least 1 year.

Transformation procedures. The two transformation procedures developed in this laboratory for use with phage group 2 strains of *S. aureus* are depicted in Fig. 1.

Optical density was measured with the red filter in a Klett-Summerson photoelectric colorimeter (Klett

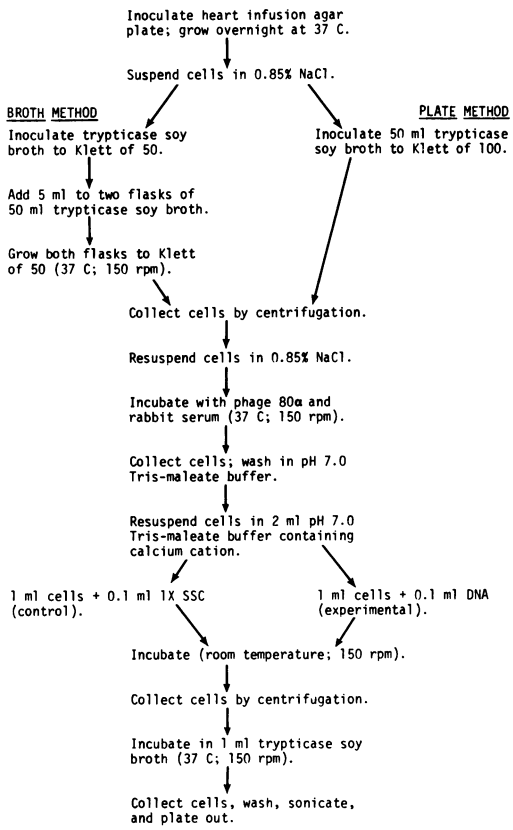

FIG. 1. *Flow chart of the transformation procedures employed for phage group 2 strains UT0002-19 and UT0017 of S. aureus.*

Mfg. Co., New York, N.Y.). In the broth method, cultures were incubated at 37°C in a New Brunswick Scientific (New Brunswick, N.J.) gyratory shaker until they reached an OD of about 50 Klett units. Cells from the cultures were then collected by centrifugation (10,400 × *g*, 20 min). All centrifugations were performed at 4°C using a Sorvall RC-5 refrigerated centrifuge (Dupont Instruments, Newton, Conn.). The cell pellet, which was obtained by either the broth or plate method, was suspended in 1 ml of 0.85% NaCl and transferred to a 125-ml triple-baffled flask that contained 1 ml of rabbit serum and 10 ml of 1×10^{10} to 5×10^{10} plaque-forming units per ml of 80α. This mixture was shaken (37°C, 150 rpm) for 10 min to effect adsorption of the phage. The cells were collected by centrifugation, washed in 10 ml of 50 mM Tris-maleate buffer (pH 7.0), and then suspended in 2 ml of this same buffer that contained 100 mM $CaCl_2$. Approximately 25 μg of DNA was used for transformation. After exposure to DNA, the cells were separately collected by centrifugation (3,640 × *g*, 7 min), suspended in 1 ml of TSB, and incubated (37°C, 150 rpm) for 30 to 60 min to allow for the complete integration and expression of the transformed trait(s). The cells were again separately collected by centrifugation, washed in 0.85% NaCl, suspended in 1 ml of saline, gently sonicated to disperse cell clumps, and plated onto appropriate media to detect transformants and revertants. The wash step was omitted if only an antibiotic resistance marker were being transformed. The experimental cells were diluted in saline and titrated on HIA that was enriched with the appropriate growth factor, if necessary (Table 2). Plates were incubated at 37°C in a humid atmosphere. Recombinants were scored after 1 to 3 days of incubation, depending upon the selected marker, and titers were determined after 1 to 2 days.

Propagation of phage 80α. Phage 80α was propagated on strain ISP8 in TSB that contained 2 mM $CaCl_2$. The phage preparation was filter sterilized and stored at 4°C. The titer, which remained stable for several weeks, was determined after dilutions through TSB and using strain ISP8 for the indicator cells in a standard plate plaque assay.

Scoring unselected markers. Cotransformation of an unselected marker within a class of transformants was usually determined by individually streaking 100 to 200 recombinants onto appropriate media to test for linkage. In some cases, replica plating was performed. Transformation of the *pen*-1 marker from strain UT0002-19 into strain UT0017 was confirmed by using a modified CBAP plate method (10) to assay for the production of inducible β-lactamase. Pen^r transformants, along with control strains, were inoculated onto HIA plates that contained 0.3% soluble starch (Difco) plus 3.5 μg of CBAP per ml as a gratuitous inducer of β-lactamase. After 2 days of incubation, the plates were flooded with 30 mg of penicillin per ml that was dissolved in 80 mM I_2 plus 40 mM KI (made soluble in 20 mM phosphate buffer, pH 7.2). After a few minutes at room temperature, a zone of clearing appeared around those colonies that were synthesizing β-lactamase. Cotransformation frequency was calculated simply as $(A/B \times 100\%)$, where A was the number of double transformants among the total number of transformants (B) examined. Map units were equivalent to 100 − percent cotransformation.

