JOURNAL OF BACTERIOLOGY, Feb. 1972, p. 844-847

Vol. 109, No. 2
Printed in U.S.A.

Transformation of Chromosomal and Plasmid Characters in *Staphylococcus aureus*

MARTIN LINDBERG, JAN-ERIC SJÖSTRÖM, AND THOMAS JOHANSSON
Department of Microbiology, The Wallenberg Laboratory, University of Uppsala, Uppsala, Sweden

Received for publication 22 November 1971

Plasmid and probably also chromosomal characters have been genetically transformed in *Staphylococcus aureus*. Recipient cells show competence throughout the exponential growth phase with a maximum at early times.

Recently Nomura et al. (11) reported on induction of hemolysin synthesis by transformation in *Staphylococcus aureus*. Earlier reports on transformation in *S. aureus* were limited to transmission of resistance markers like streptomycin and metal ions (5–9, 19). Nomura et al. (11) performed transformation in hypertonic broth containing low levels of penicillin and streptomycin.

Riggs and Rosenblum (16) reported transfection of two strains of *S. aureus* with phage deoxyribonucleic acid (DNA) in hypertonic medium only when the cell walls of the recipients had been partially disrupted with lysostaphin. A system for transfection of *S. aureus* with intact cell walls was recently developed, with 0.1 M $CaCl_2$ in distilled water used as the incubation medium for uptake of phage DNA (18).

The present communication describes methods for genetic transformation of *S. aureus* with phenol-extracted DNA. We used erythromycin, penicillin, and cadmium resistance, which are plasmid-coded characters of the donor strain (12), and the characters thymine and cytidine independence, which probably are coded by chromosomal genes as selective genetic markers in the transformation experiments.

MATERIALS AND METHODS

S. aureus strains 8325 (N) and 8325 (PI_{258}) were obtained from M. H. Richmond, Department of Bacteriology, The Medical School, Bristol. The auxotrophic mutants 8325 (N) *thy*$^-$ and 8325 (N) *cyt*$^-$ were isolated after mutagenic treatment of strain 8325 (N) with NTG (1-methyl-3-nitro-1-nitrosoguanidine; reference 1); 8325 (PI_{258}) *thy*$^-$ was a spontaneous mutant of strain 8325 (PI_{258}). The thymine-requiring mutants were isolated after enrichment in a minimal medium (CHM-medium) in the presence of aminopterin and thymine (15).

CHM-medium, used for isolation of pyrimidine-requiring mutants and as selective medium in transformation experiments, consisted of: KH_2PO_4, 0.1 g; NH_4Cl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; L-proline, 40 mg; L-arginine, 40 mg; and vitamin-free (acid) casein hydrolysate (Nutritional Biochemical Corp., Cleveland, Ohio), 10 g; dissolved in 1,000 ml of 0.1 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, *p*H 8.0. The following components were sterilized separately and added after autoclaving (per liter of medium): glucose (50%), 20 ml; thiamine, 1 mg; biotin, 0.005 mg; nicotinic acid, 1.2 mg; Ca-pantothenate, 0.25 mg; L-cystine, 40 mg; and a trace element solution (per 100 ml of water: $MnSO_4 \cdot 4H_2O$, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.06 g; and citric acid, 0.06 g), 10 ml; 1.5% agar was added to solid media.

Trypticase Soy Agar (TSA; BBL, Cockeysville, Md.) was used as selective medium in transformation experiments with plasmid-coded markers, i.e., resistance to erythromycin, penicillin, and metal ions. All resistance marker components were added in optimal concentration for genetic selection (12, 13).

Transforming DNA was prepared from cells grown in TSB (Trypticase Soy broth) overnight. The cells from 150 ml of culture were washed and suspended in 5 ml of 0.1 M Tris-hydrochloride buffer (*p*H 7.5) containing 0.15 M NaCl and 0.1 M ethylenediaminetetraacetic acid. Lysostaphin (Schwarz/Mann, Division of Becton, Dickinson and Co.) was added to a final concentration of 10 units/ml, and the suspension was incubated with shaking at 37 C for 30 min, followed by treatment with Pronase (final concentration, 2 mg/ml) at 37 C for an additional 60 min. A 0.4-ml amount of sodium dodecyl sulfate (5%, w/v, in 45% ethanol) was added, and the mixture was shaken by hand for 30 min at room temperature.

