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Factors Affecting Competence for Transformation in *Staphylococcus aureus*

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A chemically defined medium has been developed for isolation of amino acid-requiring mutants of *Staphylococcus aureus* strain 8325, and for use as a selective medium in transformation assays. Variables affecting transformation of both plasmid and chromosomal markers have been studied. The optimal pH and temperature for transformation are 6.75 to 7.0 and 30 C, respectively. Ca ions are required for transformation, and only cells lysogenic for the phage ϕ11 can be transformed. Superinfection of competent cells with ϕ11 does not increase the transformation frequency. Maximal number of transformants is obtained after 20 min of contact between cells and deoxyribonucleic acid. The transformation frequencies for the plasmid marker erythromycin resistance (*ero*) and the chromosomal markers *trp*, *thy*, and *cyt* are of the same order of magnitude, whereas the frequency for the chromosomal marker *tyr* is approximately one order of magnitude lower.

Transformation in *Staphylococcus* has been described (7, 8), and transfection has been used to determine the requirements for competence in this species (19, 20). Competence is generally defined as the ability of a bacterial strain to take up biologically active deoxyribonucleic acid (DNA) and undergo transformation. The competent state not only requires genetic information for expression of this ability, but special physiological conditions must also be prevailing to obtain high frequency of transformation (21, 22). The present study is concerned with the genetic and physiological factors which influence transformability of *Staphylococcus aureus* strain 8325 and different derivatives of this strain. A defined synthetic medium has been developed and used for isolation of amino acid-requiring mutants and as a selective medium in transformation experiments.

MATERIALS AND METHODS

Bacterial strains and phages. The *S. aureus* strains used are listed in Table 1. Phage ϕ11 was propagated on strain 8325-4 or strain 8325-4 *thy* in Trypticase soy broth (BBL, Cockeysville, Md.). The phages were pelleted at 107,000 $\times$ *g* for 120 min and suspended in nutrient broth (Difco) to give 10^{12} plaque-forming units per ml. Trypticase soy agar (TSA) and Trypticase soy broth (TSB) with 0.5% agar were used as bottom and soft agar, respectively, in the phage assay (20). Strain 8325-4 was used as indicator strain for phage titration.

Media. The composition of the synthetic minimal medium (AAM) is presented in Table 2. This medium was used for isolation of amino acid-requiring mutants and for selection of prototrophic transformants. In transformation experiments with pyrimidine and tryptophan-requiring mutants, we also used a casein-hydrolysate medium (CHM) as described earlier (8), except that L-arginine and L-proline were omitted.

Erythromycin-resistant transformants were selected on TSA medium with erythromycin at 5 μg/ml as described earlier (7). TSA medium or TSA medium with added pyrimidines at a final concentration of 5 μg/ml was used for determination of viable counts.

Transformation procedure. In our standard procedure for transformation, the recipient cells were grown on TSA plates and pyrimidine-requiring mutants were grown on TSA plates with added pyrimidine. Both cultures grew at 37 C overnight and then were suspended in TSB medium to an optical density at 524 nm (OD_{524}) of 0.100, which equals 5 $\times$ 10^7 colony-forming units (CFU) per milliliter. The cell suspension was then diluted 10 times in TSB medium and incubated on a rotary shaker at 37 C. Maximal competence is reached after 1.5 to 2 h of growth (somewhat dependent on the strain used; see Fig. 1 and 2). The competent cells were washed once in 0.15 M NaCl and then suspended in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-maleate buffer (pH 7.0) containing 0.1 M $CaCl_2$ at a cell density of approximately 10^9 CFU/ml.

In the transformation experiments, 0.9 ml of this cell suspension was mixed with 0.1 ml of DNA solution to give saturating concentration of DNA, i.e., 10 μg/ml (8). After incubation at 30 C for 20 min, the cells were centrifuged and suspended in 1 ml of TSB

TABLE 1. *Strains of Staphylococcus aureus used*

Strain	Lysogeny			Characteristics	Strain obtained from:[a]	Reference
	ϕ11	ϕ12	ϕ13			
8325	+	+	+	Wild type	M. Richmond	
8325 *nuc*	+	+	+	Mutant of 8325 lacking extracellular nuclease activity	Our laboratory	20
8325-4	−	−	−	Wild type; 8325 cured of prophages ϕ11, ϕ12, ϕ13	R. Novick	12
8325-4	+	−	−	Strain 8325-4 lysogenized with phage ϕ11	Our laboratory	20
8325-4	−	+	−	Strain 8325-4 lysogenized with phage ϕ12	R. Novick	12
8325-4	−	−	+	Strain 8325-4 lysogenized with phage ϕ13	R. Novick	12
8325(PI_{258})	+	+	+	Wild type harboring the penicillinase plasmid PI_{258}	M. Richmond	
8325(PI_{258}) *thy*	+	+	+	Thymine-requiring mutant of 8325(PI_{258})	Our laboratory	8
8325-4(PI_{258}) *thy*	−	−	−	Thymine-requiring mutant of 8325-4(PI_{258})	Our laboratory	
8325-4(PI_{258}) *thy*	+	−	−	Strain 8325-4(PI_{258}) *thy* lysogenized with ϕ11	Our laboratory	
8325 *cyt-1*	+	+	+	Cytidine- or uracil-requiring mutant of 8325	Our laboratory	8
8325 *cyt-1, trp-1*	+	+	+	Tryptophane-requiring mutant of 8325 *cyt-1*	Our laboratory	
8325 *cyt-1, trp-1, tyr-1*	+	+	+	Tyrosine-requiring mutant of 8325 *cyt-1, trp-1*	Our laboratory	

[a] M. Richmond, Department of Bacteriology, University of Bristol, Bristol BS8 ITD, England, and R. Novick, Public Health Research Institute of the City of New York, New York, have kindly supplied some of the strains used in this study.

before being plated on appropriate media. The plates were incubated at 37 C for 48 h and the colonies were counted.