ET assay. ET was assayed in neonatal mice by the same procedure previously described by this laboratory (14). Toxin-producing strains were identified by their ability to produce a positive Nikolsky sign in neonatal mice (11).

RESULTS

Necessity of isolating novel staphylococcal mutants for this study. A number of antibiotic resistance and nutritional markers have been isolated in the naturally competent phage group 3 strain 8325 (4, 9, 10). Initially, it seemed convenient to utilize the markers available in strain 8325 to map a genetic locus for ET production by transforming DNA from the phage group 2 strain UT0002-19 into specific recipients of the phage group 3 strain 8325. However, transformation of strain 8325 with DNA from either strain UT0002-19 or its parent, strain UT0002, could not be achieved. Transformation of phage group 2 strains with DNA from strain 8325 was likewise ineffective. This inability to perform

intergroup transformations in *S. aureus* may be ascribed to restriction barriers, which are the basis for phage group typing of staphylococci (7). It was also not possible to transform strain 8325 with DNA from the phage group 3 ET-producing 47 and 55 strains.

Heating staphylococci at 56°C for a few minutes is believed to inactivate their restriction enzymes, thus allowing the strains to be lysed by phage which are normally restricted (21). To overcome restriction barriers, modifications were made in transformation experiments which utilized the strain 8325 derivatives ISP1 and ISP5, as recipients, and strains 47 and 55 as donors. These modifications included (i) heating recipient cells at 56°C for 5 min before incubation with transforming DNA, (ii) exposing the cells to DNA for 60 min at 4°C, and (iii) using phage 55 to superinfect the recipient strain (20). All of these procedures failed to effect transformation. In addition, two restriction-deficient mutants, 3A-R1 and 80C-R3, which were susceptible to lysis by all typing phage of groups 1, 2, and 3 (19) could not be transformed by DNA from strain 8325. Thus, markers from strain 8325 could not be introduced into strains 3A-R1 or 80C-R3.

It was now obvious that if a locus for ET synthesis or any other markers on the phage group 2 chromosome were to be mapped, recombination studies would have to be confined to group 2 strains. Therefore, a large pool of auxotrophic and antibiotic-resistant mutants was isolated from the phage group 2 strains, UT0002-19 and UT0017 (Tables 1 and 2).

Isolation of phage group 2 staphylococcal mutants. The *pen*-1 marker is a genetic determinant for an inducible β-lactamase. This chromosomal marker is indigenous to strain UT0002-19 and all of its derivatives (Table 1). All other resistance markers used in this study were isolated as spontaneous mutations by using antibiotic gradient plates.

The *thy*-1 marker in strain UT0002-19 was isolated as a spontaneous mutation by selection of strain MR1 with 40 μg/ml of trimethoprim in CDS broth. This was an especially clean and stable marker. Strain MR23*thy*-1 grew similarly to wild-type strains when the growth medium was fortified with 20 μg of thymine per ml. A Thy⁻ auxotroph of strain UT0017 could never be isolated upon treatment with trimethoprim because this strain failed to grow in effective concentrations of the drug. The *thy*-4 marker in mutant MR136 was isolated by NTG mutagenesis, followed by penicillin enrichment.

The isolation of NTG-induced, auxotrophic mutants was enhanced when the mutagenized cells were subjected to enrichment by penicillin (for strain UT0017) or methicillin (for strain UT0002-19) before the clones were replica plated onto selective media (Tables 1 and 2). Penicillin enrichment could not be employed for strain UT0002-19, since it produced penicillinase. None of the auxotrophs of strain UT0002-19 (Table 1) lost the ability to synthesize ET in vivo when injected into neonatal mice. Arg⁻, Cys⁻, or Pro⁻ auxotrophs could not be isolated, because both wild-type strains had a natural requirement for the pertinent amino acids.

The wild-type phage group 2 strains of *S. aureus* had a remarkable propensity to repair genetic damage effected by NTG, so reversion was a frequently encountered phenomenon. Also, not all stable auxotrophs could be transformed.