The lysed suspension was mixed with an equal volume of redistilled phenol (saturated with 0.01 M Tris-hydrochloride buffer, *p*H 8.1), and the mixture was shaken by hand for 15 min at room temperature. The resulting emulsion was broken by slow-speed centrifugation. The aqueous phase was collected, and the phenol treatment was repeated five times. The aqueous phase was extracted with ethylether several times to remove the phenol. Finally, the ether was removed by nitrogen. The nucleic acids were precipitated by gently mixing the solution with two volumes of cold ethanol. The thread-like precipitate was collected on a glass rod and dissolved in 5 ml of a 0.01 dilution of SSC (0.15 M sodium chloride and 0.015 M sodium citrate, *p*H 7.2). To remove ribonucleic acid, ribonuclease was added to a final concentration of 50 μg/ml, and the mixture was incubated for 30 min at 37 C. DNA was precipitated by addition of two volumes of ethanol, collected on a glass rod, and dissolved in 2.5 ml of 0.01 $\times$ SSC. An equal volume of 0.1 $\times$ SSC was then added. The concentration of DNA was determined by the diphenylamine method (2) with calf thymus DNA as the standard. The ultraviolet spectrum of the DNA preparation was read in a Zeiss spectrophotometer, and the ratio of 260 to 280 nm was 1.70 to 1.85.

To study the development of competence, the recipient cells [8325 (PI_{258}) *thy*$^-$] were grown on TSA plates plus thymine (5 μg/ml) at 37 C overnight and then suspended in TSB to an optical density at 524 nm (OD_{524}) of 0.100, which equals 5 $\times$ 10^7 colony-forming units (CFU)/ml. The cell suspension was then diluted 10 times in TSB plus thymine and incubated on a rotary shaker at 37 C. Samples were withdrawn at intervals during growth (Fig. 1), washed in 0.1 M $CaCl_2$, and then resuspended in an appropriate volume of 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, and the mixture was incubated at 37 C on a rotary shaker for 20 min. At the end of this period, the cells were centrifuged and resuspended in 1 ml of TSB and plated on CHM-plates to select for transformants. Appropriate dilutions were plated on TSA plates with thymine for determination of viable counts. The plates were incubated at 37 C for 48 hr, and the colonies were counted. The controls in all transformation experiments included: (i) plating the recipient culture without DNA treatment, (ii) treatment of DNA with deoxyribonuclease (50 μg/ml containing 5 mM $MgCl_2$) for 10 min before the addition of cells. As shown in Fig. 1, there are no transformants until after (or in connection with) the first division, when the cells suddenly reach the competent state. The competence remains at the same level up to OD_{524} = 0.250 and then decreases to about one-third of the maximum level at the end of the exponential phase. In the following transformation experiments, the recipient cells were harvested at OD_{524} = 0.100 to obtain maximal competence. Samples treated with deoxyribonuclease showed no transformants.

RESULTS

Table 1 shows the effect of DNA concentration on transformation. A competent culture of strain 8325 (PI_{258}) *thy*$^-$ was divided into a number of parallel cultures and treated with DNA from strain 8325 (N) at various concentrations. The DNA concentration becomes limiting between 9.5 and 1.9 μg/ml of the reaction medium.

Tables 2 and 3 summarize representative transformation data using different recipient and donor strains. When resistance to antibiotics or metal ions was used as the selective marker, phenotypic expression was allowed for 60 min in TSB before plating the cells. The transformation frequencies both for chromosomal and plasmid markers are at least 1,000 times lower than those reported by Nomura (11) and also lower than the Russian reports (5, 6, 7, 8, 9) with the exception of Smirnova (19) who reported frequencies tenfold lower than ours. The difference in transformation efficiency for the thymine marker between the two recipient strains, as reported in Table 2, is reproducible.

Erythromycin resistance was the only plasmid marker which could be successfully used for selection of transformants (Table 3). The failure to use cadmium or penicillin resistance as selective markers probably depends on the distance between these markers and the maintenance (*mc*) region on the circular plasmid, but the erythromycin resistance marker is close to the *mc* region (17). This hypothesis is supported by the low cotransformation frequencies of cadmium and penicillin resistance among selected erythromycin-resistant transformants (Table 4). The *mc* region is probably included when transforming DNA is established in the recipient either as a complete or deleted plasmid.