The controls in the experiments included: (i) plating of the recipient culture without DNA treatment, (ii) sterility control of DNA, and (iii) treatment of the DNA with deoxyribonuclease (DNase; 50 μg/ml in the presence of 5 mM $MgCl_2$) for 10 min before the addition of cells.

Preparation of DNA. Transforming DNA was prepared by phenol extraction of lysostaphin-lysed cells as described earlier (8).

Isolation of auxotrophic mutants. The mutants 8325 *cyt-1, try-1* and 8325 *cyt-1, try-1, tyr-1* were isolated after mutagenic treatment of strain 8325 *cyt-1* and 8325 *cyt-1, try-1*, respectively, with 1-methyl-3-nitro-1-nitrosoguanidine (NTG; 4) and enrichment by the penicillin method (6). After treatment with NTG, phenotypic expression was allowed for 20 h in AAM medium supplemented with the respective amino acid. After being washed in 0.15 M NaCl, the cells were suspended in AAM medium to an OD_{524} of 0.100, which equals 5×10^7 CFU/ml. After growth for one generation, penicillin G was added to a final concentration of 15 U/ml. After 2 to 3 h, when the culture started to lyse, the cells were harvested, washed in 0.15 M NaCl, and plated at appropriate dilution on amino acid-supplemented AAM agar. After 48 h at 37 C, colonies were picked with sterile toothpicks to trays with small wells containing 0.15 M NaCl, and point-inoculated on AAM plates and on AAM plates with the respective amino acid added. Colonies that did not grow on the AAM plates were isolated, and the amino acid requirement was tested a second time.

RESULTS

Synthetic minimal medium. To be able to isolate amino acid-requiring mutants of strain 8325, we investigated the basal amino acid requirement of this strain. Several authors (1, 9, 11) have reported on various salt solutions for culturing *S. aureus*. The salt mixture reported in Table 2 supported the best growth in media containing amino acids, glucose, and vitamins. Starting with a mixture of 20 amino acids, the number of amino acids necessary for growth in a liquid medium was reduced to a minimum of 3 (L-cystine, L-arginine, and L-proline). However, growth was slow and the lag period was 24 to 48 h. The addition of L-glutamic acid, L-valine, L-leucine, and L-phenylalanine was necessary to obtain acceptable growth rates and short lag

TABLE 2. *Composition of the synthetic minimal medium (AAM)*[a]

Ingredients	Amounts per 1,000 ml
Salts:	
KCl	3.0 g
NaCl	9.5 g
$MgSO_4 \cdot 7H_2O$	1.3 g
$(NH_4)_2SO_4$	4.0 g
$CaCl_2 \cdot 2H_2O$[b]	22 mg
KH_2PO_4[b]	140 mg
$FeSO_4 \cdot 7H_2O$[c]	6 mg
$MnSO_4 \cdot 4H_2O$[c]	10 mg
Citric acid[c]	6 mg
Tris	12.1 g
Carbon source:	
Glucose[b]	5 g
Amino acids:	
L-Arginine	125 mg
L-Proline	200 mg
L-Glutamic acid	250 mg
L-Valine	150 mg
L-Threonine	150 mg
L-Phenylalanine	150 mg
L-Leucine	150 mg
L-Cystine[b]	80 mg
Vitamins:	
Biotin[b]	0.1 mg
Thiamin[b]	2 mg
Nicotinic acid[b]	2 mg
Calcium pantothenate[b]	2 mg

[a] When a solid medium was required, 15 g of agar (Difco) per ml was added and the pH was adjusted to 7.9 with 4 M HCl before autoclaving. When L-tryptophan or L-tyrosine is required, these amino acids are added after autoclaving to a final concentration of 200 mg/1000 ml. When uracil or thymine is required, these pyrimidines are added after autoclaving to a final concentration of 5 mg/1000 ml.

[b] Sterilized by filtration and added separately after autoclaving.

[c] Sterilized by filtration and added separately after autoclaving as a solution containing 100 mg of $MnSO_4 \cdot 4H_2O$, 60 mg of $FeSO_4 \cdot 7H_2O$, and 60 mg of citric acid per 100 ml.

periods in liquid medium. When agar was added to the minimal medium, L-threonine had to be included to obtain plating efficiencies of the same order of magnitude as on TSA plates, although this amino acid did not affect growth in liquid medium. Table 3 shows the generation time in TSB medium as compared to the AAM medium for different derivatives of strain 8325. Plating efficiencies on AAM agar and CHM agar, expressed as percentage of the number of CFU on TSA plates, are also presented. The percentages presented in Table 3 are from dilutions giving about 100 CFU on a TSA plate. The plating efficiency on the AAM medium is, however, enhanced slightly when the number of colonies per plate is increased.

Isolation and characterization of amino acid-requiring mutants. Four tryptophan-requiring mutants were isolated in two different experiments after NTG treatment of strain 8325 *cyt-1*. L-Tryptophan was nonlimiting at a final concentration of 1.5 to 2 μg/ml. The mutant with the lowest frequency of reversion (8325 *cyt-1, try-1*) was used in transformation experiments and mutagenized further with NTG. From these experiments, one tyrosine-requiring mutant was isolated and characterized further. L-Tyrosine was nonlimiting at a final concentration of 2 μg/ml in liquid AAM medium.