TABLE 1. *Strains of S. aureus that produce ET*

Strain[a]	Genetic traits	Origin
UT0002-19	*pen*-1	Rogolsky et al. (13)
MR1	*pen*-1	Single clone from strain UT0002-19
MR2	*ery*-3	MR1 after selection on erythromycin gradient plate
MR3	*lin*-3	MR1 after selection on lincomycin gradient plate
MR5	*nov*-1	MR1 after selection on novobiocin gradient plate
MR6	*ole*-3	MR1 after selection on oleandomycin gradient plate
MR7	*rif*-1	MR1 after selection on rifampin gradient plate
MR8	*tet*-4	MR1 after selection on tetracycline gradient plate
MR9	*ala*-8	MR1 after NTG mutagenesis and methicillin enrichment
MR10	*gly*-1	MR1 after NTG mutagenesis
MR11	*his*-3, *nov*-9	MR1 after transformation with DNA from MR112
MR12	*ilv*-1	MR1 after NTG mutagenesis
MR18	*lys*-5	MR23 after transformation with DNA from MR126
MR19	*lys*-7	MR1 after NTG mutagenesis
MR20	*met*-1	MR1 after NTG mutagenesis and methicillin enrichment
MR21	*pur*-6	MR1 after NTG mutagenesis
MR22	*thr*-4	MR23 after transformation with DNA from MR135
MR23	*thy*-1	MR1 after selection with trimethoprim (40 μg/ml)
MR24	*trp*-3	MR1 after NTG mutagenesis
47	*pen*-3	Arbuthnott[b] and Billcliffe (1)
55	Pen[a]	Arbuthnott and Billcliffe (1)

[a] Strain UT0002-19 and its derivatives belong to phage group 2. The *pen*-1 marker, which encodes for a β-lactamase, is indigenous to all of these phage group 2 strains. Strain MR1 was selected for its high sensitivity to erythromycin, lincomycin, novobiocin, oleandomycin, rifampin, and tetracycline. Strains 47 and 55 are combination phage group 1 and 3 staphylococci.

[b] J. P. Arbuthnott, Department of Microbiology, Trinity College, University of Dublin, Ireland.

TABLE 2. *Strains of S. aureus which do not produce ET*

Strain[a]	Genetic traits	Origin
UT0017	Pen^s	Rogolsky et al. (14)
MR100	Pen^s	Single clone from strain UT0017
MR101	*ery*-1	MR100 after selection on erythromycin gradient plate
MR103	*nov*-9	MR100 after selection on novobiocin gradient plate
MR104	*ole*-1	MR100 after selection on oleandomycin gradient plate
MR105	*rif*-10	MR100 after selection on rifampin gradient plate
MR106	*tet*-3	MR100 after selection on tetracycline gradient plate
MR107	*ala*-10	MR100 after NTG mutagenesis and penicillin enrichment
MR108	*ala*-12	MR100 after NTG mutagenesis and penicillin enrichment
MR111	*his*-3	MR100 after NTG mutagenesis
MR112	*his*-3, *nov*-9	MR111 after transformation with DNA from MR103
MR115	*ilv*-9	MR100 after NTG mutagenesis and penicillin enrichment
MR126	*lys*-5	MR100 after NTG mutagenesis
MR127	*pen*-1	MR115 after transformation with DNA from MR8
MR128	*phe*-4	MR100 after NTG mutagenesis
MR129	*pur*-3	MR100 after NTG mutagenesis
MR130	*pyr*-26	MR100 after NTG mutagenesis and penicillin enrichment
MR131	*ser*-4	MR100 after NTG mutagenesis and penicillin enrichment
MR134	*ser*-14	MR100 after NTG mutagenesis and penicillin enrichment
MR135	*thr*-4	MR100 after NTG mutagenesis
MR136	*thy*-4	MR100 after NTG mutagenesis and penicillin enrichment
MR137	*trp*-21	MR100 after NTG mutagenesis and penicillin enrichment
3A-R1	*hsd*R	Stobberingh[c] and Winkler (19)
80C-R3	*hsd*R	Stobberingh and Winkler (19)
ISP1	*thy*-101, *pig*-131	Pattee[c] and Neveln (9)
ISP5	*thy*-101, *thr*-106 *ilv*-129, *pig*-131	Pattee and Neveln (9)
ISP133	*thy*-133	Thompson and Pattee (20)
SP134	*thy*-134	Thompson and Pattee (20)
ISP135	*thy*-135	Thompson and Pattee (20)
ISP136	*thy*-136	Thompson and Pattee (20)

[a] Strain UT0017 and its derivatives, as well as strains ISP133, ISP134, ISP135, and ISP136, belong to phage group 2. Strains ISP1 and ISP5 belong to phage group 3. Strains 3A-R1 and 80C-R3 are restriction-deficient mutants that are sensitive to all group 1, 2, and 3 phage (19). All derivatives of strain UT0017 are sensitive to penicillin except for strain MR127. Strain MR100 was selected for its high sensitivity to erythromycin, lincomycin, novobiocin, oleandomycin, rifampin, and tetracycline. Mutants MR111, MR112, MR126, MR129, and MR135 were routinely subcultured and titered on heart infusion agar that was fortified with the required growth factor.

[b] E. E. Stobberingh, Biomedlisch Centrum, Rijksuniversiteit Limburg, Maastrict, The Netherlands.

[c] P. A. Pattee, Department of Bacteriology, Iowa State University, Ames, Iowa.