DISCUSSION

The competence curve for transformation reported in Fig. 1 shows that the cells can be transformed during the whole exponential growth phase, but the maximum is in the beginning of this phase. The competence for transfection (18) is also confined to this period. In the transfection system, however, there is a very narrow competence peak. Thus, already 1 hr after the competence maximum has been reached, the competence level has dropped to zero. The difference between the two systems may be explained by a gradual increase in nuclease to which the bacterial DNA is more resistant than the bacteriophage DNA. The high nuclease activity has often been considered the main obstruction to demonstrating transformation in *S. aureus*. This obstacle may be by-

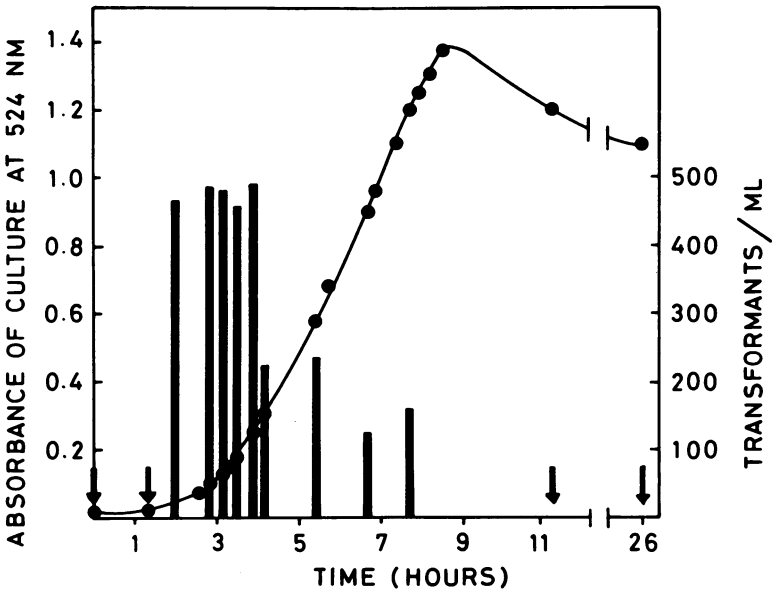

FIG. 1. *Ability of 8325 (PI_{258}) thy⁻ cells to be transformed by DNA from strain 8325 (N) at different points on the growth curve. Symbols: growth, ●; bars, number of transformants; arrows, samples with no competent cells.*

TABLE 1. *Effect of the concentration of DNA [donor: 8325 (N)] on the frequency of transformation of Staphylococcus aureus 8325 (PI_{258}) thy⁻*

DNA (μg/ml)	CHM-medium (CFU/ml)	Complete medium[a] (CFU/ml)
47.2	600	2.5×10^8
23.6	490	
9.5	500	
1.9	210	
0.95	5	
0.19	0	
0.04	0	
0	3	5×10^8

[a] Viable count before incubation in $CaCl_2$, approximately 10^9 CFU/ml.

TABLE 2. *Transformation of pyrimidine-requiring mutants of Staphylococcus aureus with DNA from wild-type cells*

Donor	Recipient	CHM-medium (CFU/ml)		Complete medium (CFU/ml)
		DNA[a] present	DNA absent	
8325 (N)	8325 (N) *thy*⁻	98	0	3×10^8
8325 (PI_{258})	8325 (N) *thy*⁻	160	0	3.2×10^8
8325 N	8325 (N) *cyt*⁻	164	20	1.8×10^8
8325 N	8325 (PI_{258}) *thy*⁻	655	20	2.5×10^8

[a] DNA concentration, 30 to 40 μg/ml.

TABLE 3. *Transformation of the plasmid marker erythromycin resistance (ero^r) by DNA from strain 8325 (PI_{258}); recipient strain: 8325 (N)*

Expt no.	TSA + ero[a] (ero^r transformants/ml)		TSA (CFU/ml)
	DNA present[b]	DNA absent	
1	116	0	9×10^8
2	194	0	9.7×10^8
3	450	0	6.1×10^9

[a] Trypticase Soy Agar (BBL) with erythromycin.
[b] DNA concentration, 24 μg/ml. The same DNA preparation was used in all three experiments.

passed by using mutants lacking extracellular nuclease activity (14). We are at present isolating such mutants.

The method of DNA preparation is a critical step, since no biological activity was observed with DNA isolated by the method of Marmur (10). Another important factor is the requirement for bivalent ions of which calcium ions are the most effective. $CaCl_2$ at 0.1 M seems a rather unphysiological reaction medium, but we have not as yet been able to find any alternative to this salt solution.