Competence curve. The high extracellular nuclease activity, which is characteristic for *S. aureus*, has been considered the main obstruction in transformation in this species (14). To investigate whether extracellular nuclease affects transformation, we isolated a mutant of strain 8325, defective in production of extracellular nuclease (20). The growth and development of competence of the wild-type 8325 and the mutant 8325 *nuc* are shown in Fig. 1. Growth was started at an OD_{524} of 0.010 (ca. 5×10^6 CFU/ml) as described above. Samples were withdrawn at intervals and concentrated to approximately 10^9 CFU/ml before DNA was added. The donor strain was 8325 (PI_{258}), and erythromycin-resistant transformants were selected. The *nuc* mutant has a lower growth rate than the wild type. Both strains have a marked competence maximum close to the doubling of the number of CFU. The competence of the wild type then gradually decreases, whereas the mutant shows two additional competence maxima in the early exponential growth phase. The production of extracellular nuclease was followed during growth both for the wild type and mutant (20). The mutant does not produce measurable extracellular nuclease during the active growth cycle, whereas, for the wild type, nuclease activity can be detected in the growth medium just after the competence maximum is passed. Since erythromycin resistance is a plasmid-coded marker, the growth and competence curves were also established for a chromosomal marker by using a thymine-requiring mutant of strain 8325(PI_{258}) as recipient and strain 8325(PI_{258}) as donor (Fig. 2). A comparison of Fig. 1A and 2 reveal no significant differences in the competence pattern. It should be empha-

TABLE 3. *Growth rates and plating efficiencies on complex and defined media for different strains of S. aureus*

Strain	Generation time in liquid medium (min)		Plating efficiency	
	TSB[a]	AAM[b]	(CFU on CHM plates)/(CFU on TSA plates) × 100	(CFU on AAM plates)/(CFU on TSA plates) × 100
8325	70	220	100	77
8325-4	65	195	98	85
8325(PI_{258})	90	150	100	64
8325(PI_{258}) *thy*	80	150	81	55
8325 *cyt-1*	60	150	96	88
8325 *cyt-1, trp-1*	75	180	91	45
8325 *cyt-1, trp-1, tyr-1*	75	210	81	45

[a] TSB, Trypticase soy broth. For thymine- and cytidine-requiring mutants, the respective pyrimidine was added to the medium.

[b] Synthetic minimal medium (Table 2) with appropriate additions when used for auxotrophic mutants.

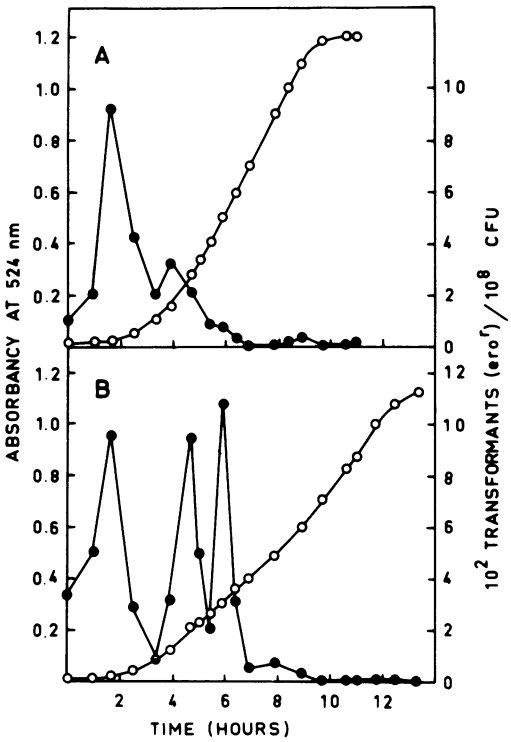

FIG. 1. *Development of competence for (A) S. aureus wild-type (8325) and for (B) S. aureus 8325 nuc grown in TSB medium. Growth was started at an OD_{524} of 0.010, corresponding to 5 × 10^6 CFU/ml. Donor strain: 8325(PI_{258}). Symbols: ○, growth; ●, number of erythromycin-resistant transformants per 10^8 CFU of the recipients.*

sized that we have at present no information on the gross structure of the staphylococcal genome, but we assume that the thymine locus is chromosomal.

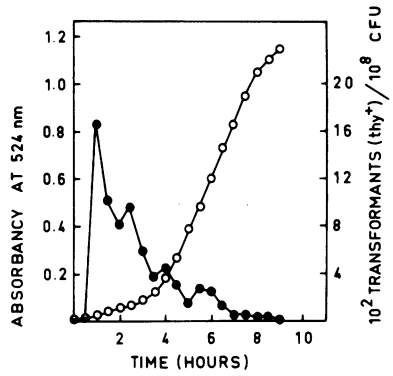

FIG. 2. *Development of competence for S. aureus 8325(PI_{258}) thy grown in TSB medium plus thymine (5 μg/ml). Growth was started at an OD_{524} of 0.010, corresponding to 5 × 10^6 CFU/ml. Donor strain: 8325(PI_{258}) thy$^+$. Symbols: ○, growth; ●, number of thy$^+$ transformants per 10^8 CFU of the recipients.*

Effect of pH on transformation. Figure 3 shows the number of transformants as a function of pH of the buffer used in the transformation experiments. The recipient cells [8325(PI_{258}) *thy*] were washed in 0.15 M NaCl and suspended in Tris-maleate buffer (at various pH values) containing 0.1 M $CaCl_2$. After 20 min of contact between cells and DNA, the mixtures were divided into two samples. One sample was centrifuged directly. The other sample was treated with DNase (final concentration 50 μg/ml in presence of 10^{-3} M $MgCl_2$) for 10 min at 30 C and then centrifuged. Before being plated on CHM medium, the cells from both samples were suspended to equal volumes in TSB broth. The maximal number of transformants was obtained at pH 6.75 to 7.0. The lower curve of the figure shows that treatment of