Transformation of regimen for phage group 2 staphylococci. Figure 1 is a flowchart for performing DNA-mediated transformation in phage group 2 *S. aureus*. The plate method made it easier to perform multiple transformations concurrently, but certain markers (e.g., *met*-1 and *thr*-4 could not be transformed by this procedure. Also strain 8325 could not be transformed by the plate method. A mutant which grew slowly in TSB was more easily transformed by the plate method than by the broth method. Transformation frequencies, regardless of the method used, generally fell within the range of 10^{-6} to 10^{-8} (Tables 3 and 4). However, mutants of strain UT0017 typically exhibited a plate method transformation frequency 1 or 2 magnitudes lower than the broth method frequency. Most mutants of strain UT0002-19 transformed well by the plate procedure.

A key step in the transformation regimen was the incubation with 80α helper phage. If phage were omitted, no transformation occurred. Inclusion of about 8% rabbit serum in the phage adsorption step approximately doubled the number of transformants obtained. Substitution of bovine, fetal calf, or lamb serum also increased the number of transformants but with a greater variation than when rabbit serum was used. Another key step in the transformation protocol was the incubation with DNA in the presence of calcium cations. If calcium were excluded, no transformation occurred even if the cells had previously been adsorbed with helper phage.

Competence curves. Figures 2 and 3 depict

TABLE 3. *Frequency of transformation of some genetic markers in recipients derived from strain UT0002-19*

Donor	Recipient	Selective medium	Transformation frequency[a]
ery-1	Ery^s	HIA (plus erythromycin at 0.5 μg/ml)	2.2×10^{-6}
nov-9	Nov^s	HIA (plus novobiocin at 2 μg/ml)	8.1×10^{-6}
ole-1	Ole^s	HIA (plus oleandomycin 5 μg/ml)	3.6×10^{-6}
rif-10	Rif^s	HIA (plus rifampin at 10 μg/ml)	2.4×10^{-7}
tet-3	Tet^s	HIA (plus tetracycline at 2 μg/ml)	4.3×10^{-8}
Ala$^+$	*ala*-8	CDS (minus alanine)	6.3×10^{-7}
Gly$^+$	*gly*-1	CDS (minus glycine)	4.8×10^{-7}
His$^+$	*his*-3	CDS (minus histidine	9.6×10^{-8}
Ilv$^+$	*ilv*-1	CDS (minus isoleucine)	1.9×10^{-6}
Lys$^+$	*lys*-5	CDS (minus lysine)	3.0×10^{-8}
Met$^+$	*met*-1	CDS (minus methionine and plus homoserine at 30 μg/ml	1.1×10^{-8}
Pur$^+$	*pur*-6	CDS (minus adenine and guanine)	7.3×10^{-7}
Thr$^+$	*thr*-4	CDS (minus threonine)	1.2×10^{-7}
Thy$^+$	*thy*-1	CDS (minus thymine)	2.7×10^{-6}
Trp$^+$	*trp*-3	CDS (minus tryptophan)	1.6×10^{-7}

[a] Expressed as the number of transformants per number of CFU. All crosses were heterologous, the DNA donors being derivatives of strain UT0017. The *his*-3, *lys*-5, and *thr*-4 markers in mutants of strain UT0002-19 are isogenic to the wild-type loci in strain UT0017. These markers were originally isolated in strain UT0017 and later transformed into strain UT0002-19 (Table 1).

the development of competence of strains UT0002-19 and UT0017, respectively, in TSB. In both cases, the selected donor marker was *nov*. Both strains exhibited a peak in competence after 2 h of incubation, when they were in early log phase. Strain UT0002-19 produced 720 transformants per 10^8 colony-forming units (CFU) at this point. Strain UT0017 produced 400 recombinants per 10^8 CFU at maximal competency. After 2 h of growth, the transformation frequency of both strains dropped considerably, but they both remained competent throughout the growth curve. After maximal competency was achieved, the transformation frequency of the two strains hovered around 100 recombinants per 10^8 CFU for the next 6.5 h. It was the unexpectedly high degree of competence exhibited at zero time by strain UT0002-19 (Fig. 2) that prompted the development of the plate method routine.

MOI. The effect of the multiplicity of infection (M.O.I.) of 80α in conferring competence to strain UT0002-19 is shown in Fig. 4. In this instance, mutant MR23*thy*-1 was interacted with 80α at different MOIs and then transformed with heterologous DNA from strain MR103*nov*-9 by using the plate method. Both Nov^r and Thy$^+$ recombinants were selected, but only the former are depicted in Fig. 4. The two classes of recombinants exhibited identical profiles, but the Thy$^+$ transformants were not as numerous.

The optimal MOI for inducing competence in mutant MR23 was 4, but this MOI reduced the viable cell titer by 60% (Fig. 4). An MOI of 2 yielded the same number of Nov^r transformants but caused a lethality of only 20%. At an MOI of 4, those cells which remained viable were proportionately more competent than those exposed to a lower MOI. On the other hand, the degree of competence at an MOI of 8 dropped about 30% although lethality remained at 60%.