Methods for separation of chromosomal and plasmid DNA from plasmid-carrying strains of

TABLE 4. *Cotransformation of penicillin (pen) and cadmium (cad) resistance among selected erythromycin-resistant (ero^r) transformants*

Expt no.[a]	No. of *ero^r* transformants tested	No. of cotransformants		
		cad^r	*pen^r*	*cad^r + pen^r*
1	28	2	6	2
2	32	0	0	0
3	72	0	4	0

[a] Experiment number corresponds to experiment number in Table 3.

S. aureus have been reported (3, 17). A recurrent problem in the field of staphylococcal genetics is to determine whether a marker is coded by a chromosomal or a plasmid gene (4). Provided that the two categories of DNA are biologically active after the separation procedures, transformation should be a useful tool for solving problems of this type. Work along these lines is in progress.

ACKNOWLEDGMENTS

We would like to express our appreciation for helpful discussion and support from Lennart Philipson. The excellent technical assistance of Solveig Karlsson is also gratefully acknowledged.

LITERATURE CITED

1. Altenbern, R. A. 1967. Genetic studies of pigmentation of *Staphylococcus aureus*. Can. J. Microbiol. **13**:389–395.
2. Burton, K. 1956. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315–323.
3. Cannon, M. C., and L. K. Dunican. 1970. The separation of plasmid and chromosomal DNA from *Staphylococcus aureus* using poly-L-lysine Kieselguhr columns. Biochem. Biophys. Res. Commun. **39**:423–428.
4. Dornbusch, K., H. O. Hallander, and F. Löfquist. 1969. Extrachromosomal control of methicillin resistance and toxin production in *Staphylococcus aureus*. J. Bacteriol. **98**:351–358.
5. Imshenetiskii, A. A., K. Z. Perova, T. A. Zaitseva, and A. N. Belozerskii. 1959. Transmission of streptomycin resistance in staphylococci by means of DNA. Mikrobiologiya **28**:187–190.
6. Imshenetskii, A. A., and K. Z. Perova. 1959. Transformation carried out by cell-free extracts. Mikrobiologiya **29**:505–511.
7. Komarov, P. V. 1962. Some physiological pecularities of the streptomycin-resistant variants of *Staphylococcus*. Mikrobiologiya **31**:277–281.
8. Komarov, P. V. 1962. A study of some quantitative indicators of DNA-mediated transmission of streptomycin resistance in staphylococci. Mikrobiologiya **31**:454–458.
9. Lambina, V. A. 1961. Induction in staphylococci of resistance to streptomycin, rivanol, and copper by cell-free extracts. Mikrobiologiya **30**:60–66.
10. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208.
11. Nomura, H., T. Udou, K. Yoshida, Y. Ichikawa, Y. Naito, and M. R. Smith. 1971. Induction of hemolysin synthesis by transformation in *Staphylococcus aureus*. J. Bacteriol. **105**:673–675.
12. Novick, R. P., and M. H. Richmond. 1965. Nature and interactions of the genetic elements governing penicillinase synthesis in *Staphylococcus aureus*. J. Bacteriol. **90**:467–480.
13. Novick, R. P., and C. Roth. 1968. Plasmid-linked resistance to inorganic salts in *Staphylococcus aureus*. J. Bacteriol. **95**:1335–1342.
14. Omenn, G. S., and J. Friedman. 1970. Isolation of mutants of *Staphylococcus aureus* lacking extracellular nuclease activity. J. Bacteriol. **101**:921–924.
15. de Repentigny, J., S. Sonea, and A. Frappier. 1964. Selection of thymineless mutants by growing *S. aureus* pathogenic strains in the presence of aminopterin and thymine or thymidine. Rev. Can. Biol. **23**:451–454.
16. Riggs, H. G., Jr., and E. D. Rosenblum. 1969. Transfection of lysostaphin-treated cells of *Staphylococcus aureus*. J. Virol. **3**:33–37.
17. Rush, M. G., C. N. Gordon, R. P. Novick, and R. C. Warner. 1969. Penicillinase plasmid DNA from Staphylococcus aureus. Proc. Nat. Acad. Sci. U.S.A. **63**:1304–1310.
18. Sjöström, J. E., M. Lindberg, and L. Philipson. 1972. Transfection of *Staphylococcus aureus* with bacteriophage deoxyribonucleic acid. J. Bacteriol. **109**:285–291.
19. Smirnova, B. I., T. L. Gubenko, and T. M. Ribakowa. 1969. Transformation of streptomycin resistance in *Staphylococcus aureus*. Mikrobiologicheskij J. **31**:233–235.