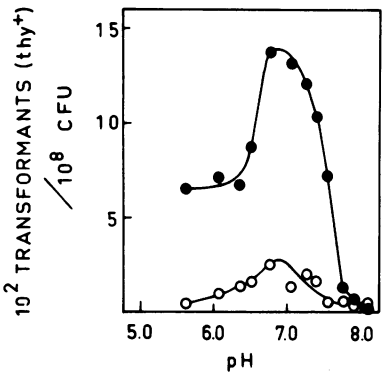

FIG. 3. *Effect of pH of the transformation buffer on the number of transformants (thy$^+$ per 10^8 recipient CFU). Donor strain: 8325(PI$_{258}$) thy$^+$. The competent cells (8325(PI$_{258}$) thy) were mixed with DNA at different pH in Tris-maleate buffer containing 0.1 M $CaCl_2$. After 20 min of contact between cells and DNA at 30 C, the cells were centrifuged for 10 min at 4 C and either suspended in TSB medium before plating (upper curve) or treated with DNase (50 μg/ml in the presence of 10^{-3} M $MgCl_2$) for 10 min, centrifuged, and then resuspended in TSB medium before plating (lower curve).*

the transformation mixture with DNase before plating results in a decreased number of transformants. The reduction in number of transformants after DNase treatment varies between 30 and 70% in different experiments. Plating of cells directly after the addition of DNA gives no transformants. Thus, adsorption of DNA cannot occur on the agar surface, but transport of already adsorbed DNA continues after plating.

Effect of divalent ions. Transformation in Tris-maleate buffer occurred only if one of the divalent cations Mg^{2+}, Ba^{2+}, or Ca^{2+} was present. The frequency of transformation obtained with Mg^{2+} and Ba^{2+} as competence-promoting ions was 10% of that with Ca^{2+}. The optimal concentration of Ca^{2+} was 0.1 M if the cells were plated directly from the transformation mixture (Fig. 4, lower curve). Centrifugation of the transformation mixture at 4 C for 10 min and suspension of the cells in TSB before plating resulted in an increased number of transformants and a broader effective concentration range of Ca^{2+} (Fig. 4, upper curve). The cations Fe^{2+} and Fe^{3+} could not promote transformation.

Temperature optimum. Competent cells of strain 8325(PI$_{258}$) *thy* were incubated for 20 min at different temperatures with DNA in 0.1 M Tris-maleate buffer (pH 7.0) containing 0.1 M $CaCl_2$. When cells were plated on agar medium directly from the transformation mixture, the optimal temperature for transformation was 27 to 30 C (Fig. 5, lower curve). Centrifugation of the transformation mixture at 4 C for 10 min and suspension in TSB or 0.15 M NaCl before plating resulted in an increased number of transformants with a plateau in the temperature range from 4 to 27 C (Fig. 5, upper curve).

Duration of DNA exposure. Competent cells of strain 8325(PI$_{258}$) *thy* and DNA were mixed and samples were removed at intervals. The samples were plated directly on CHM agar. Transformation reached a maximal level after 20 min of exposure to DNA (Fig. 6). A plateau was also reached in 20 min when the cells were treated with DNase prior to plating (not shown).

Effect of lysogeny on competence. In a recent paper on transfection in *S. aureus*, it was shown that development of competence requires that the cells are lysogenic for phage φ11 (20). Table 4 shows that lysogeny with the same phage is necessary also for development of competence for uptake of plasmid and chromosomal DNA. The wild-type strain 8325 is lysogenic for phages φ11, φ12, and φ13. When this strain is cured of the prophages (strain 8325-4), it also loses the ability to express competence. Lysogenization of the cured strain with phage φ11 re-established the competence. Phages φ12

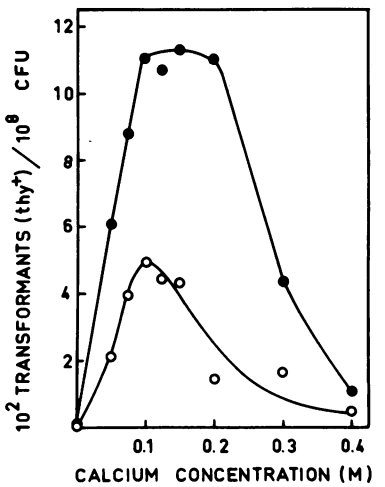

FIG. 4. *Effect of the concentration of $CaCl_2$ on the number of transformants (thy$^+$ per 10^8 recipient CFU). Donor strain: 8325(PI$_{258}$) thy$^+$. Competent cells [8325(PI$_{258}$) thy] were suspended in Tris-maleate buffer (pH 7.0) containing different concentrations of $CaCl_2$ before DNA was added. After 20 min of contact between cells and DNA, the cells were plated directly (lower curve) or centrifuged and suspended in TSB medium before plating (upper curve).*

and ϕ13 have no competence-inducing capacity.

In two experiments, ϕ11 phages at a multiplicity of infection of 1 were added to cells of strain 8325-4 *thy* suspended in transformation buffer with DNA from the donor strain 8325(PI_{258}). Phage ϕ11 was propagated on the recipient strain. After 20 and 40 min of incubation at 30 C, samples were removed and centrifuged at 4 C for 10 min. The cells were suspended in 0.15 M NaCl and plated on CHM plates. A few transformants were obtained (Table 5). The transformants were reisolated on TSA plates and then tested for sensitivity for phage ϕ11. All transformants were immune to phage ϕ11, which indicates that competence has been expressed in some of the cells which have been lysogenized during simultaneous exposure to phages and DNA. The frequency of lysogenization in the reported experiments was 1.4×10^{-1}.