Representative transformation crosses. Transformation frequencies of some of the genetic markers in the phage group 2 strains UT0002-19 and UT0017 are listed in Tables 3 and 4, respectively, along with the media used in their selection. Only heterologous crosses are

TABLE 4. *Frequency of transformation of some genetic markers in recipients derived from strain UT0017*

Donor	Recipient	Selective medium	Transformation frequency[a]
ery-3	Ery^s	HIA (plus erythromycin at 0.5 μg/ml)	2.4×10^{-8}
lin-3	Lin^s	HIA (plus lincomycin at 2 μg/ml)	4.3×10^{-8}
nov-1	Nov^s	HIA (plus novobiocin at 2 μg/ml)	1.6×10^{-6}
ole-3	Ole^s	HIA (plus oleandomycin at 5 μg/ml)	2.9×10^{-7}
pen-1	Pen^s	HIA (plus penicillin at 0.075 μg/ml)	3.3×10^{-7}
rif-1	Rif^s	HIA (plus rifampin at 10 μg/ml)	2.2×10^{-7}
tet-4	Tet^s	HIA (plus tetracycline at 3 μg/ml)	5.4×10^{-7}
Ala$^+$	*ala*-10	CDS (minus alanine)	1.7×10^{-7}
His$^+$	*his*-3	CDS (minus histidine)	3.3×10^{-7}
Ilv$^+$	*ilv*-9	CDS (minus isoleucine)	1.7×10^{-8}
Lys$^+$	*lys*-5	CDS (minus lysine)	1.0×10^{-6}
Phe$^+$	*phe*-4	CDS (minus phenylalanine)	1.0×10^{-6}
Pur$^+$	*pur*-3	CDS (minus adenine and guanine)	1.1×10^{-7}
Pyr$^+$	*pyr*-26	CDS (minus cytosine and uracil)	4.3×10^{-8}
Ser$^+$	*ser*-4	CDS (minus serine)	1.5×10^{-7}
Thr$^+$	*thr*-4	CDS (minus threonine)	4.7×10^{-7}
Thy$^+$	*thy*-1	CDS (minus thymine)	3.2×10^{-7}
Trp$^+$	*trp*-21	CDS (minus tryptophan)	7.2×10^{-8}

[a] Expressed as the number of transformants per number of CFU. All crosses were heterologous, the DNA donors being derivatives of strain UT0002-19.

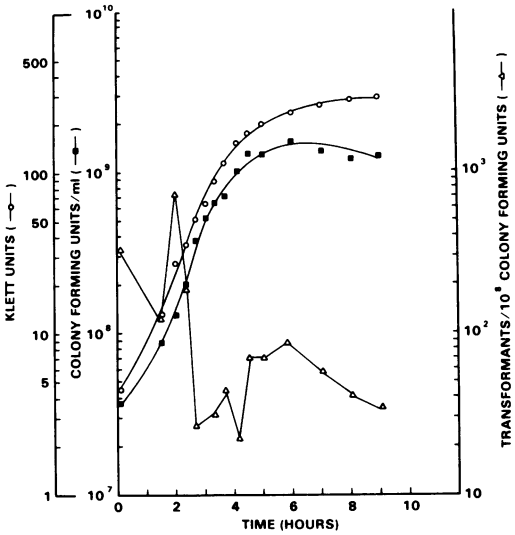

FIG. 2. *Development of competence in strain UT0002-19 during growth in TSB. Using a gyratory shaker, the culture was incubated at 37°C and shaken at 150 rpm in an initial volume of 800 ml. At each time point, a sample that contained 10^9 CFU of strain MR1, an antibiotic sensitive derivative of strain UT0002-19, was removed and transformed with heterologous DNA from mutant MR103nov-9. Recombinants which received the nov marker were selected on heart infusion agar that contained 2 μg of novobiocin per ml. CFU/ml were titrated on heart infusion agar alone. Turbidity readings, expressed as Klett units, were measured on a Klett-Summerson colorimeter (red filter).*

depicted, although a large number of homologous crosses were also carried out in the process of mapping markers. Transformation frequencies generally ranged from 10^{-6} to 10^{-8}, regardless of whether the cross was homologous or heterologous or if the broth or plate method was used. Heterologous crosses were frequently done because of the attempt to transfer either a Tox^+ marker from strain UT0002-19 into strain UT0017 or a Tox^- locus in the reciprocal cross.

When transforming antibiotic resistance markers into sensitive recipients, it was necessary to select the recombinants on HIA that contained an antibiotic concentration less than that for which the donor marker conferred resistance. Once transformants were obtained on a low concentration of antibiotic, they then could be subcultured onto a higher concentration. This technique was used to differentiate true Ery^r transformants from contaminating background colonies in the recipient strains that were resistant to low levels of erythromycin.