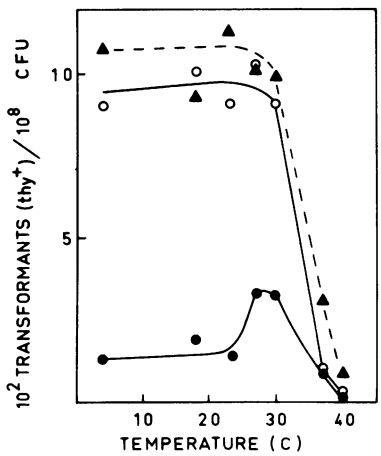

FIG. 5. *Effect of temperature on the number of transformants (thy$^+$ per 10^8 recipient CFU). Donor strain: 8325(PI_{258}) thy$^+$. Competent cells [8325(PI_{258}) thy] were suspended in Tris-maleate buffer (pH 7.0) containing 0.1 M $CaCl_2$ and incubated for 10 min at different temperatures for temperature equilibration before DNA was added. After 20 min of contact between cells and DNA, the cells were plated directly from the transformation mixtures (●) or centrifuged for 10 min at 4 C and suspended in TSB medium (○) or 0.15 M NaCl (▲) before plating.*

Effect of superinfection with phage ϕ11 on transformation frequency. Since superinfection of competent cells of strain 8325-4(ϕ11) at high multiplicities increased the frequency of transfection (20), we also tested the effect of superinfection on the transformation. The addition of phage ϕ11 to the transformation mixture did not increase the transformation frequency (Table 6).

Effect of centrifugation of the transformation mixture. Centrifugation of the transformation mixture before plating results in an increase of the number of transformants by a

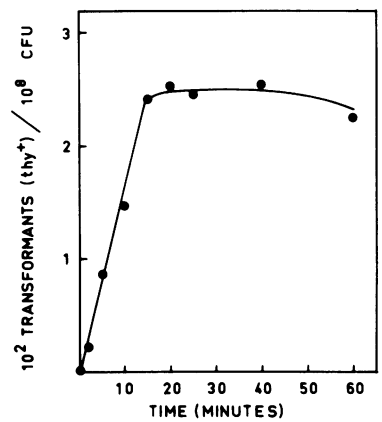

FIG. 6. *Effect of DNA exposure time on the number of transformants. Competent cells of strain 8325(PI_{258}) thy were mixed with DNA, and samples were removed at different times for assay of the number of transformants. Donor strain: 8325(PI_{258}) thy$^+$.*

TABLE 4. *Requirement of lysogeny for competence induction*

Recipient strain	Lysogeny			Donor strain	Transformants/10^8 recipient CFU	
	ϕ11	ϕ12	ϕ13		*ero*^{r} [a]	*thy*$^+$
8325	+	+	+	8325(PI_{258})	4.3×10^2	
8325-4	+	−	−	8325(PI_{258})	5.5×10^2	
8325-4	−	+	−	8325(PI_{258})	<3	
8325-4	−	−	+	8325(PI_{258})	<3	
8325-4	−	−	−	8325(PI_{258})	<3	
8325-4(PI_{258}) *thy*	−	−	−	8325(PI_{258})		18[b]
8325-4(PI_{258}) *thy*	+	−	−	8325(PI_{258})		6×10^2

[a] Erythromycin resistance.

[b] Equal to frequency of spontaneous reversion.

factor of 2 to 3 (Fig. 4 and 5). A 20-fold dilution of the transformation mixture in fresh buffer before centrifugation eliminates the enhancement caused by centrifugation. The addition of DNA at the same concentration as in the original incubation mixture restored the effect of centrifugation. Irrespective of whether TSB broth, 0.15 M NaCl, or fresh transformation buffer was used for resuspension after centrifugation, the same increase was observed (Fig. 5), whereas resuspension in the supernatant of the original transformation mixture reduced the enhancement twofold.

The concentration of recipient cells was also varied while the DNA concentration was kept constant (40 μg/ml). There is a maximum in transformation frequency at 6×10^8 recipient CFU/ml (Table 7). The enhancement of transformation by centrifugation in the presence of DNA may thus depend on a higher collision frequency between the cells and DNA during centrifugation. These results suggest also that the cells do release a factor into the transformation buffer, which results in a reduction of the number of transformants when the cell concentration is increased.

Transformation of amino acid-requiring mutants. Table 8 presents transformation data for different amino acid-requiring mutants. All strains and characters were transformed at frequencies significantly different from the spontaneous reversion frequencies. In all experiments, the tyrosine locus was transformed at a lower frequency than the other markers studied. This is not due to low competence of the triple mutant, since the tryptophane marker was transformed at the same efficiency to both the double and the triple mutants. The lower number of transformants on AAM plates than on CHM plates is explained by differences in plating efficiencies (Table 3). In all experiments, the number of viable recipient bacteria

TABLE 5. *Competence induction by simultaneous addition of φ11 phages and DNA from strain 8325(PI_{258}) to cells of strain 8325-4 thy*[a]

Determination	Incubation time (min)	No. of transformants (*thy*⁺/ml)	Viable count (CFU/ml)
Cells without DNA and φ11	20	<3	3.5×10^8
	40	<3	3.2×10^8
Cells + φ11 (MOI = 1)	20	<3	8×10^7
	40	<3	1.5×10^8
Cells + DNA	20	<3	1.2×10^8
	40	<3	2.2×10^8
Cells + φ11 (MOI = 1) + DNA	20	60	9×10^7
	40	30	8×10^7

[a] Recipient cells were grown under the standard conditions for competence induction. φ11 phages propagated on 8325-4 *thy* were added at multiplicity of infection (MOI) of 1 to cells in transformation buffer containing DNA (40 μg/ml). After 20 and 40 min of incubation at 30 C, samples were plated on CHM plates for selection of *thy*⁺ transformants and on TSA plates with thymine for determination of viable count.