Some markers, such as *nov*-1 and *nov*-9, were easy to transform, whereas others, such as *pur*-3 and *his*-3, were often difficult to repair by transformation. Transformation of the *met*-1 marker in mutant MR20 could not be effected unless the selective medium were devoid of me-

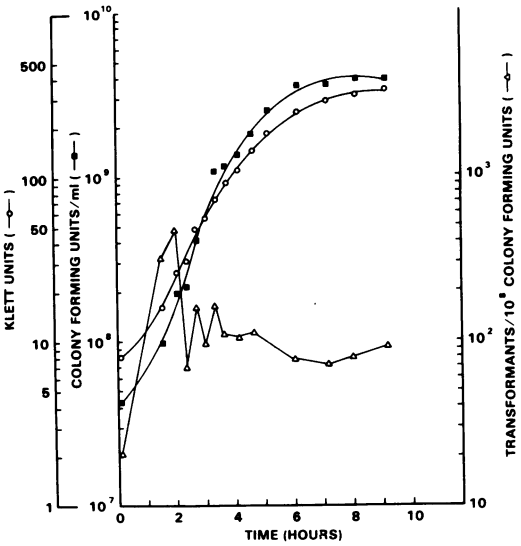

FIG. 3. *Development of competence in strain UT0017 during growth in TSB. All conditions were identical to those described in the legend to Fig. 2 except that, in this case, a cell sample of strain MR100, an antibiotic sensitive derivative of strain UT0017, was transformed at each time point with heterologous DNA from mutant MR5nov-1.*

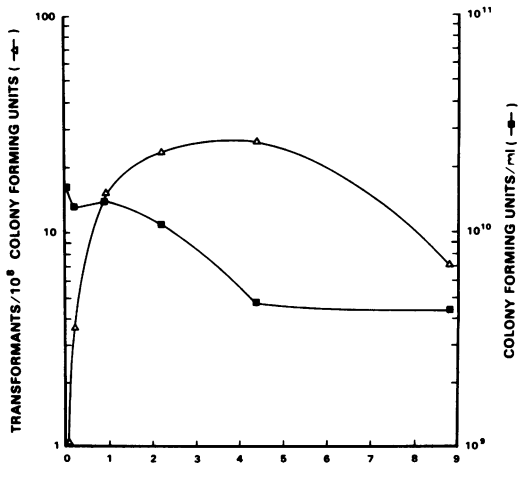

FIG. 4. *Ability of phage 80α to confer competence to a derivative of strain UT0002-19 at differing MOIs. Mutant MR23thy-1 was transformed with heterologous DNA from mutant MR103nov-9 using the plate method (Fig. 1). Recombinants which received the nov marker were selected on HIA that contained 2 μg of novobiocin per ml. CFU/ml were titrated on heart infusion agar alone.*

thionine but enriched with homoserine. Homoserine was essential for transformation.

Gene linkages on the chromosomes of strains UT0002-19 and UT0017. Two linkage groups were identified on the chromosome of strain UT0002-19 (Table 5 and Fig. 5). The first linkage group was *thy*-1-*lys*-5-*trp*-3-*thr*-4-*ala*-8. The second consisted of *nov*-9 and *his*-3, both of which were transformed together from mutant MR112 into strain MR1 (Table 5). The data for the first linkage group were compiled from both homologous and heterologous crosses. The *lys*-5 and *thr*-4 markers in strains MR18 and MR22, respectively, were transformed from mutants of strain UT0017 (Table 1). Pattee and Neveln (9) have linked *nov* and *his* markers in strain 8325, and they also have defined a linkage group 1 similar to the one in strain UT0002-19 with the exception that no *ala* marker was linked to *thr*.

Three linkage groups were identified on the chromosome of strain UT0017 (Table 6 and Fig. 6). 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. All of these linkages were detected by homologous crosses except for the latter, which involved transfer of the *pen*-1 marker from mutant MR8 into mutant MR115 (Table 6). The β-lactamase synthesized by the Pen^r recombinants, as well as by the donor strain, was inducible. Pattee et al. (10) found that a gene for β-lactamase synthesis also mapped next to an *ilv* marker on the strain 8325 chromosome.

Linkage group 1 in strain UT0017 was similar to that found in strain UT0002-19 (Fig. 5 and 6), except that an *ala* marker could not be linked proximal to *thr*-4. The *lys*-5 and *thr*-4 markers were the same genes as those mapped in linkage group 1 on the strain UT0002-19 chromosome. Likewise, the *nov*-9 and *his*-3 markers were the same as those transformed together into strain UT0002-19, but these markers were also associated with the *pyr*-26 marker on the strain UT0017 chromosome. Pattee and Neveln (9) also found linkage among these three markers on the strain 8325 chromosome.

Chromosomal map distances were not additive (Fig. 5 and 6). This incongruity is believed to occur because markers differ in their efficiency of integration (2). For example, the *nov*-1 and *nov*-9 markers were much easier to transform than the *his*-3 marker. This phenomenon of nonadditive map distances has also been observed during mapping in strain 8325 (4, 8–10). Moreover, in some reciprocal crosses, cotransformation of linked genetic markers cannot even be detected. This is the reason why some of the map distances depicted in Fig. 5 and 6 do not have two-way arrows. When markers that differ in integration efficiency are distantly linked, as are *thy*-4 and *thr*-4 in strain UT0017 (Fig. 6), cotransformation may go undetected in one direction.