TABLE 7. *Influence of recipient cell concentration on the transformation frequency*[a]

No. of recipients (CFU/ml)	No. of *thy*⁺ transformants/10^8 recipient CFU	
	+DNA	−DNA
3.5×10^9	28	17
1.7×10^9	102	Not tested
6×10^8	334	<3
2.5×10^8	184	<3
1.1×10^8	126	<3
4×10^7	150	<3
3×10^7	13	<3
2×10^7	3	<3

[a] Donor DNA was prepared from strain 8325(PI_{258}). Recipient cells of strain 8325(PI_{258}) *thy* were grown under standard conditions for competence induction. Cells were suspended and serially diluted in transformation buffer. DNA was added (final concentration 40 μg/ml), and after 20 min of incubation at 30 C samples were plated on CHM plates for selection of *thy*⁺ transformants and on TSA plates with added thymine for determination of viable count.

TABLE 6. *Effect of superinfection with φ11 on transformation frequency*

Recipient strain	Donor strain	Multiplicity of infection	No. of transformants (*ero*[r][a]/ml)	Viable count (CFU/ml)	Transformation frequency
8325-4(φ11)	8325(PI_{258})	0	1.9×10^3	6.5×10^8	2.9×10^{-6}
8325-4(φ11)	8325(PI_{258})	0.1	2.3×10^3	6.5×10^8	3.5×10^{-6}
8325-4(φ11)	8325(PI_{258})	1.0	7.0×10^2	6.0×10^8	1.2×10^{-6}
8325-4(φ11)	8325(PI_{258})	10	1.3×10^3	3.3×10^8	3.9×10^{-6}

[a] Erythromycin resistance.

(CFU) was determined after plating on TSA with the necessary additions.

Transformation of plasmid markers. The plasmid markers erythromycin (*ero*) and cadmium (*cad*) resistance were used as selective markers to demonstrate transformation of plasmid markers. Donor DNA was isolated from strain 8325(PI_{258}) by phenol extraction of crude lysates. The number of transformants was, in an average of five experiments, approximately 100 times lower when selected for cadmium resistance than for erythromycin resistance as reported previously (7). When selection was for *ero* resistance, the transformants received, as a rule, only the *ero* marker, whereas the cad^R transformants carried most of the resistance markers of the donor plasmid. The reasons for these differences have been discussed (7).

The kinetics of transformation of the drug-sensitive strain of *S. aureus* 8325 to erythromycin resistance by DNA from strain 8325(PI_{258}) is shown in Fig. 7. After 20 min of incubation at 30 C, the transformation mixture was plated on TSA plates. The plates were incubated at 37 C and overlaid at different times with 3 ml of soft agar containing erythromycin to give a final concentration of 1 μg/ml of agar medium. The number of recipient bacteria exhibiting erythromycin resistance increases more than 1,000-fold during subsequent incubation on antibiotic-free plates and reaches a maximum in 75 min.

DISCUSSION

The effects of lysogeny upon transfection and transformation have been reported in several bacterial species. Lysogenic streptococci cannot be made highly competent, as measured by irreversible DNA uptake (15); thus, in this species transformation frequencies are decreased owing to lysogeny. *Bacillus subtilis* lysogenic for bacteriophage φ105 retains its ability to be made competent (23). Transfection is hardly affected, but the frequency of transformation is drastically reduced (24). The differences in effects of lysogeny on transfection and transformation could possibly depend on an abnormality in a recombination system induced by lysogeny with phage φ105, and this defect may discriminate between bacterial and phage DNA. Peterson and Rutberg (16), on the other hand, observed a decrease both in the frequency of DNA-mediated transfection with bacteriophage φ1 and transformation in *B. subtilis* 168 lysogenic for phage φ105, results which were interpreted to suggest that the cells were less capable of assimilating the DNA. Setlow et al. (17) reported on the relationship between transformation and lysogeny with the phages HP1cl, S2, and a defective phage of *Haemophilus*

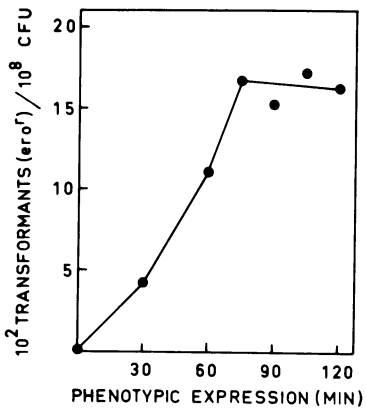

FIG. 7. *Kinetics of expression of erythromycin resistance in transformed S. aureus 8325-4(φ11). Competent cells of strain 8325-4(φ11) were exposed to DNA from strain 8325(PI_{258}). After 20 min the cells were plated on TSA. The plates were incubated at 37 C, and at different times soft agar containing erythromycin was poured onto the plates. The final concentration of erythromycin in the plates was 5 μg/ml.*

TABLE 8. *Transformation of auxotrophic mutants by DNA from wild-type strain 8325(PI_{258})*