Attempt to map a chromosomal genetic determinant for ET A synthesis. If ET A synthesis is controlled by a single genetic locus in strain UT0002-19, then this Tox$^+$ locus should cotransform with antibiotic resistance or nutritional markers to which it is linked in crosses that utilize strain UT0017 as the recipient. However, if multiple, unlinked genes for ET A synthesis exist, then cotransformation of the Tox$^+$ genotype with a linked chromosomal marker

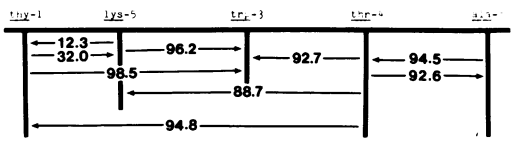

FIG. 5. *Linkage group on the chromosome of phage group 2 strain UT0002-19. Map units are equivalent to (100 − percent cotransformation) and are derived from the data in Table 5. Arrows point in the direction of the selected markers.*

TABLE 5. *Linkage relationships of genetic markers in strain UT0002-19 determined by two-factor transformation crosses*

Linkage group	Donor	Recipient	Selected trait	% Cotransformation[a]
1	MR126 *lys*-5 Thy$^+$	MR23 Lys$^+$ *thy*-1	Thy$^+$	87.7 (*lys*-5 Thy$^+$)
	MR23 *thy*-1 Lys$^+$	MR18 Thy$^+$ *lys*-5	Lys$^+$	68.0 (*thy*-1 Lys$^+$)
	MR126 *lys*-5 Trp$^+$	MR24 Lys$^+$ *trp*-3	Trp$^+$	3.8 (*lys*-5 Trp$^+$)
	MR23 *thy*-1 Trp$^+$	MR24 Thy$^+$ *trp*-3	Trp$^+$	1.5 (*thy*-1 Trp$^+$)
	MR135 *thr*-4 Trp$^+$	MR24 Thr$^+$ *trp*-3	Trp$^+$	7.3 (*thr*-4 Trp$^+$)
	MR135 *thr*-4 Lys$^+$	MR18 Thr$^+$ *lys*-5	Lys$^+$	11.3 (*thr*-4 Lys$^+$)
	MR135 *thr*-4 Thy$^+$	MR23 Thr$^+$ *thy*-1	Thy$^+$	5.2 (*thr*-4 Thy$^+$)
	MR135 *thr*-4 Ala$^+$	MR9 Thr$^+$ *ala*-8	Ala$^+$	7.4 (*thr*-4 Ala$^+$)
	MR9 *ala*-8 Thr$^+$	MR22 Ala$^+$ *thr*-4	Thr$^+$	5.5 (*ala*-8 Thr$^+$)
2	MR112 *nov*-9 *his*-3	MR1 Nov^s His$^+$	*nov*-9	31.0 (*nov*-9 *his*-3)

[a] Cotransformation frequencies were based on examination of at least 200 recombinants.

TABLE 6. *Linkage relationships of genetic markers in strain UT0017 determined by two-factor transformation crosses*

Linkage group	Donor	Recipient	Selected trait	% Cotransformation[a]
1	MR126 *lys*-5 Thy$^+$	MR136 Lys$^+$ *thy*-4	Thy$^+$	13.3 (*lys*-5 Thy$^+$)
	MR126 *lys*-5 Trp$^+$	MR137 Lys$^+$ *trp*-21	Trp$^+$	20.4 (*lys*-5 Trp$^+$)
	MR135 *thr*-4 Lys$^+$	MR126 Thr$^+$ *lys*-5	Lys$^+$	3.8 (*thr*-4 Lys$^+$)
	MR135 *thr*-4 Trp$^+$	MR137 Thr$^+$ *trp*-21	Trp$^+$	33.6 (*thr*-4 Trp$^+$)
2	MR103 *nov*-9 His$^+$	MR111 Nov^s *his*-3	*nov*-9	36.2 (*nov*-9 His$^+$)
	MR103 *nov*-9 His$^+$	MR111 Nov^s *his*-3	His$^+$	87.0 (*nov*-9 His$^+$)
	MR5 *nov*-1 His$^+$	MR111 Nov^s *his*-3	*nov*-1	21.8 (*nov*-1 His$^+$)
	MR5 *nov*-1 His$^+$	MR111 Nov^s *his*-3	His$^+$	70.7 (*nov*-1 His$^+$)
	MR103 *nov*-9 Pyr$^+$	MR130 Nov^s *pyr*-26	*nov*-9	11.0 (*nov*-9 Pyr$^+$)
	MR103 *nov*-9 Pyr$^+$	MR130 Nov^s *pyr*-26	Pyr$^+$	27.7 (*nov*-9 Pyr$^+$)
3	MR8 *pen*-1 Ilv$^+$	MR115 Pen^s *ilv*-9	Ilv$^+$	3.4 (*pen*-1 Ilv$^+$)

[a] Cotransformation frequencies were based on examination of at least 200 recombinants.