Expt[a] no.	Recipient strain	Selected marker	Transformants per 10^8 recipient cells selected on		Spontaneous revertants per 10^8 cells selected on	
			CHM[b]	AAM[c]	CHM	AAM
1.	8325(PI_{258}) *thy*	*thy*$^+$	1,350	750	<1	<1
2.	8325 *cyt-1*	*cyt*$^+$	460	200	4.0	3.8
3.	8325 *cyt-1, trp-1*	*trp*$^+$	400	320	2.1	1.4
4.	8325 *cyt-1, trp-1, tyr-1*	*tyr*$^+$		38		2.0
5.	8325 *cyt-1, trp-1*	*trp*$^+$	Not tested	120	Not tested	<1
6.	8325 *cyt-1, trp-1, tyr-1*	*trp*$^+$	Not tested	150	Not tested	7

[a] Separate DNA preparations were used in experiments 1 through 4 and 5 through 6, respectively.
[b] Casein-hydrolysate medium.
[c] Synthetic minimal medium (Table 2).

influenzae. The presence of any of the prophages did not appreciably alter transformation frequencies in various *rec*$^+$ and *rec*$^-$ strains. However, exposure of competent lysogens to transforming DNA may induce phage, but only in *rec*$^+$ strains, which are able to integrate transforming DNA into their genome.

The absolute requirement of lysogeny for phage ϕ11 for competence induction in *S. aureus* 8325 both for transfection (20) and transformation (Table 4) may also constitute an example of lysogenic conversion, but in this case results in an increased permeability for macromolecules. Since superinfection with phage ϕ11 at high multiplicities markedly increased the frequency of transfection (20), a helper function of the phage directly at the cell envelope may be involved. The transformation frequency, however, is not affected significantly by superinfection (Table 6). The difference between transfection and transformation in this respect may depend upon the fact that superinfected cells allow phage propagation, which results in cell lysis, and the cells cannot therefore score as transformants. Another possible explanation to the enhancement of transfection by superinfecting ϕ11 phages which has not been ruled out is marker rescue by incoming ϕ11 DNA. Simultaneous incubation of nonlysogenic and thus noncompetent cells of strain 8325-4 *thy* with DNA and phage ϕ11 in transformation buffer results in competence induction and transformation. All transformants were lysogenic for phage ϕ11. The step controlled by the ϕ11 genome in the transformation process is, however, still unresolved.

The competence pattern of the *nuc* mutant, which produces no extracellular nuclease, is more complex than that of the wild type (Fig. 1). The recurrent competence maxima of the *nuc* mutant occur at short time intervals. Extracellular nuclease is detected in the medium of the wild type just after the first competence maximum is reached (20), which may explain the decrease in competence late in the growth curve. However, nuclease production is probably not the only factor regulating competence, since competence of the mutant is also confined to the early exponential growth phase, although extracellular nuclease is lacking throughout the growth curve in this mutant (20). It should be emphasized that the *nuc* mutant is not free from intracellular nuclease activity. Cosloy and Oishi (3) recently reported on genetic transformation in $CaCl_2$-treated *Escherichia coli* K-12 cells which lack adenosine triphosphate-dependent DNase and exonuclease I. It is conceivable that in the *E. coli* system the activities of these nucleases damage the incoming DNA before it can participate in recombination. At present, we cannot define in absolute terms the physiological conditions necessary for induction of competence in *S. aureus*, and no explanation for the repeated competence maxima of the *nuc* mutant can be offered. Since the growth rate of the wild type is higher and the *nuc* mutant was isolated after mutagenization with nitrosoguanidine, it is possible that the *nuc* mutant is a double mutant. However, a similar competence pattern with two early maxima has been reported in *Diplococcus pneumoniae* (18). In this system, maximum competence was displaced to a higher cell concentration by adding tryptic peptides of casein to the competence medium. Hotchkiss (4) observed correlation between transformability and cell division in a partially synchronized pneumococcal culture which he referred to as competence "waves."

Divalent cations, such as Sr^{2+}, Ba^{2+}, Ca^{2+}, or Mg^{2+} are required for DNA uptake in *Bacillus subtilis* (25) and *Micrococcus lysodeikticus* (5). Treatment of recipient cells with $CaCl_2$ is also necessary for uptake of DNA into *E. coli* as first reported by Mandel and Higa (10) for transfection and, subsequently, applied for transformation in this organism (3, 13). The results from our studies show that transformation in *S. aureus* occurs in Tris-maleate buffer only if one of the divalent cations Mg^{2+}, Ba^{2+}, or Ca^{2+} is present out of which Ca^{2+} at a concentration of 0.1 to 0.2 M is most efficient (Fig. 4).

A contact period of 20 min between recipient cells and DNA is optimal, but treatment of the cells with DNase after attachment of DNA reduces the number of transformants 30 to 70%. The percentage varies in different experiments. The DNase may reach and destruct DNA attached to the cell surface and possibly also DNA located in the periplasmic space.

There is a temperature optimum at 27 to 30 C for assimilation of DNA when cells are plated directly from the transformation mixture (Fig. 5, lower curve). Centrifugation of the transformation mixture at 4 C and suspension of the cells in TSB medium or NaCl resulted in an enhancement of the transformation frequency irrespective of the temperature between 4 and 30 C (Fig. 5, upper curves). The enhanced transformation frequency by centrifugation probably depends on a higher frequency of collision between cells and DNA during centrifugation. This interpretation is supported by the observation that a dilution of the transformation mixture eliminated the enhancement, but the effect was restored by adding DNA to the same concentration as in the original mixture

before dilution. There is also an optimal cell concentration around 6×10^8 cells per ml for transformation (Table 7), which may suggest that competent cells release a nuclease which destructs donor DNA at high cell concentrations.

To be able to isolate amino acid-requiring mutants and study transformation of these auxotrophic characters, we developed a defined minimal medium (Table 2). Plating efficiencies on the constructed AAM medium vary between 45 and 90% of those on a complex medium for different strain derivatives of strain 8325. The use of the AAM medium makes it possible to isolate and study pyrimidine-, purine-, vitamin-, and amino acid-requiring mutants, which is required for a more detailed analysis of the *S. aureus* genome.

The transformation frequencies for chromosomal markers and the plasmid erythromycin marker are of the same order of magnitude. The number of transformants was approximately 100 times lower when cadmium resistance was used for selection of transformants. The reasons for this difference have been discussed (7).

Although we have managed to increase the competence level significantly by refining the conditions during the transformation procedure, the low transformation frequencies of this system are still a serious disadvantage for genetic studies aiming at a map of the chromosome. However, the observation that competence induction requires lysogeny may suggest a route to define conditions for a high level of competence. Temperature-sensitive mutants of phage $\phi 11$ have now been isolated (J.-E. Sjöström and L. Philipson, manuscript in preparation). Some of these mutants cannot express competence at the nonpermissive temperature. Genetic and biochemical studies of these mutants may elucidate the mechanism of competence induction by phage $\phi 11$.

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LITERATURE CITED

1. Altenbern, R. A. 1971. An expanded genomic map of *Staphylococcus aureus*. Can. J. Microbiol. **17**:1239-1242.
2. Cohen, S. N., A. C. Y. Chang, and L. Hsu. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-factor DNA. Proc. Nat. Acad. Sci. U.S.A. **69**:2110-2114.
3. Cosloy, S. D., and M. Oishi. 1973. The nature of the transformation process in *Escherichia coli* K12. Mol. Gen. Genet. **124**:1-10.
4. Hotchkiss, R. D. 1954. Cyclical behaviour in pneumococcal growth and transformability occasioned by environmental changes. Proc. Nat. Acad. Sci. U.S.A. **40**:49-55.
5. Kloos, W. E. 1969. Factors affecting transformation of *Micrococcus lysodeikticus*. J. Bacteriol. **98**:1397-1399.
6. Lederberg, J. 1950. Isolation and characterization of biochemical mutants of bacteria, p. 5. *In* J. H. Courrie, Jr. (ed.), Methods in medical research, vol. 3. Year Book Publishers, Chicago.
7. Lindberg, M., and R. P. Novick. 1973. Plasmid-specific transformation in *Staphylococcus aureus*. J. Bacteriol. **115**:139-145.
8. Lindberg, M., J. E. Sjöström, and T. Johansson. 1972. Transformation of chromosomal and plasmid characters in *Staphylococcus aureus*. J. Bacteriol. **109**:844-847.
9. Mah, R. A., D. Y. C. Fung, and S. A. Morse. 1967. Nutritional requirements of *Staphylococcus aureus* S-6. Appl. Microbiol. **15**:866-870.
10. Mandel, M., and A. Higa. 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53**:159-162.
11. Miller, R. D., and D. Y. C. Fung. 1973. Amino acid requirements for the production of enterotoxin B by *Staphylococcus aureus* S-6 in a chemically defined medium. Appl. Microbiol. **25**:800-806.
12. Novick, R. P. 1967. Properties of a cryptic high frequency transducing phage in *Staphylococcus aureus*. Virology **33**:155-166.
13. Oishi, M., and S. D. Cosloy. 1972. The genetic and biochemical basis of the transformability of *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **49**:1568-1572.
14. Omenn, G. S., and J. Friedman. 1970. Isolation of mutants of *Staphylococcus aureus* lacking extra cellular nuclease activity. J. Bacteriol. **101**:921-924.
15. Parsons, C. L., J. M. Ranhand, C. G. Leonard, A. E. Colon, and R. M. Cole. 1973. Inhibition of transformation in group H streptococci by lysogeny. J. Bacteriol. **113**:1217-1222.
16. Peterson, A. M., and L. Rutberg. 1969. Linked transformation of bacterial and prophage markers in *Bacillus subtilis* 168 lysogenic for bacteriophage $\phi 105$. J. Bacteriol. **98**:874-877.
17. Setlow, J. K., M. E. Boling, D. P. Allison, and K. L. Beattie. 1973. Relationship between prophage induction and transformation in *Haemophilus influenzae*. J. Bacteriol. **115**:153-161.
18. Sirotnak, F. M. 1971. Marked enhancement of *Diplococcus pneumoniae* competence for transformation by tryptic peptides of casein. Biochem. Biophys. Res. Commun. **45**:63-69.
19. Sjöström, J. E., M. Lindberg, and L. Philipson. 1972. Transfection of *Staphylococcus aureus* with bacteriophage deoxyribonucleic acid. J. Bacteriol. **109**:285-291.
20. Sjöström, J. E., M. Lindberg, and L. Philipson. 1973. Competence for transfection in *Staphylococcus aureus*. J. Bacteriol. **113**:576-585.
21. Spizizen, J., B. E. Reilly, and A. H. Evans. 1966. Microbial transformation and transfection. Annu. Rev. Microbiol. **20**:371-400.
22. Tomasz, A., and J. L. Mosser. 1969. On the nature of the competent state in genetic transformation. Annu. Rev. Genet. **3**:217-232.
23. Yasbin, R. E., G. A. Wilson, and F. E. Young. 1973. Transformation and transfection in lysogenic strains of *Bacillus subtilis* 168. J. Bacteriol. **113**:540-549.
24. Yasbin, R. E., and F. E. Young. 1972. The influence of temperate bacteriophage $\phi 105$ on transformation and transfection in *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **47**:365-371.
25. Young, F. E., and J. Spizizen. 1963. Incorporation of deoxyribonucleic acid in the *Bacillus subtilis* transformation system. J. Bacteriol. **86**:392-400.