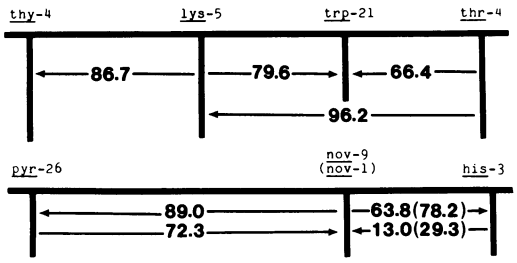

FIG. 6. *Linkage groups on the chromosome of phage group 2 strain UT0017. Map units are equivalent to (100 − percent cotransformation) and are derived from the data in Table 6. Arrows point in the direction of the selected markers. Values in parentheses of the nov-his linkage represent data of a heterologous cross.*

into strain UT0017 might not be possible. Therefore, donor DNA from the ET-negative UT0017 strain was transformed into the toxinogenic UT0002-19 recipient to detect linkage between a Tox$^-$ marker and either a trait for nutritional biosynthesis, or antibiotic resistance. Recombinants from these crosses were streaked for isolation, and only a single clone was injected into a mouse to check for cotransfer of a Tox$^-$ gene. This precaution was undertaken when it was discovered that the transformant clones were frequently mixed with true recombinants and nontransformed cells. Therefore, if a transformant clone contained some Tox$^+$ cells, the injection of this mixed clone into a mouse would yield a Tox$^+$ phenotype, and the presence of a Tox$^-$ marker would go undetected. On the other hand, it was not necessary to streak for isolation those transformant clones of strain UT0017 that received DNA from the ET-producing strain UT0002-19. If such clones were mixed after cotransfer of a Tox$^+$ marker, a Tox$^+$ phenotype would still be detectable in the mouse bioassay.

The efforts to map either a Tox$^+$ or Tox$^-$ locus were unsuccessful. In crosses that utilized strain UT0017 as recipient, a Tox$^+$ marker could be linked neither to the *ery*-3, *lin*-3, *nov*-1, *ole*-3, *pen*-1, *rif*-1, or *tet*-4 resistance markers, nor to the *ala*-12, *his*-3, *ilv*-9, *lys*-5, *phe*-4, *pur*-3, *ser*-14, *thr*-4, or *trp*-21 auxotrophic markers. Also, a Tox$^+$ marker could not be linked to the *thy*-136 marker in strain ISP136. In reciprocal crosses that utilized strain UT0002-19 as recipient, a Tox$^-$ marker from strain UT0017 could be linked neither to the *ery*-1, *nov*-9, *ole*-1, *rif*-10, or *tet*-3 resistance markers, nor to the *ala*-8, *ilv*-1, *lys*-7, *pur*-6, *thr*-4, or *thy*-1 auxotrophic markers.

DISCUSSION

Rudin et al. (15) reported that transformation in strain 8325 occurred only if one of the divalent cations, Mg^{2+}, Ba^{2+}, or Ca^{2+}, were present during the incubation with DNA. It was found in this study that calcium cations were also an absolute requirement for transformation in phage group 2 staphylococci. The calcium probably plays some important role in DNA uptake. The mode of action of rabbit serum in enhancing transformation is not known.

The gene linkages detected on the strains UT0002-19 and UT0017 chromosome are in general agreement with those found on the strain 8325 chromosome. An *ala* marker was detected in linkage group 1 of strain UT0002-19 but no comparable marker has been found in the corresponding linkage group of strains 8325 and UT0017. Strain 8325 contains a *pur* marker in linkage group 2 and *tet* and *pur* markers in linkage group 3. No comparable markers could be found on the group 2 chromosome.

The transformation frequencies observed for

S. aureus are several orders of magnitude lower than those observed in other systems. These low frequencies are probably a reflection of both the molecular mechanism of competence and the mechanics of integration of transforming DNA. Map distances within linkage groups were observed not to be additive (Fig. 5 and 6). This might be explained by another observation in this study showing that group 2 markers differ in their ability to be transformed. Cotransfer of linked markers is a reflection of the integration of these markers. If one of a pair of donor-linked markers is integrated inefficiently or reverted by repair enzymes, then observed linkage values would not represent true physical distances.

A genetic determinant for ET synthesis could not be mapped on the group 2 chromosome. It is believed that more than 50% of the group 2 chromosome was scanned to locate an ET A marker. Such markers can be distantly separated from the major loci that govern antibiotic resistance or amino acid biosynthesis. Another possibility is that a Tox^+ or Tox^- marker could have been loosely linked to a marker that was selected in a specific transformation cross, but the locus which governed the ET A trait reverted before it could be detected in the animal model.

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Thanks are extended to P. A. Pattee of Iowa State University for providing instruction and helpful suggestions about the isolation of staphylococcal mutants and the development of a regimen for DNA-mediated transformation.

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